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A comparative approach using biomarkers in feral and caged Neotropical fish: Implications for biomonitoring freshwater ecosystems in agricultural areas

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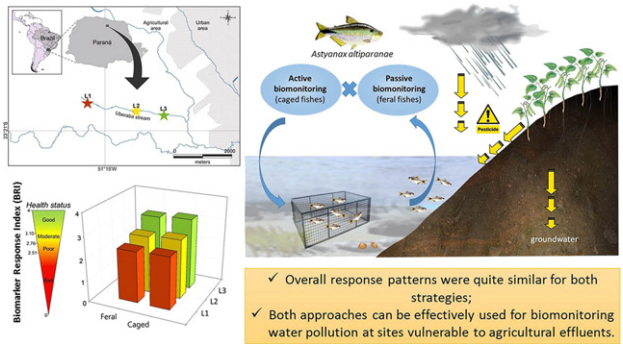
HIGHLIGHTS

- Biomarkers responses in feral and caged *Astyanax altiparanae* were determined.
- Fish were collected or confined (for 168 h) along a stream in an agricultural area.
- Most sensitive biomarkers were DNA breaks, lipid peroxidation and acetylcholinesterase activity.
- Both approaches were effective for discriminating contamination levels along the stream.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to investigate the responses of biomarkers in feral and caged fish and the capacity of these biomarkers to discriminate contamination levels along a stream located in an agricultural area in Southern Brazil. Specimens of the Neotropical fish, *Astyanax altiparanae*, were confined for 168 h in three lakes along the stream. Additionally, during the weeks of *in situ* exposure, wild specimens of this species were collected from the same sites. Biochemical biomarkers were analyzed, such as phase I biotransformation enzyme 7-ethoxyresorufin-*O*-deethylase (EROD) and phase II biotransformation enzyme glutathione *S*-transferase, and we also determined hepatic and branchial levels of non-protein thiols (NPSH), oxidative damage such as lipid peroxidation (LPO), and acetylcholinesterase (AChE) activity in muscle and brain. Genetic biomarkers such as DNA breaks (comet assay), frequency of micronuclei (MN) and erythrocytic nuclear abnormalities (ENA) were also examined. The results indicate that the most sensitive biomarkers for discriminating contamination levels are DNA breaks, LPO and AChE activity. Similar results were obtained for both caged and feral fish. The biomarkers that reflect the results of cumulative events, such as ENA, were more discriminative for chronically exposed specimens (feral fishes). Analyzing biomarkers using an integrated response index showed that both approaches (using feral and caged *A. altiparanae*) were effective for discriminating contamination levels along the stream, corroborating the results of chemical analyses for selected pesticides. Taken together, these results

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highlight the importance of biomarker selection and show that both approaches (caged and feral fish) are satisfactory for evaluating water quality in streams impacted by agricultural activities.

1. Introduction

According to a recent review conducted by Albuquerque et al. (2016), Brazil is responsible for 20% of the total pesticides consumed in the world and is the world's largest consumer of pesticides. The process of agricultural expansion has resulted in domestic growth in pesticides of 194% over the last decade (IBAMA, 2013). In terms of Brazilian states, Paraná (Southern Brazil) is the third largest consumer of pesticides, with sales of approximately 55,000 tons of active ingredients in 2012 alone (IBAMA, 2013). The presence of pesticides in water bodies in agricultural areas of Paraná state was already detected (Vieira et al., 2016) at levels far in excess of those set by Brazilian environmental regulation (Resolution 357/2005 of the National Council for Environment - CONAMA).

The occurrence of pesticides in freshwater ecosystems has been well registered worldwide and is a major issue, causing concern at different geographic scales (Konstantinou et al., 2006). However, in Brazil, is difficult to evaluate the risks posed by pesticides to native aquatic fauna considering that data in peer-reviewed literature are still scarce (Albuquerque et al., 2016). Thus, environmental studies reporting the effects of these contaminants on aquatic biota are important, increasing the need for integrative approaches to the assessment of freshwater quality and the biological effects of pesticides on aquatic ecosystems (Vieira et al., 2016).

Integrated and multidisciplinary studies combining biological and chemical evaluations represent a valuable approach for management and monitoring of heterogeneous aquatic environments (Bebianno et al., 2015). In this context, biomarkers can be valuable in providing information on the effects of contaminants that may impair the health of the organism (Moreira et al., 2004). As such, alongside chemical analysis, biomarkers could be incorporated into environmental monitoring programs as a fast-screening tool, prior to the implementation of preventive bioremediation strategies (Bebianno et al., 2015).

In situ tests using caged fish are a useful approach for assessing contaminant effects on the aquatic biota (Schlenk et al., 2008; Klobucar et al., 2010; Vieira et al., 2014, 2016). The active biomonitoring presents some advantages compared to passive approach, such as: the investigation of sites where feral specimens are difficult to capture or occur in unsatisfactory number; the exact knowledge of the exposure period, thus avoiding the possibility of organisms to acclimate to the new situation; and the standardization of organisms used in the tests (size, age, sex, reproductive stage of development), making it feasible the comparison of results from different sites (Wepener, 2013). According to Smolders et al. (2003), the parallel comparison between the wild and caged species may indicate to what extent the native organisms have adapted to conditions at the particular environment. Moreover, the approach using caged fishes can exclude adaptive factors reducing the influence of genetic and adaptive phenomena, which can impair the efficiency of biomonitoring to distinguish different levels of environmental contamination (Regoli and Principato, 1995).

Parallel feral and caged fish studies can allow comparison between the responses of both experimental approaches and provide information on susceptibility and/or possible adaptations of either group. Nevertheless, there are still few studies in which biomarker determinations have been undertaken in caged fish of any species, and there are even fewer studies in which comparisons with feral specimens of the same species have been made (Barra et al., 2001; de la Torre et al., 2002; Winter et al., 2004, 2005).

In Brazil, studies emphasizing the application of biomarkers in indigenous fish species as tools for assessing water quality are scarce (Wilhelm-Filho et al., 2001; Winkaler et al., 2001; Akaishi et al., 2004; Ramsdorf et al., 2009). Freshwater fish of the genus *Astyanax* have been used in environmental monitoring studies for determining biomarkers at sites affected by different levels of contamination (Winkaler et al., 2001; Silva and Martinez, 2007; Lemos et al., 2008; Trujillo-Jiménez et al., 2011; Vieira et al., 2014; Yamamoto et al., 2016). *Astyanax altiparanae* (Garutti and Britski, 2000) was selected for this study due to its reported biomarker sensitivity (Vieira et al., 2014; Bettim et al., 2016), its abundance at the studies sites and its availability as hatchery-reared specimens for a parallel caged-feral fish study.

Therefore, the goal of this study was to investigate multiple biomarker responses in *A. altiparanae* simultaneously caged at and collected from sites along a stream subject to pesticide contamination in order to understand how these organisms respond to acute and chronic exposure to environmental contaminants. In addition, the biomarker responses of feral and caged fish were integrated into a Biomarker Response Index (BRI) to grade the level of contamination along the stream and to identify the biomarkers that show the strongest responses to the environmental stressors present.

2. Material and methods

2.1. Study area

The sites investigated in this study are located along the Uberaba stream (Fig. 1), a small watercourse in the municipality of Londrina (Northern Paraná). This region shows intense rural activity, with a prevalence of non-perennial monocultures grown using heavy mechanization and pesticides, with the potential risk of contaminating the soil as well as ground and surface waters. Three sampling sites, characterized as artificial lakes, were delimited along the stream, from source to mouth, denoted lake 1 (L1), lake 2 (L2) and lake 3 (L3). In particular, L1, which is the source of the stream and surrounded by farmland, seems to be the location most susceptible to pesticide contamination (Fig. 1). During the study period, there was a transition from corn to soy monoculture, and different herbicides were applied and recorded, such as glyphosate and atrazine.

2.2. Test organisms and experimental design

Adults of *A. altiparanae* ($n = 120$, 5.6 ± 0.4 g body mass; 7.6 ± 0.1 cm total length, no sex differentiation) supplied by a local fish farming facility (Aqualina, Rancho Alegre, PR) were used in the *in situ* tests, carried out in May and June 2013. *In situ* tests were conducted for 168 h, at each site (L1, L2 and L3), along three subsequent weeks. At each site, sampling of caged fish was performed simultaneously with resident fish collection and sampling.

Before the *in situ* tests, one group of the 40 fish, for each experimental site, was acclimated under controlled laboratorial conditions (data are shown in Table 1) during one week in tanks with dechlorinated water and oxygenation and photoperiod of 12 h:12 h. During acclimation fish were fed every 2 days with commercial fish food (Guabi®, protein content 36%).

After acclimation, a group of fish ($n = 20$) was sampled in the laboratory (basal group) to determine biomarker baseline levels for this species. The results were used to calculate the Biomarker Response Index (BRI), according to Hagger et al. (2008, 2010). Another group of fish ($n = 20$ for each site) was transported (in transit for <1 h) to the selected experimental site (L1, L2 or L3) in plastic bags containing water and

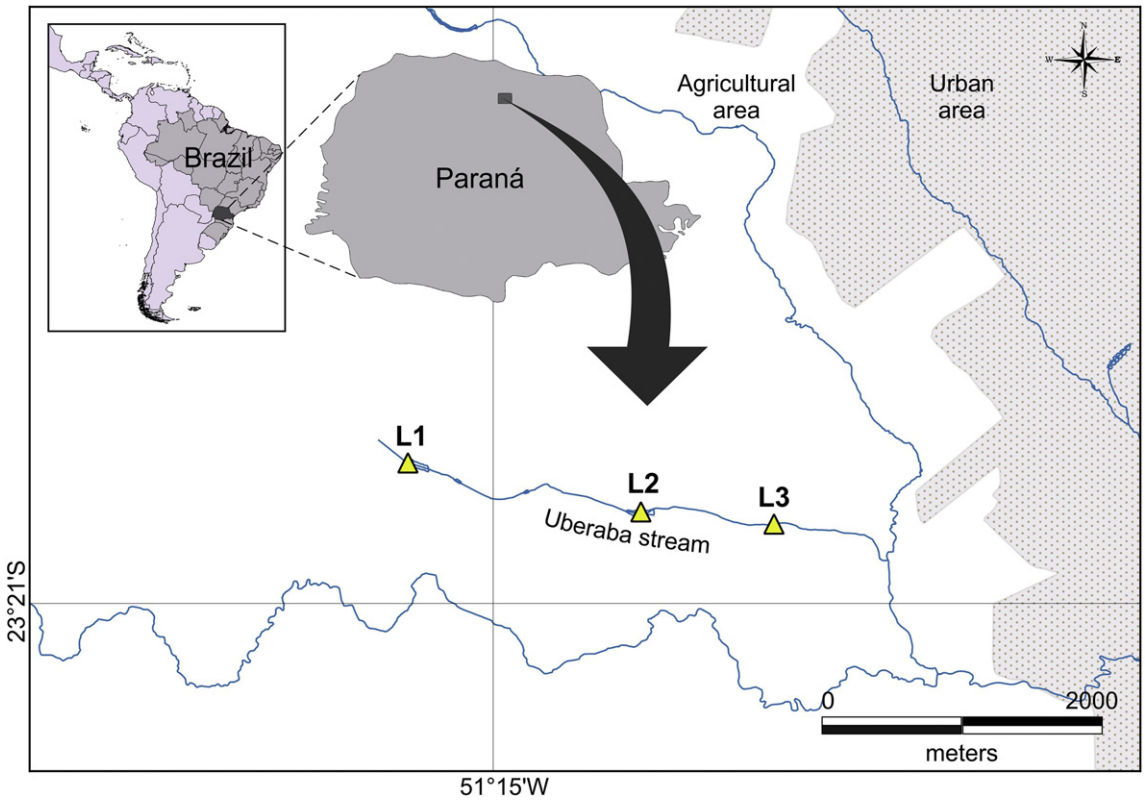

Fig. 1. Map of Brazil showing the Paraná State and the location of the municipality of Londrina (Northern Paraná). In detail the three sites where experiments were carried out: lake 1 (L1), lake 2 (L2) and lake 3 (L3), along the Uberaba stream, located in an area characterized by the intensive use of pesticides throughout the years.

oxygen, and transferred to 120-L cages that were kept submerged for seven days (168 h). The cages (60 × 50 × 40 cm) coated with 5 mm mesh allowed water circulation and were completely submerged. During confinement fish were not fed with commercial fish food. *A. altiparanae* is an omnivorous species with a great capacity to adapt to all kinds of diets. Thus, during caging period, fish feed on suspended material entering through the cage (*e.g.* plankton, micro invertebrates, leaves detritus) and on detritus, as cages remained in contact with the sediment.

During the week that the cage remained at each experimental site, feral specimens of *A. altiparanae* (n = 26, 3.8 ± 0.6 g body mass; 6.3 ± 0.4 cm total length, both sexes) were collected at the same sites using fishing nets and trawl nets. Following capture or after removal from the cages, the fish were transported to the laboratory in isothermal boxes containing aerated stream water. On the first and last day of fish confinement, the physical and chemical parameters of the water were determined using a multiparameter probe (HORIBA-U52). During the caging operations, water and sediment samples were collected for subsequent chemical analysis.

The fish used in the experiments were adults and the proportion of males: females was relatively equivalent, although it was difficult to determine sex accurately because of the initial stage of development of the gonads and the period of non-reproduction of this species. The fish collected in the field, even slightly smaller, also had initial development of gonads, but were not sexually mature. Estimates for the sexual maturation size for the genus *Astyanax* range from 7.8 to 10.4 cm in total length (Nomura, 1975; Agostinho et al., 1984), presenting split spawning during the rainy seasons (Sep-Mar) and peaks that are directly influenced by water temperature and rainfall indices (Porto-Foresti et al., 2010). The *in situ* experiments occurred in the period of May/June, which corresponds to the dry season and therefore to the non-breeding season for the species.

2.3. Fish handling and tissue sampling

In the laboratory, the animals were kept alive in plastic bags containing water from the caging/sampling sites. Initially fish were anaesthetized in water (benzocaine 1%) and blood samples were withdrawn from the caudal vein. After that, fish were killed by medullar section and samples of gills, liver, axial muscle and brain were removed. Samples of blood were conserved in fetal bovine serum (Gibco®) and kept cool for the comet assay. The samples of the other tissues were stored at −72 °C for the biochemical analyses. These procedures were

Table 1
Abiotic parameters of the water from the acclimation tanks, in the laboratory, and water collected from experimental sites in the first and last day of caging. The values for the water from acclimation are represented as mean ± SD.

		Caging sites		
Parameters	Acclimation	L1	L2	L3
Temperature (°C)	20.55 ± 1.36	22.15–19.46	20.13–19.57	20.8–20.5
pH	6.82 ± 0.23	5.47–5.61	5.81–6.25	6.8–6.08
Dissolved oxygen (mg $O_2 \cdot L^{-1}$)	7.54 ± 0.87	5.55–5.8	6.87–6.41	8.4–8.81
Conductivity ($\mu S \cdot cm^{-1}$)	83 ± 11.0	33–34	41–37	43–51
Hardness (mg $CaCO_3 \cdot L^{-1}$)	57.8 ± 3.2	36	38	38

approved by the Animal Ethics Committee of the State University of Londrina (Process 19559.2012.01).

2.4. Analysis of pesticides in water and sediments

In order to characterize the pesticide content of the Uberaba stream, samples of surface water and sediment were collected at each sampling site during the *in situ* experiments. Tests were carried out for 29 legacy pesticides (organochlorine insecticides - OCs) and 7 currently used pesticides (CUPs), such as trifluralin, glyphosate, atrazine, ametrine, simazine, irgarol and clomazone.

The sediment samples were tested for OCs according to the protocols described by Niencheski and Fillmann (2006) and detailed by Vieira et al. (2016), using a gas chromatograph (Perkin Elmer Clarus 500) with a ^{63}Ni electron capture detector (ECD) and an ELITE 5MS capillary column.

The determinations of the herbicides triazine, irgarol and clomazone were carried out as described by Demoliner et al. (2010). The final organic extracts were directly analyzed by LC-ESI-MS-MS (Waters Alliance 2695 Separations Module HPLC). To test the water and sediment for glyphosate, samples were prepared as described by Harayashiki et al. (2013). The extracts were determined by ion chromatography (IC Compact 881, Metrohm, Herisau, Switzerland).

2.5. Biochemical analyses

Samples of liver and gills were weighed, homogenized (1:10 w/v) in K phosphate buffer (0.1 M, pH 7.0) and centrifuged (16,000*g*, 20 min, 4 °C). The supernatant was separated to analyze the subsequent biochemical parameters: activity of 7-ethoxyresorufin-*O*-deethylase (EROD) and glutathione-*S*-transferase (GST), concentration of non-protein thiols (NPSH) and the occurrence of lipoperoxidation (TBARS).

EROD activity was determined according to Eggens and Galgani (1992), by the rate of conversion of 7-ethoxyresorufin to resorufin. The activity of glutathione *S*-transferase (GST) was estimated according to Keen et al. (1976), by monitoring the complexation of reduced glutathione (GSH) with the 1-chloro-2,4-dinitrobenzene (CDNB). The concentration of non-protein thiols (NPSH) was determined according to the method described in Beutler et al. (1963), which based on the reaction of the —SH groups with the color reagent 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), forming thiolate (TNB). Lipid peroxidation (LPO) was determined using TBARS assay (thiobarbituric acid reactive substances), by measuring malondialdehyde (MDA), which is one of the final subproducts of LPO, according to Camejo et al. (1998).

Samples of brain and muscle were homogenized in K phosphate buffer (0.1 M; pH 7.5; 1:10 w/v) and centrifuged (16,000*g*, 20 min, 4 °C). The supernatant was used to determine acetylcholinesterase (AChE) activity according to Ellman et al. (1961), adapted for microplates by Alves Costa et al. (2007).

Protein concentration was determined using the Bradford (1976) method, which is based on the reaction of proteins with Coomassie Brilliant Blue G-250 stain.

2.6. Genotoxic analyses

The alkaline comet assay (Singh et al., 1988) with erythrocytes was performed according to previously published protocols (Vieira et al., 2014, 2016).

The micronuclei (MN) and erythrocytic nuclear abnormalities (ENA) tests with acridine orange fluorescence were performed according to the technique described by Ueda et al. (1992), with some modifications. A small drop of blood was mixed on the slide with fetal bovine serum (1:1), and a blood smear performed. The slides were dried overnight and then fixed with methanol for 10 min. Before examination, each slide was stained with acridine orange (0.003%) in Sorenson's buffer at pH 6.8 for 2–3 min and immediately examined under an epifluorescence microscope at 1000× magnification. For each fish, 3000 cells were examined to evaluate the presence of micronuclei and alterations in the normal elliptical shape of the nuclei of blood cells that did not fit the micronuclei concept, described as ENA (Carrasco et al., 1990). ENAs were categorized in three types: segmented nucleus, lobulated nucleus and kidney-shaped nucleus, and the presence of binucleated cells was also noted.

Immature erythrocytes (IE) were identified according to Çavas and Ergene-Gözükara (2005). Acridine orange selectively stains immature or polychromatic erythrocytes. Under a fluorescence microscope, micronuclei and the main nuclei display a strong green-yellow fluorescence. IEs can be easily identified among all the cells by their cytoplasmic RNA content, appearing as a red fluorescence in the cytoplasm.

2.7. Biomarker Response Index (BRI)

The Biomarker Response Index (BRI) proposed by Hagger et al. (2008, 2010) was calculated for each site, with some modifications proposed by Parolini et al. (2013). The basal group (sampled at the laboratory, just after acclimation) was used as a reference and the experimental sites (L1, L2 and L3) classified according to biological status. Biomarkers were categorized in terms of the molecular, cellular and physiological alterations according to the level of biological organization, and different weights were established for each category. According to Parolini et al. (2013), the cellular biomarkers included in the present study (DNA damage, occurrence of MNs, ENAs and lipoperoxidation) were weighted as 2, whereas molecular assays (EROD and GST activity, and NPHS concentration) were weighted as 1, based on the assumption that an alteration at the cellular level will have a greater impact on the health status of the organism than variations at the molecular level. AChE activity was categorized as a physiological biomarker, and was assumed to have a greater impact on the performance of the fish, and therefore weighted as 3. The percentage alteration was calculated for each biomarker as the % deviation of experimental site mean values from the basal group value, and then ranked in categories with scores from 1 to 4. Biomarkers with small differences (±20%) were assigned a score of 4, with differences between ±20% to ±50% a score of 3, with major differences (±50–100%) a score of 2 and deviations that significantly exceeded the corresponding recorded baseline level (±100%) a score of 1. Finally, the BRI was calculated using the following equation:

$$\mathrm{BRI} = \sum(\mathrm{biomarker_n\,score}^{*}\,\mathrm{biomarker_n\,weight}) \Big/ \sum \mathrm{biomarker_n\,weight}$$

The BRI was related with a biological health status representing the degree of variation from normal/basal levels, according to the following categories, as defined by Hagger et al. (2008): *good* - none or minor alterations from normal response (BRI value 3.01–4.0); *moderate* - moderate alterations (BRI value 2.76–3.00); *poor* - major alterations (BRI value 2.51–2.75); *bad* - severely altered responses (BRI value 0–2.5).

2.8. Statistical analyses

The results of biomarker were verified for normality and homogeneity of variance by applying the Shapiro Wilk and Levene tests respectively. The results for biomarkers examined in fish tested *in situ* and in feral fish collected from the caging sites were compared separately (L1 × L2 × L3). Analysis of variance parametric or non-parametric (ANOVA or Kruskal-Wallis test, respectively) was applied according to the data distribution, followed by a multiple comparison test whenever necessary. Values of $p < 0.05$ were considered significant. The data shown in the graphs represent means ± SEM.

3. Results

3.1. Analysis of water and sediment

The physical and chemical parameters of the water did not vary greatly from one study site to another, except for dissolved oxygen (DO) values observed at L1, which were lower than at the other sites (Table 1).

Of the 7 CUPs and 29 legacy pesticides appraised in the sediments from the experimental sites along Uberaba stream (Table 2), 11 were above detection limits, including the herbicides atrazine, simazine and trifluralin, along with the organochlorine pesticides hexachlorocyclohexanes (*a* and *b*-HCH), hexachlorobenzene (HCB), heptachlor, dichlorodiphenyltrichloroethane (*pp*-DDT), endrin ketone, dieldrin and endosulfan II. The highest pesticide concentrations in the sediment samples were found at L1, indicating a possible decreasing contamination gradient from up to downstream. In the water samples, only atrazine and ametrine were detected, and the highest concentrations of both herbicides were also observed at L1.

3.2. Biomarkers

The biomarker results of comparisons across the three sites (L1 × L2 × L3), taking each experimental approach (feral or caged fish) separately, are described below. Although the two approaches (caged × feral) could not be statistically compared due limitations such as distinct fish origin, exposure time and size and age differences, some important considerations were noted regarding the differences observed between the caged and feral fish in the responses of some biomarkers. In general, overall response patterns were quite similar for most biomarkers, and variations in these responses are highlighted below.

In terms of biotransformation enzymes, hepatic EROD activity did not vary significantly for caged and feral fish among the study sites (Fig. 2.A). In the gills, EROD activity was not detected by the method used. Hepatic GST activity (Fig. 2.B) was significantly lower in the fish caged at L1 than in the other lake sites. Similarly, GST activity was lower in the liver of L1 feral fish. In the gills (Fig. 2.C), GST activity was lower in the fish confined at L1 and L2 than in fish at L3. The opposite was observed for feral fish at L1 and L2, which had higher gill enzyme activity levels than the fish collected at L3. Note that GST levels were higher in feral fish compared to caged fish, especially in the liver.

In regard to antioxidants, NPSH levels were lower in the liver (Fig. 3.A) of fish confined at L1 compared to those at L3. There was no significant difference in hepatic concentration of NPSH in the feral fish collected from different sites. In the gills (Fig. 3.B), there were no significant changes in NPSH levels among the sites for both caged and feral fish. In common with GST, feral fish showed higher levels of NPSH compared to caged fish, especially in the liver. Lipid peroxidation was evidenced by the increased TBARS in the liver of caged and feral fish from L1 compared to L3 (Fig. 3.C). The feral fish at L2 also showed higher levels of lipid peroxidation compared to L3. Lipid peroxidation in the

Table 2
Concentrations of selected pesticides in sediment ($\mu g \cdot kg^{-1}$ dry weight) and water ($\mu g \cdot L^{-1}$) samples collected from the experimental sites (L1, L2 and L3) at Uberaba stream during experimental period. The maximum permitted concentrations (MPC) for some pesticides, set by the Brazilian guidelines (BRASIL, 2005) for inland waters, and for sediment set by the sediment Canadian Environmental Quality Guidelines (CEQG) that represent probable effect level with adverse biological effects, are indicated.

Pesticides	Sediment				Water			
	L1	L2	L3	MPC	L1	L2	L3	MPC
Herbicides								
Atrazine	0.471	0.336	0.272		0.31	0.13	0.13	2
Glyphosate	BDL	BDL	BDL		nd	nd	nd	65
Irgarol	nd	nd	nd		nd	nd	nd	
Simazine	0.597	0.419	0.486		BDL	BDL	BDL	2
Ametrine	BDL	BDL	BDL		0.666	0.397	0.399	
Clomazone	BDL	BDL	BDL		nd	nd	nd	
Trifluralin	3.71	2.918	1.967		nd	nd	nd	0.2
Organochloride insecticides								
α-Hexachlorocyclohexane	5.13	3.198	1		nq	nq	nq	
β-Hexachlorocyclohexane	1.23	0.97	0.829		nq	nq	nq	
γ-Hexachlorocyclohexane	BDL	BDL	BDL		nq	nq	nq	
δ-Hexachlorocyclohexane	BDL	BDL	BDL	1.38	nq	nq	nq	
Hexachlorobenzene	2.18	2.197	2.194		nq	nq	nq	
Chlorothalonil	BDL	BDL	BDL		nq	nq	nq	
Oxychlordane	BDL	BDL	BDL		nq	nq	nq	
Heptachlor	0.06	0.052	BDL	2.74	nq	nq	nq	
Heptachlor epoxide	BDL	BDL	BDL		nq	nq	nq	
Dichlofluanid	BDL	BDL	BDL		nq	nq	nq	
Transnonachlor	nd	nd	nd		nq	nq	nq	
α-Chlordane	nd	nd	nd	8.87	nq	nq	nq	
γ-Chlordane	BDL	BDL	BDL		nq	nq	nq	
op′-DDD	BDL	BDL	BDL	8.81	nq	nq	nq	
pp′-DDD	nd	nd	nd		nq	nq	nq	
op′-DDE	BDL	BDL	BDL	6.75	nq	nq	nq	
pp′-DDE	nd	nd	nd		nq	nq	nq	
op′-DDT	nd	nd	nd	4.77	nq	nq	nq	
pp′-DDT	0.021	0.022	0.003		nq	nq	nq	
Aldrin	BDL	BDL	BDL		nq	nq	nq	
Endrin	nd	nd	nd	62.4	nq	nq	nq	
Endrin aldehyde	nd	nd	nd		nq	nq	nq	
Endrin ketone	0.008	nd	0.003		nq	nq	nq	
Dieldrin	0.056	0.078	0.032	6.67	nq	nq	nq	
Endosulfan I	nd	nd	nd		nq	nq	nq	
Endosulfan II	0.001	0.004	BDL		nq	nq	nq	
Endosulfan sulfate	nd	nd	nd		nq	nq	nq	
Methoxychlor	nd	nd	nd		nq	nq	nq	
Mirex	nd	nd	nd		nq	nq	nq	

BDL — below detection limit of the method; nd = not detected; nq = not quantified.

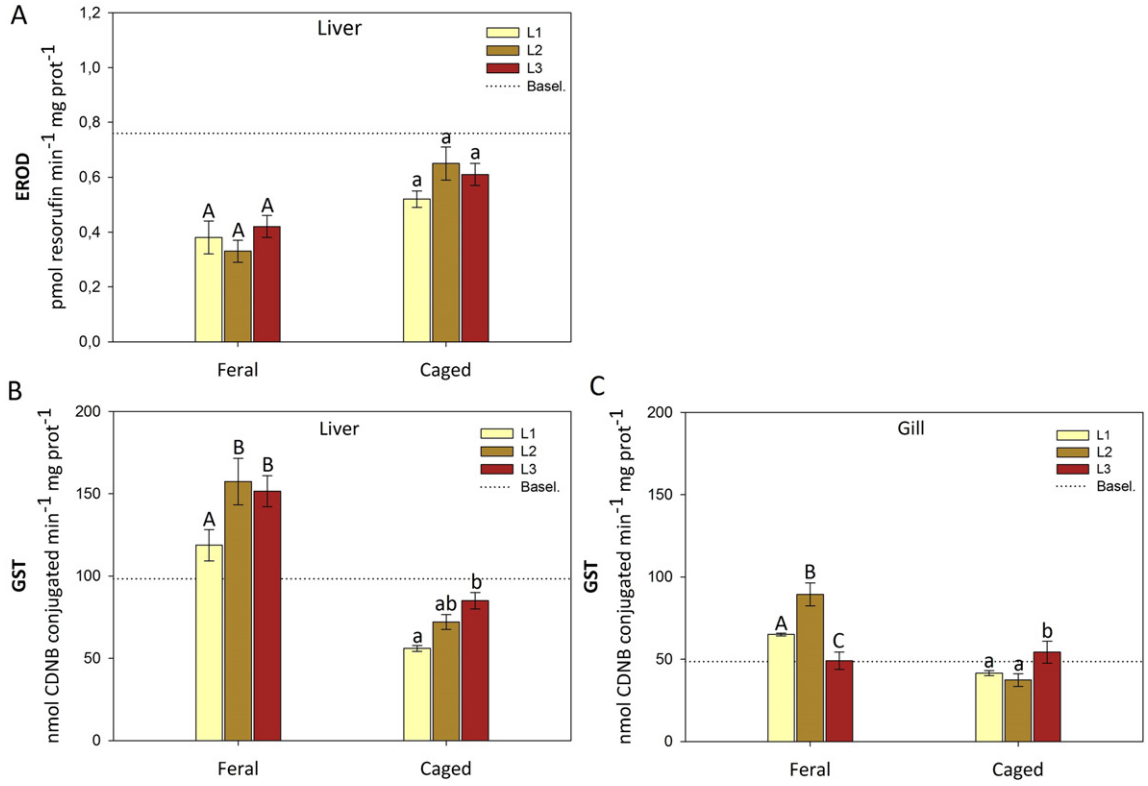

Fig. 2. Activity of 7-ethoxiresorufina-*O*-deetilase (EROD) in liver (A) and glutathione-*S*-transferase (GST) in liver (B) and gill (C) of *A. altiparanae* caged and collected at the three lakes (L1, L2 and L3) along the Uberaba stream. The dashed line represents the baseline value of the biomarker measured in animals kept under controlled conditions. Results are mean ± SEM. Different letters indicate significant differences between sites ($p < 0.05$).

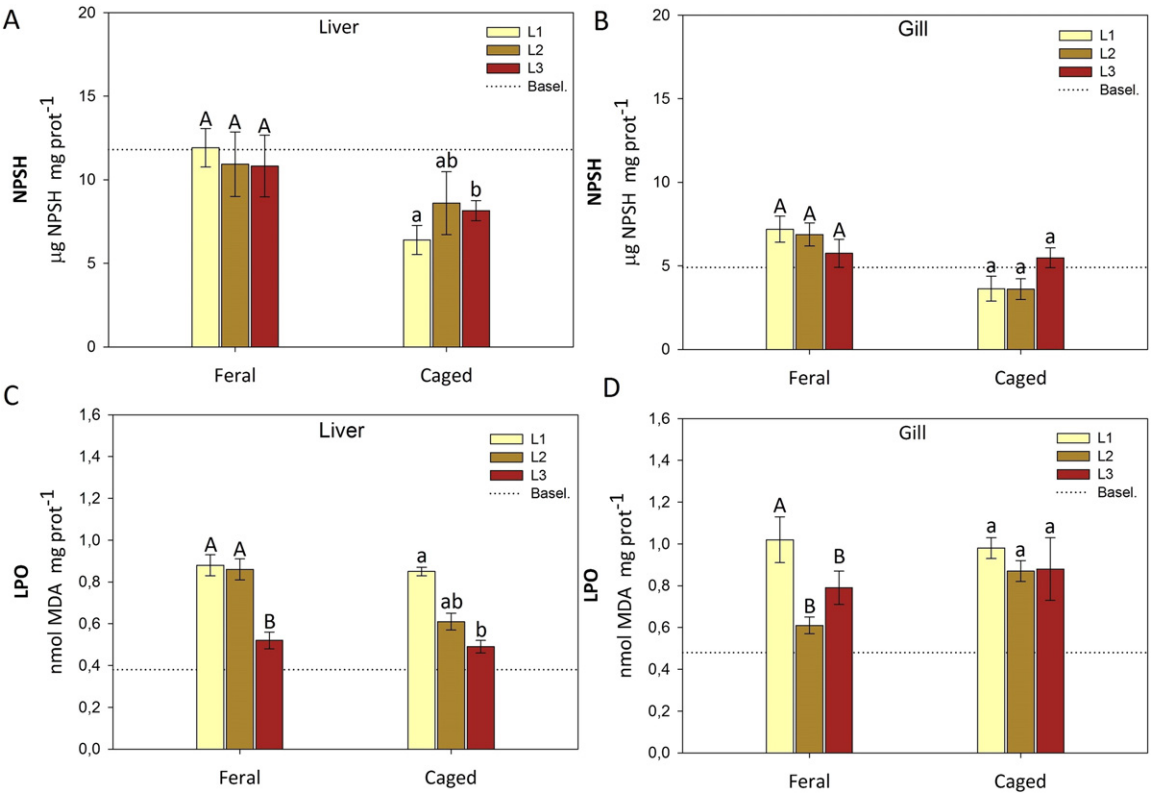

Fig. 3. Concentrations of non-protein thiols (NPSH) in liver (A) and gill (B) and lipid peroxidation (LPO) in liver (C) and gill (D) of *A. altiparanae* caged and collected at the three lakes (L1, L2 and L3) along the Uberaba stream. The dashed line represents the baseline value of the biomarker measured in animals kept under controlled conditions. Results are mean ± SEM. Different letters indicate significant differences between sites ($p < 0.05$).

gills resulted in an increase in TBARS levels only in the feral fish from L1, compared to fish from the other sites (Fig. 3.D).

The results for AChE activity in the brain and muscle of the fish are shown in Fig. 4. In the muscle, there were significant decreases in AChE activity in fish caged at L1 and L2, as well as in feral fish collected from the same sites, compared to L3 (Fig. 4.A). There were significant decreases in the AChE activity in the brains of both caged and feral fish from L1 compared to the other sites (Fig. 4.B).

In regard to genotoxic biomarkers, the feral fish from L1 exhibited significantly higher DNA damage scores compared to those from the other experimental sites (Fig. 5.A) and DNA damage was greater in caged fish at L1 compared to those at L2. The MN frequency was no significant difference for any of the specimen groups analyzed (Fig. 5.B). On the other hand, significantly higher ENA frequencies were observed in both caged and feral fish from L1 compared to those from L3 (Fig. 5.C). There was no significant difference in the frequency of IEs (Fig. 5.D) among any of the experimental groups. The main alterations found in the erythrocytes of *A. altiparanae* are shown in Fig. 6.

3.3. Biomarker Response Index

The biomarker weights and scores for different tissues of both caged and feral fish from each experimental site (L1, L2, L3) used for calculating the Biomarker Response Index (BRI) are shown in Table 3. BRI values representing the overall general health status of the fish are shown in Fig. 7. For the caged fish, a possible relationship with the contamination gradient along Uberaba stream was observed. Fish caged at L1 had the lowest BRI value (L1 = 2.42), followed by site 2 (L2 = 2.74) and then site 3 (L3 = 3.26). The BRI values for the feral fish were slightly lower than those obtained for caged fish, but followed the same pattern (L1 = 2.37 < L2 = 2.63 < L3 = 3.16). Based on these BRI values, caged and feral fish from L1 were in bad health; those from L2 in poor health, and those from L3 in good health. Thus, the health status of caged and feral fish from the same site was similar. L1 fish showed the most severe changes in biomarker responses, mainly in terms of higher levels of cell damage (DNA damage and lipid peroxidation), as well as decreased AChE activity compared to fish from the other sites.

4. Discussion

In the present work, a set of biomarker responses measured in feral and caged fish in a stream subjected to agricultural inputs was applied in order to understand how these organisms respond to acute and chronic exposure to environmental contaminants, and how these responses reflect environment quality. Some similar response patterns were observed in feral and confined fish, such as the biomarkers that reflect oxidative, genotoxic and neurotoxic effects. Conversely, the response patterns for exposure biomarkers, such as NPSH levels and the activity of enzymes involved in phase II biotransformation, were not very consistent when both methodological approaches are compared, and this could indicate different adaptive responses to long-term exposure.

Water variables, such as pH, temperature and dissolved oxygen (DO), may affect organism responses. Normal DO values in water are approximately 8 $mg \cdot L^{-1}$ at 25 °C (Derisio, 2000) and lower values were detected in the waters at sites L1 and L2. Despite concentrations below 8 $mg \cdot L^{-1}$, the values detected are considered acceptable, and the possibility of hypoxia was discarded. Because L1 is an artificial reservoir close to the source of the stream, water flow can be very slow, which may explain the low DO concentration. Furthermore, the environment contains a large amount of organic matter, and of the three lake sites, L1 is closer to cropping areas and therefore the most susceptible to chemical fertilizer input. Variations in temperature may also affect the metabolism of ectothermic animals and may induce oxidative stress (Lushchak, 2011). Mean temperatures varied from 19.5 to 22.15 °C and are within the expected range for the time of year. The pH also influences the aquatic ecosystem and may affect the physiology of several organisms. The indirect effects of pH, especially on the speciation of toxic metals within the aquatic environment, should also be considered. The pH values at the three sites varied from 5.4 to 6.8 and are within, or close to, the range recommended by the Brazilian National Environmental Council (BRASIL, 2005) in resolution 357/2005, relating to the protection of aquatic communities.

Exposure of organisms to environmental contaminants may result in biochemical impairments and/or adaptive responses (Masfaraud et al., 1992). The metabolic processes of biotransformation Phases I and II are often necessary for the detoxification and excretion of toxic compounds in aquatic animals (Goksøyr and Förlin, 1992). In fish, organic compounds such as OCPs can be oxidized by phase I reactions catalyzed by cytochrome P450 isoenzymes, particularly CYP1A subfamily (Karaca et al., 2014). CYP1A activity can be changed in the presence of pesticides and other pollutants in the aquatic ecosystems (van der Oost et al., 2003). Although any significant difference was observed in EROD activity in the liver of both caged and feral fish from the three experimental sites, the levels of enzymatic activity in caged and feral specimens were lower than the baseline levels for this parameter. In the literature, the inhibition of EROD activity promoted by OCPs in field studies is reported (Couillard et al., 2005; Kolankaya, 2006). Also, the inhibition of P450 enzymes by others pesticides classes, like organophosphates and carbamates is a well-known effect of these chemicals (Fabrizi et al., 1999; Hernández-Moreno et al., 2011).

GST acts in the second phase of biotransformation, conjugating toxic compounds or their metabolites with endogenous molecules, such as GSH, to be eliminated from the organism (Hermes-Lima, 2004). In our

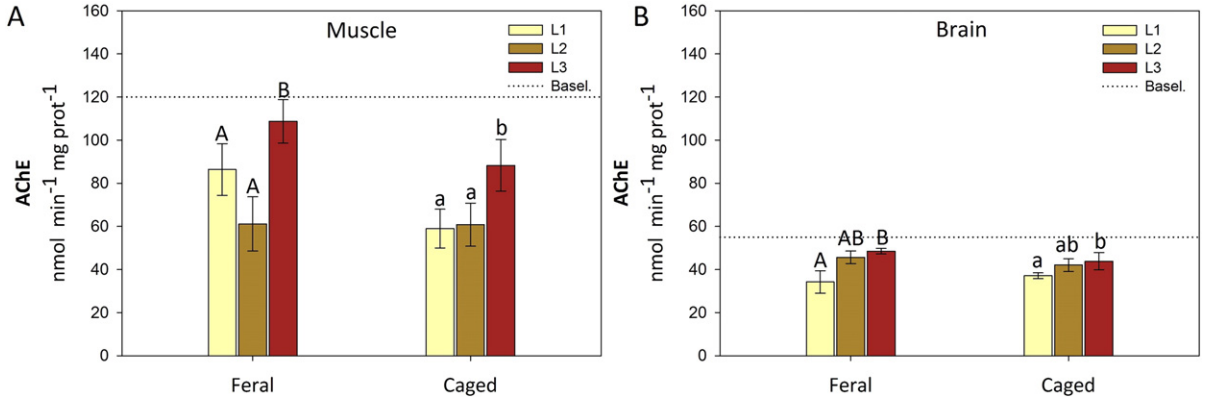

Fig. 4. Activity of acetylcholinesterase (AChE) in muscle (A) and brain (B) of *A. altiparanae* caged and collected at the three lakes (L1, L2 and L3) along the Uberaba stream. The dashed line represents the baseline value of the biomarker measured in animals kept under controlled conditions. Results are mean ± SEM. Different letters indicate significant differences between sites ($p < 0.05$).

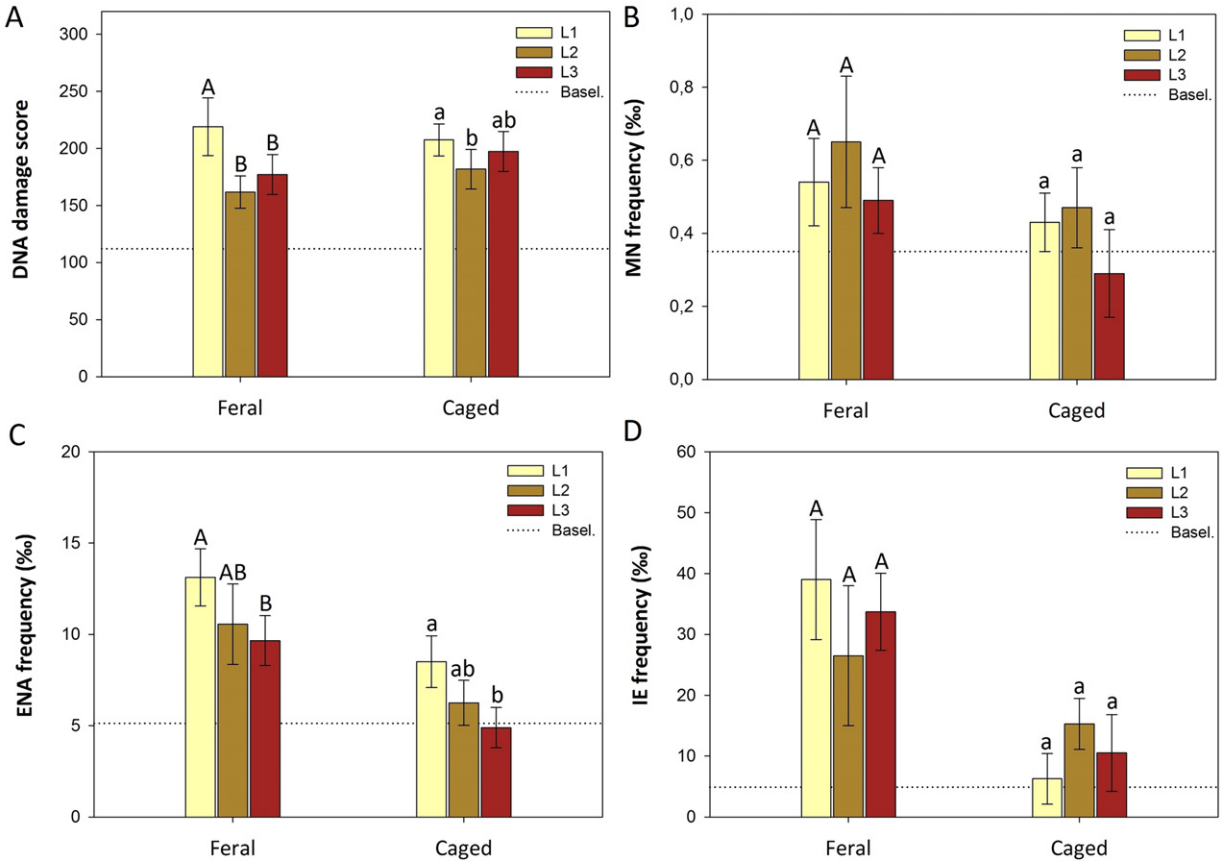

Fig. 5. Score of DNA damage (A), frequency of micronuclei (MN) (B), nuclear abnormalities (ENA) (C) and immature erythrocytes (IE) frequency (D) of *A. altiparanae* caged and collected at the three lakes (L1, L2 and L3) along the Uberaba stream. The dashed line represents the baseline value of the biomarker measured in animals kept under controlled conditions. Results are mean ± SEM. Different letters indicate significant differences between sites ($p < 0.05$).

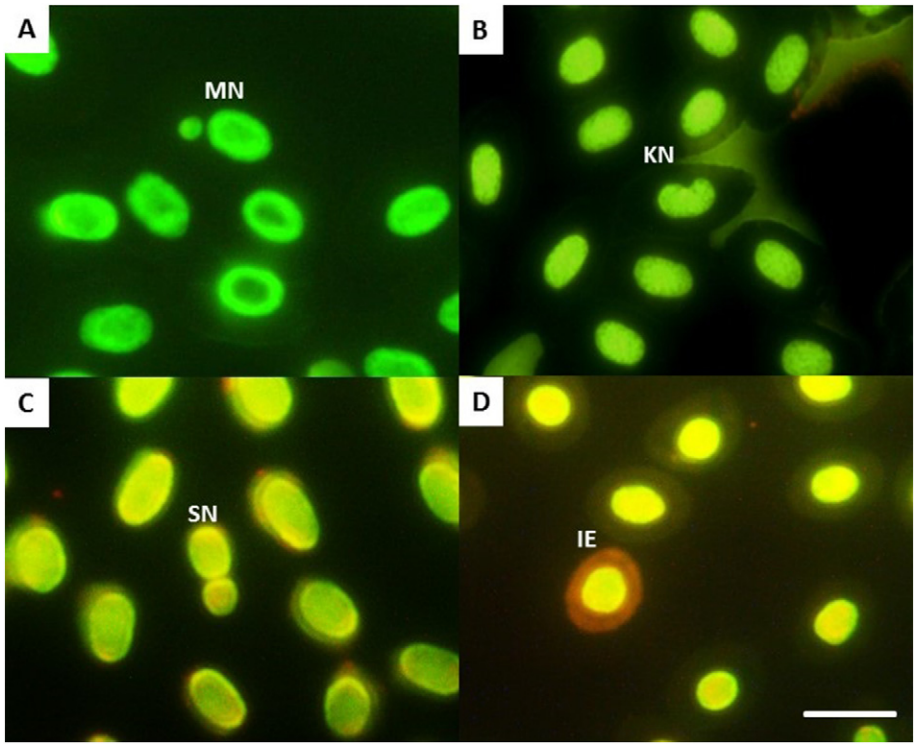

Fig. 6. Photomicrographs of *A. altiparanae* erythrocytes showing in A) cell with micronuclei (MN), B) kidney-shaped nucleus (KN), C) segmented nucleus (SN) and D) immature erythrocyte (IE). Acridine orange staining, bar = 20 μm, magnification of 1,000×.

Table 3
Biomarker weights and scores for different tissues of both caged and feral fish from each experimental site (L1, L2, L3) used for calculating the Biological Response Index (BRI). The scores correspond to the deviation of experimental site mean values from the basal group value and then ranked in categories with scores from 1 to 4. Biomarkers with small differences (±20%) were assigned a score of 4, with differences between ±20% to ±50% a score of 3, with major differences (±50–100%) a score of 2 and deviations that significantly exceeded the corresponding recorded baseline level (±100%) a score of 1.

Biomarker	Organ/tissue	Level of biological organization	Weighting biomarker	Scores					
				Caged			Feral		
				L1	L2	L3	L1	L2	L3
EROD	Liver	Molecular	1	3	4	4	3	2	3
GST	Liver	Molecular	1	3	3	4	4	2	3
	Gill		1	4	3	4	3	2	4
NPSH	Liver	Molecular	1	3	3	3	4	4	4
	Gill		2	3	3	4	3	3	4
LPO	Liver	Cellular	2	1	2	3	1	1	3
	Gill		2	1	2	2	1	3	2
DNA damage	Erythrocytes	Cellular	2	2	2	2	2	3	2
MN/ENA	Erythrocytes	Cellular	3	2	3	4	1	1	2
AChE	Brain	Physiological	3	3	3	4	3	4	4
	Muscle		3	3	3	3	3	3	4

study, there were decreases in the GST activity levels in the liver of both caged and feral fish from L1, compared to the other study sites. However, in feral fish, GST activity levels in the liver and gills were higher than in caged fish. This high level of GST activity in feral fish may be an adaptive response to chronic exposure to the mixture of pesticides present in these environments. It should be emphasized that when considering the effects of stress in organisms chronically exposed to chemical products in their natural environment, no simple or general response can be described (Biagianti-Risbourg et al., 2013).

Previous studies have already reported increases in the activity of GST activity in fish exposed to pesticides, some of which are extensively applied close to the Uberaba stream, such as endosulfan (Dong et al., 2013), 2,4-D (Oruc et al., 2004), glyphosate (Modesto and Martinez, 2010a, 2010b) and atrazine (Paulino et al., 2012), one of the most widely used herbicides in the region around the experimental sites selected for this study. Although at low concentrations, atrazine was detected in water and sediment samples from all the sites evaluated along Uberaba stream.

Reduced glutathione (GSH) is a tripeptide with fundamental roles in redox reactions, transport of amino acids and elimination of several toxic agents, and is the main line of protection against cell lesions

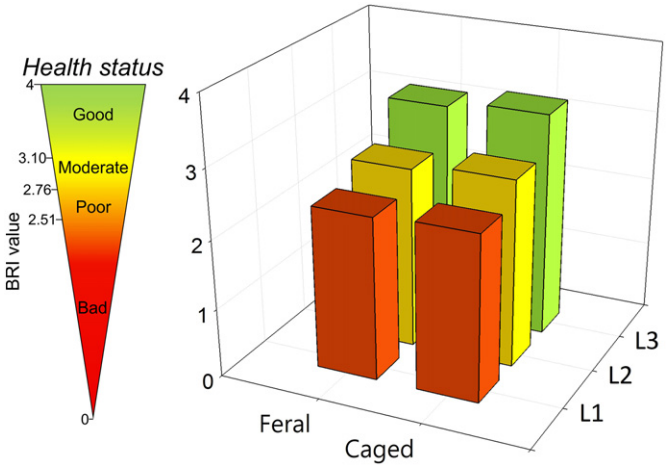

Fig. 7. Biomarker Response Index (BRI) and the res6pective health status calculated for caged and feral *A. altiparanae* collected at the three lakes (L1, L2 and L3) along the Uberaba stream. The colors of the bars indicate the following categories, as defined by Hagger et al. (2008): *green*- no or slight alterations from normal response (BRI value 3.01–4.0); *yellow* - moderate alterations (BRI value 2.76–3.00); *orange* - major alterations (BRI value 2.51–2.75); *red* - severely altered responses (BRI value 0–2.5).

mediated by reactive oxygen species (ROS) (van der Oost et al., 2003). In our study the concentration of non-protein thiols (NPSH) was measured in order to estimate GSH content. In the liver of fish caged at L1, the drop in the NPSH content may indicate a depletion of the reserves of this antioxidant, due to boosted production of ROS, or even interference in the synthesis or recycling of GSH by GR (glutathione reductase), not measured in this study. According to Lushchak (2016), the GSH content is adjusted to specific physiological or environmental conditions *via* several regulatory pathways, but is mainly regulated at the level of biosynthesis in the cells. Oxyradical-generating compounds such as pesticides can alter GSH metabolism in several ways (Otto and Moon, 1995). The drop in hepatic NPSH levels in the caged fish could also have indirectly affected liver GST activity, since this enzyme uses GSH (one of the NPSH species abundantly present in cells) to conjugate with xenobiotics.

Oxidative stress occurs when there is an imbalance between ROS production and the total antioxidant capacity of the cell (Lushchak, 2016). This antioxidant capacity is due to the joint action of enzymes such as catalase, superoxide dismutase and glutathione peroxidase, and small-molecular-weight molecules such as GSH, α-tocopherol and β-carotene (Hermes-Lima, 2004). In our study, LPO was found in both caged and feral fish from some sites. In the liver, the increased occurrence of LPO may have been intensified by the decreases in NPSHs, such as GSH, the primary cell antioxidant. LPO was also found in the gills, although the NPSH levels remained unchanged.

The activity of AChE was reduced in both the brain and muscle of the feral fish from some of the study sites. This enzyme, which is present in the cholinergic synapses and motor endplates, is responsible for hydrolyzing the neurotransmitter acetylcholine into choline and acetic acid. AChE is sensitive to specific pesticides, such as organophosphates and carbamates, and some metals (Ghisi et al., 2016), although other studies have already shown the anticholinesterase effects of others pesticides classes in different fish species, such as glyphosate herbicide (Glusczak et al., 2006, 2007; Cattaneo et al., 2011, Modesto and Martinez, 2010a, 2010b, Sandrini et al., 2013). Depending on the chemical nature of the xenobiotic, AChE inhibition may be irreversible. Although glyphosate was not detected in the water and sediment samples at the study sites, AChE inhibition could be the result of previous exposure to this herbicide, since glyphosate has a half-life of 4.2 days in stream waters (Vera et al., 2010).

Regarding genotoxic biomarkers, there was an increase in the number of DNA breaks (damage score) detected by the comet assay in both caged and feral fish at site L1, which exhibited the highest concentrations of the contaminants evaluated. These results corroborate the data in the literature on fish exposed to different pesticides that are used on a large scale in the study region, such as atrazine (Ventura et al., 2008; Çavas, 2011; Nwani et al., 2011; Santos and Martinez, 2012)

and glyphosate-based herbicides, in both laboratory and field studies on exposure in potentially contaminated areas (Çavas and Könen, 2007; Cavalcante et al., 2008; Rossi et al., 2011; Ramsdorf et al., 2012).

In our study, there was no significant difference in the frequency of micronuclei between fish forming different experimental groups, although there was a trend toward an increase in the frequency of MNs in feral fish compared to the baseline group. In fishes the trophic level can influence the basal number of micronuclei as a consequence of the bioaccumulation process along the trophic chain (Porto et al., 2005). Species of the genus *Astyanax* are considered omnivorous and opportunistic, taking advantage of new environmental conditions and the availability of food items (Gomiero and Braga, 2005). The restricted movement of the caged fish may have forced them to rely more heavily on sediment-based food sources, rather than those in the water column. Thus, differences in exposure route may have played a role in the distinct responses observed in caged and feral *A. altiparanae*.

Although the occurrence of MNs was not significant, ENAs such as kidney-shaped, lobulated and segmented nuclei, in addition to binucleated cells, were observed more often in feral fish from L1 compared to those from other study sites. Several studies have shown the positive relationship between the ENA and MN frequencies, suggesting that identifying these alterations may be useful in fish genotoxicity studies (Ferraro et al., 2004; Ergene et al., 2007). Fishes living in polluted environments can develop higher rates of ENAs and necrotic and apoptotic cells (Talapatra and Banerjee, 2007).

The increased occurrence of LPO and DNA damage in *A. altiparanae* living in the agricultural areas studied may indicate an exhaustion phase. Individuals chronically exposed to chemical substances (such as organic pollutants) may show a significant increase in the responses of biochemical biomarkers (such as biotransformation metabolism and oxidative stress). However, when acute or chronic stress surpasses the compensation limits, the biomarker responses are often depleted and can even fall below basal values (Biagianti-Risbourg et al., 2013). During exhaustion, energy reserves are depleted, resulting in degenerative events such as LPO, DNA damage, and cell necrosis and lysis.

There was no significant difference in the frequency of IEs across all the groups analyzed. According to Udroi (2006), alterations in hematopoietic dynamics may generate conflicting results because several genotoxic agents can favor decreased erythropoiesis. Analyzing the frequency of IEs in fish is important for understanding erythropoietic dynamics. However, in isolation, this kind of analysis does not allow the causes of such an increase to be identified.

Besides the effects of the xenobiotics present in the water bodies investigated, several non-pollution-related variables may have an additional impact on the various enzyme systems, and may thus interfere with biomarker responses when experimental conditions are not thoroughly analyzed or controlled. Factors such as the age, developmental stage, nutritional status, sex and reproductive cycle phase of the fish can also have profound effects on some biological parameters (van der Oost et al., 2003). The difference in size between feral and caged fish may offer an explanation of the difference in the levels of substances such as GST and NPSH, however, there is a limited amount of information regarding sexual, seasonal and developmental differences in GST activity in fish (Stegeman et al., 1992). The liver NPSH level in caged fish was depleted in fish from the most contaminated sites, as would be expected under oxidative stress conditions (Lenton et al., 1999). Conversely, in feral fish, NPSH levels were similar for fish from sites with different contamination levels. However, in general, NPSH concentrations were lower in caged fish than in feral fish, suggesting that caged fish were more sensitive to contaminant exposure and/or that the indigenous fish can be more resistant to the effects of contamination.

Feral fish populations can undergo genetic adaptation, involving information transfer to the offspring. In the worst-case scenario, chemical substances may have severe effects (evasion/escape, death), leading to a population decrease. If the organisms survive previous exposure, these chemical products may exert selective pressure that leads to the presence of resistant genotypes in impacted areas (Biagianti-Risbourg et al., 2013). For this reason, our understanding of the responses of these exposure biomarkers in feral fish, compared to caged fish, leaves a lot to be desired.

The caged fish approach afforded some benefits, such as the standardization of the fish in terms of size and age, lower stress levels during handling, and the ease of obtaining a satisfactory number of individuals. The BRI values obtained for these fish indicate a contamination gradient along the stream, a fact that can be confirmed by chemical analysis of the water and sediment. The gradient observed up-to downstream (L1 > L2 > L3) indicates greater input of contaminants at the source of the stream, which is completely surrounded by agricultural crops located a few meters from the water, facilitating surface runoff and possible contamination of the groundwater that forms the stream. Since the study sites are lakes and therefore lentic environments, pesticides tend to accumulate more easily, and the water flow does not carry them downstream rapidly.

Some of these physiological parameters differed between the two experimental approaches, although it is very difficult to establish cause-and-effect relationships in resident animals from areas with a history of contamination. When all results are integrated into the BRI, these differences are attenuated and diluted, since the parameters that weigh the most on the final values of the index are those effects markers, which receive a higher weight in relation to the biomarkers of exposure (*e.g.* GST and NPSH); and the adverse effects such as lipid peroxidation and DNA damage were similar in both experimental approaches. However, it is worth mentioning that small differences in BRI values were observed in the resident fish at the three experimental sites, where the native fish showed a slight decline in health compared to the caged fish.

Recently, several authors have suggested using methods that combine various biomarkers the responses into a single value, clarifying the results and facilitating large-scale arrangement of biomarkers in environmental monitoring (Hagger et al., 2008, 2010; Sanchez et al., 2013; Vieira et al., 2014, 2016), including the BRI. The capability to interpret and simplify complex biological alterations is a major advantage of using these indexes and can provide a practical resource for risk assessment and target risk management.

In conclusion, these findings reinforce the argument in favor of selecting biomarkers for fast screening of environmental quality and the potential risks to fish health. Although some differences were observed between feral and caged fish, the results of this study indicate that both approaches can be effectively used for diagnosing and monitoring water pollution at sites vulnerable to agricultural effluents, and that the biomarkers selected were effective for assessing the contamination gradient along a stream in an agricultural area. The results also showed that rapid effect biomarkers, such as DNA breaks and LPO, were the most sensitive biomarkers in both caged and feral fish.

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