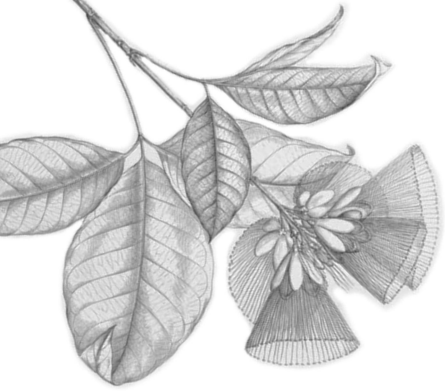

Contrasting fish biomarker responses between streams with different environmental conditions

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ABSTRACT:

Streams are environments susceptible to anthropic impacts that harm aquatic organisms by affecting their homeostasis. This study aimed to determine variations in the response of biochemical and genetic biomarkers of fish under the influence of anthropogenic impacts. Therefore, individuals of the tetra fish *Astyanax lacustris* were exposed to the water column in an impacted stream and one of its non-impacted tributaries (reference) during the dry and wet seasons. For biochemical analyzes, we evaluated the antioxidant enzymes catalase and glutathione peroxidase and lipid peroxidation in tissue samples from the gills and liver. For the genotoxicity test, we evaluated micronuclei and nuclear anomalies in blood samples. The antioxidant enzymes showed seasonal variation, regardless of the stream; lipid peroxidation did not differ between seasons or between streams. The frequency of micronuclei and nuclear abnormalities were more frequent in the impacted stream during the wet season, probably in response to the leaching of toxic compounds which tend to be increased during this period. These results support the use of genotoxicity biomarkers in biomonitoring programs.

Keywords: Ecotoxicology; Antioxidant enzymes; Genotoxicity.

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Several human activities result in the degradation of water quality in rivers, threatening the biota and negatively affecting the functioning of aquatic ecosystems (de Lima Cardoso et al. 2018). Among the factors which contribute to water degradation, pollutants from diffuse sources are particularly prevalent (Santana et al. 2018). In the context of various stressors, pollutants can affect the structure and function of biological systems, causing responses (biomarkers) at the molecular, biochemical, histological, and behavioral levels of organisms before the community level is affected (Ballesteros et al. 2017).

In addition to anthropic alterations, aquatic organisms are also exposed to natural variations in local conditions, such as changes in physico-chemical conditions across seasons (Louiz et al. 2016). In fact, seasonal changes in water condition may cause significant changes in organism's physiology and biochemical responses (Ondei et al. 2020).

Organisms exhibit a series of adjustments in biochemical, physiological, and behavioral pathways in response to stressors. These adjustments are important mechanisms which tend to maintain homeostasis and survival (Petitjean et al. 2019). These responses are important in the context of biomonitoring because they can provide early information about biological changes and show rapid responses to environmental stressors (Marques et al. 2016). For this reason, the biomarkers involved in these responses are being used to assess the quality of aquatic ecosystems (Dalzochio et al. 2019). Antioxidant enzymes are often used in biomonitoring studies since they are essential to maintain the integrity of cellular metabolism when an organism is in conditions which trigger the generation of reactive oxygen species (ROS) (van der Oost et al. 2003). The micronucleus test is also widely used for providing information about changes in genetic material due to exposure to compounds which may lead to genetic mutations, chromosomal damage, or DNA damage (Fenech 2000).

Studies on the biomonitoring of aquatic ecosystems are essential for the generation and use of information regarding the management of water resources (Buss et al. 2008). To this end, fish can be used because they present responses to xenobiotics similar to those of large vertebrates (Scalon et al. 2010) and respond to the low concentration of mutagenic agents (Mansouri et al. 2012). In addition, in fish it is possible to assess acute toxicity and stress effects while there is also a wealth of information on the life history of most fish species, fish occupy various trophic levels, have a variety of species and are at the top of the aquatic food chain (Karr 1981). Karr (1981) also suggests characteristics such as ease of identification and the fact that fish occur in practically all aquatic environments as characteristics which make fish excellent candidates for monitoring water resources.

Thus, understanding how these effects interact with anthropogenic influences is critical to improving our capability to monitor water quality in dynamic and seasonally variable ecosystems. To achieve this, a biomonitoring approach using responses from fish biomarkers, exposed in different streams and during different seasons, can demonstrate the anthropic impact on the ecosystem and how seasonality can influence responses.

In this study, we evaluated the response of biochemical biomarkers (antioxidant enzymes and lipid peroxidation) and genetoxic (micronucleus and nuclear abnormalities) of fish in streams with different levels of anthropic impacts. We therefore carried out the exposure of fish during the dry and wet seasons in cages in a reach of a stream which drains an industrial area, being subject to anthropic impacts and also in a reach of preserved (non-polluted) stream. We predicted that the activity of antioxidant enzymes, lipid peroxidation (LP), and frequency of micronuclei and nuclear anomalies (FMN and FNA) would be increased in the impacted stream during the dry season. This would occur due to the decrease in water flow during the dry season, which would affect the increase in the concentration of xenobiotics within the stream (Petrovic et al. 2011). During the wet season, this effect would be minimized by increasing the flow that would dilute the xenobiotics (Girardi et al. 2016). We used the *Astyanax lacustris* fish (Yellow-tailed tetra) as a test organism because it is native to the hydrographic basin where the study area is located (Alto Paraná, Central Brazil) and also because it is a species belonging to a genus which is sensitive to water contamination by genotoxic and mutagenic substances in the aquatic environment (Disner et al. 2017, Stevanato & Ostrensky 2018, Viana et al. 2018a, 2018b, Sposito et al. 2019).

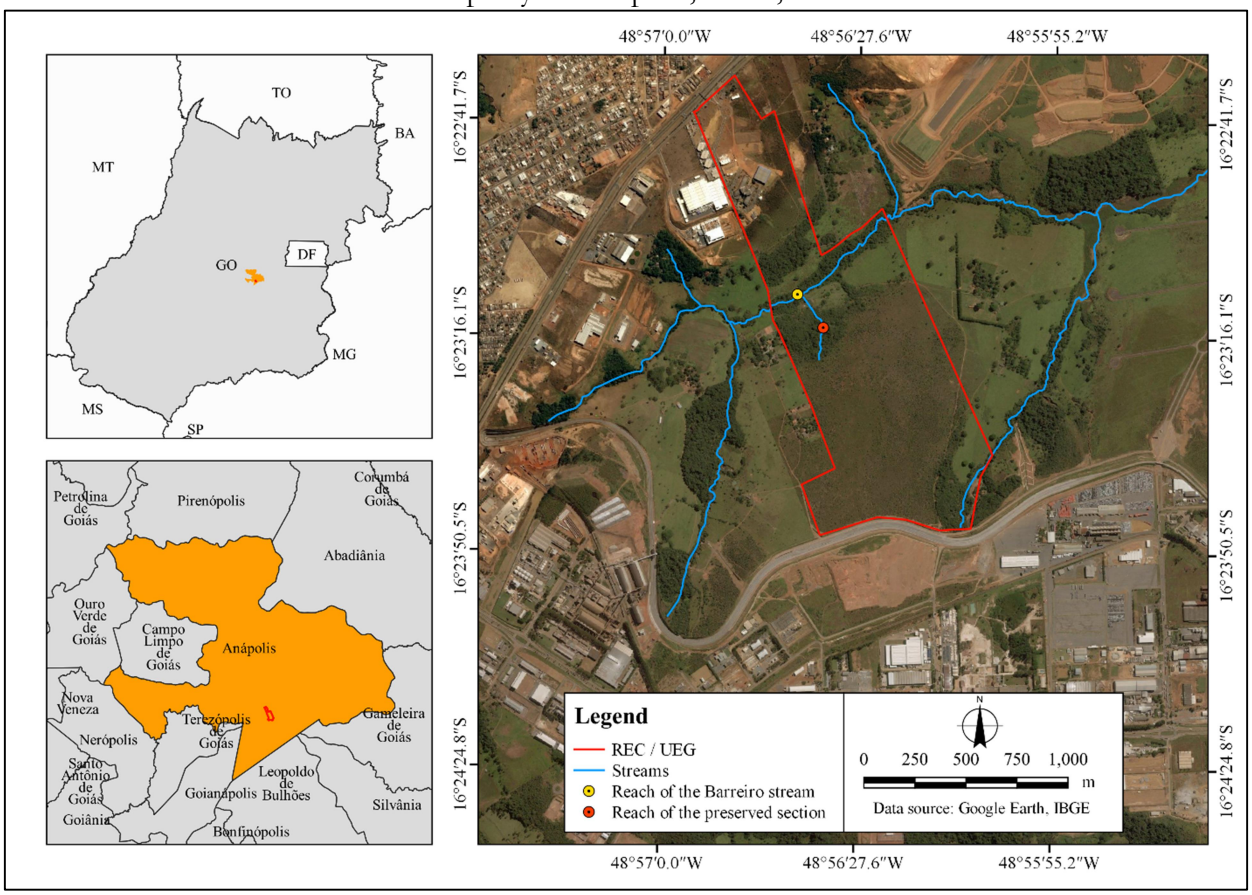
MATERIALS AND METHODS

STUDY AREA

We carried out the study in two streams located within the Ecological Reserve of the Goiás State University (REC / UEG), municipality of Anápolis, Goiás, Brazil (Figure 1). The first was a reach of the Barreiro stream, also known as the Extrema (impacted: 16°23'9.38"S 48°56' 37.72"W). The Barreiro stream is part of the Antas river basin and extends for 22.852 km. Its source springs are located within the Agro-industrial District of Anápolis (DAIA). In addition to suffering industrial effluent discharges, the stream features degraded riparian forests and intense silting, changes which result in impaired water quality, creating the potential for genotoxic damage (Bailão et al. 2020). The second stream section (reference) is a tributary of the Barreiro stream (16°23'14.65 "S 48°56'33.41" W), which emerges within a preserved area of REC / UEG. The preserved section has intact riparian forest

and is free from industrial effluent discharges, silting or leaching of contaminants from the adjacent soil. The area upstream of the preserved section also contains an area of Cerrado stricto sensu (savannah).

Figure 1. Map of the study area located in the Ecological Reserve of the Goiás State University, municipality of Anápolis, Goiás, Brazil.



Source: Prepared by the authors.

EXPERIMENTAL DESIGN

Adult individuals of *Astyanax lacustris* were acclimatized during a period of one week within the commercial establishment, specializing in live-bait and ornamental fish, from where they were originally obtained. The individuals were randomly exposed in the two streams within cages (10 fish per cage) during periods of rain and drought (March and June, respectively, of 2017) for a period of 72 hours. Five cages were maintained per stream in each season at a distance of 10 meters from each other. According to Vieira et al. (2017), the response of caged fish is similar those observed for wild fish. In addition, using caged fish makes it possible to standardize organisms, expose animals to pre-established conditions, ease of carrying out studies where native species are abundant or absent, and control of exposure time (Besse et al. 2012).

The cages were constructed from nylon, 38 cm high and 24 cm in diameter. The cages were completely submerged in marginal portions of the streams, where the speed of the water flow was reduced, without any strong current. The caged fish in the impacted and reference streams were similar in size (6.21 ± 1.15 cm in the impacted stream and 6.23 ± 4.73 cm in the reference stream; t-test, p = 0.95). Fish were not fed during the exposure period.

After the exposure period, the fish were euthanized via eugenol overdose diluted in water (200 µL / 1000 mL). We then collected the livers and gills and froze them in liquid nitrogen in the field before, in the laboratory, transferring them to a freezer maintained at -80 ºC. We collected the blood through venipuncture of the caudal vein with a syringe containing anticoagulant (heparin) and transferred the blood to a microtube. The project was approved by the Ethics Commission on the Use of Animals at the State University of Goiás, under protocol No. 004/2016.

We evaluated the environmental characteristics of the places where the cages were placed by measuring the limnological variables: dissolved oxygen (DO), pH, temperature and electrical conductivity (CE), using a multiparameter probe (YSI Professional Plus). We compared the parameters analyzed with the values established in CONAMA Resolution No. 357/2005 for Class 2 water resources.

ENZYMATIC ACTIVITY

For enzymatic analysis, we homogenized, separately, 80 mg of gills and livers in 0.2 mM Tris-HCl buffer, pH 7.5 containing 0.5 M sucrose; 1 mM EDTA; 1 mM DTT; 0.15 M KCl and 0.03 M phenylmethylsulfonyl fluoride protease inhibitor (PMSF) (1:4 mass: volume ratio) for 25 sec. The protein concentration in the samples was quantified by the method of Bradford (1976). GPx activity was analyzed in gill and liver tissues. Due to the lack of sufficient gill tissue samples, CAT was analyzed only in liver tissue.

We analyzed CAT activity by the decomposing H_2O_2 speed per decreasing absorbance at 240 to 30º C for 1 min (Beutler 1975). For the test we used a reaction medium containing 10 mM H_2O_2; 1M Tris-HCl, pH 8.0 and 5 mM EDTA, pH 8.0.

GPx's activity was analyzed per decreasing absorbance promoted by the oxidation of GSH reduced to its oxidized form (GSSG) (Sies et al. 1979). The reaction medium contained 0.1 M potassium phosphate buffer, pH 7.0; 0.005 M EDTA, pH 7.0; 0.2 mM NADPH; GR 0.2 U / ml and GSH 0.001 M. The substrate solution contained 0.52 mM NADPH; 0.1 M potassium phosphate buffer, pH 7.5 and EDTA 0.005 M, pH 7.0.

LIPID PEROXIDATION TEST

For the lipid peroxidation test, we used the product formed from malondialdehyde (MDA), the final product of lipid peroxidation, and 2-thiobarbituric acid (TBA) (Heath & Packer 1968). To accomplish this, we homogenized the tissues obtained from gills and livers in 0.1 M Tris-HCl buffer, pH 8 (1: 3 mass: volume). After homogenization, we added 150 µl of TBA to the samples, which were incubated in an acid medium at 90 ° C for 40 minutes. Subsequently, the samples were cooled and 500 µl of butanol was added. Afterwards we centrifuged the samples at 2800 RCF for 10 minutes to extract the product formed between MDA and TBA in butanol. Subsequently we pipetted 200 µl of the supernatant. We measured the levels of lipid peroxidation by reading in spectrophotometer at 535 nm. Quantification was performed by comparing with a standard curve of different concentrations of MDA obtained by hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) reacted with TBA incubated at 90 ° C for 40 minutes.

MICRONUCLEUS TEST

In the field, in order to carry out the micronucleus test and to test for other nuclear abnormalities, we pipetted 10 µl of blood from the microtube to prepare the slides, using the smear technique (blood extensions). In the laboratory, we fixed the smears in absolute methanol for 10 minutes. After 24 hours, we submitted the smears to the Feulgen reaction, which has two important steps. The first is acid hydrolysis in which the slides are placed in staining vats and immersed in 10% HCl for 11 minutes at 60 ° C. In the second stage, the slides are immersed in the Schiff's reagent (dye) for two hours in a dark place and, subsequently, washed three times. We analyzed micronuclei and performed analysis of other abnormalities using an optical microscope under the objective of immersion (1000x magnification). We analyzed two slides of a single fish from each cage. In total we analyzed 3000 erythrocytes from each fish (1500 per slide) and subsequently we calculated the frequencies of micronuclei and nuclear abnormalities.

We considered micronuclei to be a circular intracytoplasmic body with the same nuclear refrigent, not linked to it and with a smaller size. We classified nuclear abnormalities as notched, lobed, blebbed, vacuolated nuclei (Carrasco et al. 1990), binucleated nucleus and broken-eggs (Tolbert et al. 1992).

STATISTICAL ANALYSIS

To compare fish responses between streams and seasons, we used each cage as a replica (different fish tissues made up a pool). We accomplished the two-way ANOVA test complemented by Tukey's post hoc test, with streams and stations as predictor variables and antioxidant enzymes, lipid peroxidation, micronucleus and nuclear abnormalities as response variables. We tested the data for the assumptions of normality and homoscedasticity using the Shapiro-Wilk and Levene tests. Data that had the assumptions violated, even after being transformed (log), were analyzed with the ANOVA test by ranks.

RESULTS

Limnological variables varied according to streams and seasons. The electrical conductivity was higher in the impacted stream in both seasons and also showed seasonal variation in the reference stream, being higher in the wet season (interaction effect, $F_{1.16} = 21.34$, $p < 0.001$). The pH was higher in the impacted stream, but only in the dry season, with no difference between streams in the wet season (interaction effect, $F_{1.16} = 15.46$, $p < 0.01$). The temperature increased in both streams during the wet season and differed between streams in the dry season, being higher in the reference stream; there was no difference in temperature between streams in the wet season (interaction effect, $F_{1.16} = 8.18$, $p < 0.05$). Dissolved oxygen did not differ between streams and between stations ($p > 0.05$) (Table 1). DO and pH values of the streams are in accordance with the parameters established by CONAMA resolution nº 357/2005.

Table 1. Mean ± standard deviation of limnological variables of the streams evaluated.

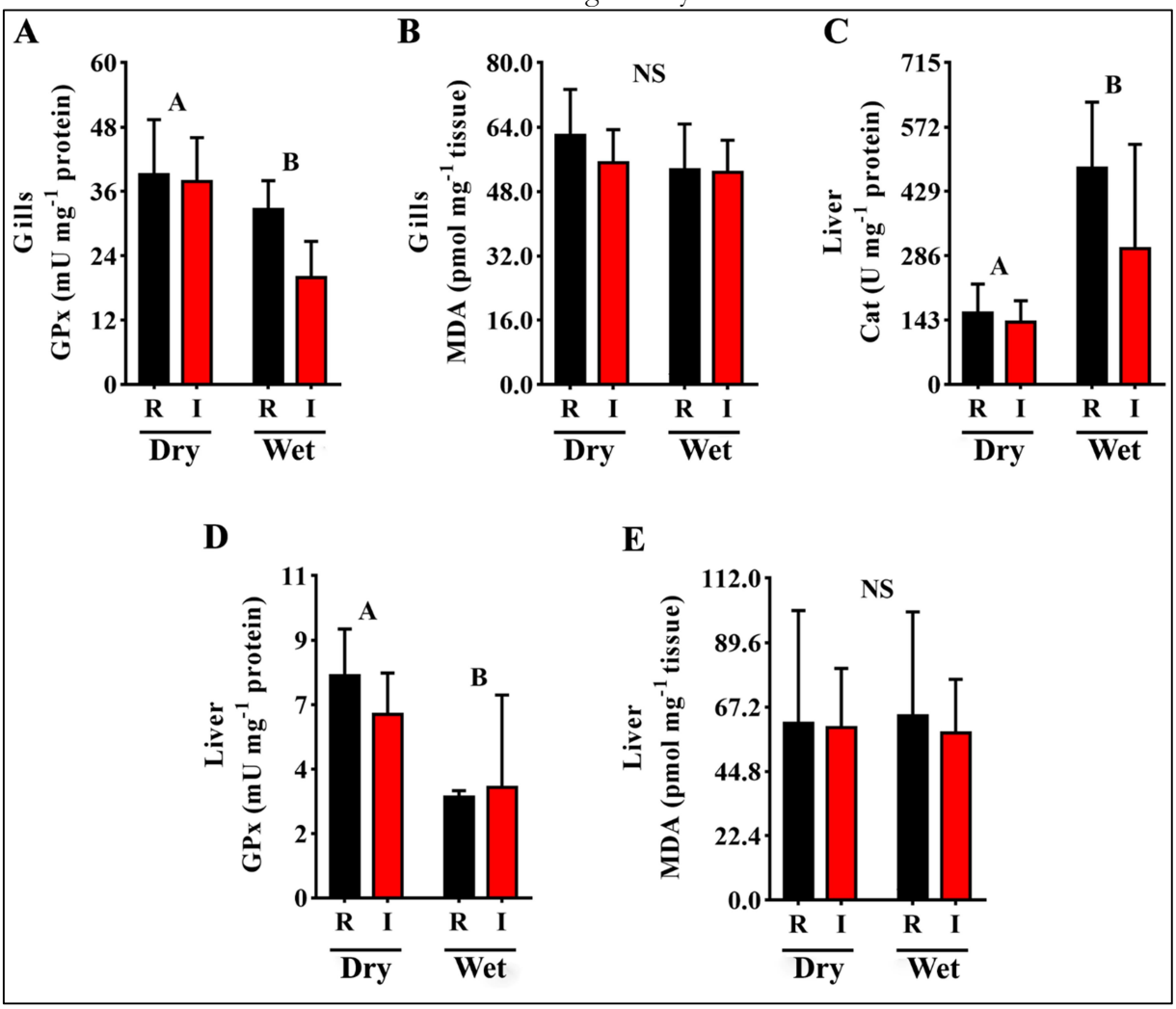
Limnological variables	CONAMA Resolution No 357/2005	Dry		Wet	
		Reference	Impacted	Reference	Impacted
Electric conductivity ($\mu S\ cm^{-1}$)	-	3.62 ± 0.94^{Aa}	40 ± 1.51^{Ab}	5.52 ± 0.18^{Ba}	38.36 ± 0.77^{Ab}
Dissolved oxygen ($mg\ L^{-1}$)	≥ 5	8.18 ± 0.75^{Aa}	7.68 ± 0.19^{Aa}	7.99 ± 0.34^{Aa}	7.65 ± 0.13^{Aa}
pH	6 a 9	7.01 ± 0.06^{Aa}	7.48 ± 0.38^{Ab}	8.02 ± 0.81^{Ba}	7.26 ± 0.12^{Aa}
Temperature (ºC)	-	21.24 ± 0.05^{Aa}	19.72 ± 0.04^{Ab}	22.36 ± 0.09^{Ba}	22.86 ± 0.88^{Ba}

Different capital letters indicate a significant difference between the dry and wet seasons for a given stream and different lower-case letters indicate a significant difference between the reference and impacted streams in a given season (Tukey's post hoc test at $p < 0.05$). The differences are shown only between the levels of the factors that had a significant effect. Source: The Authors.

GPx activity of the gills and liver were higher during the dry season in both streams (season effect, $F_{1.16} = 12.06$, $p < 0.05$; $F_{1.13} = 8.88$, $p < 0.01$, gill and liver, respectively) (Figure 2A-D). In the

liver, CAT activity was higher in the wet season (season effect, $F_{1.14}$ = 13.56) (Figure 2C). There was no seasonal or stream influence for lipid peroxidation in gills and liver (p> 0.05) (Figure 2B-E).

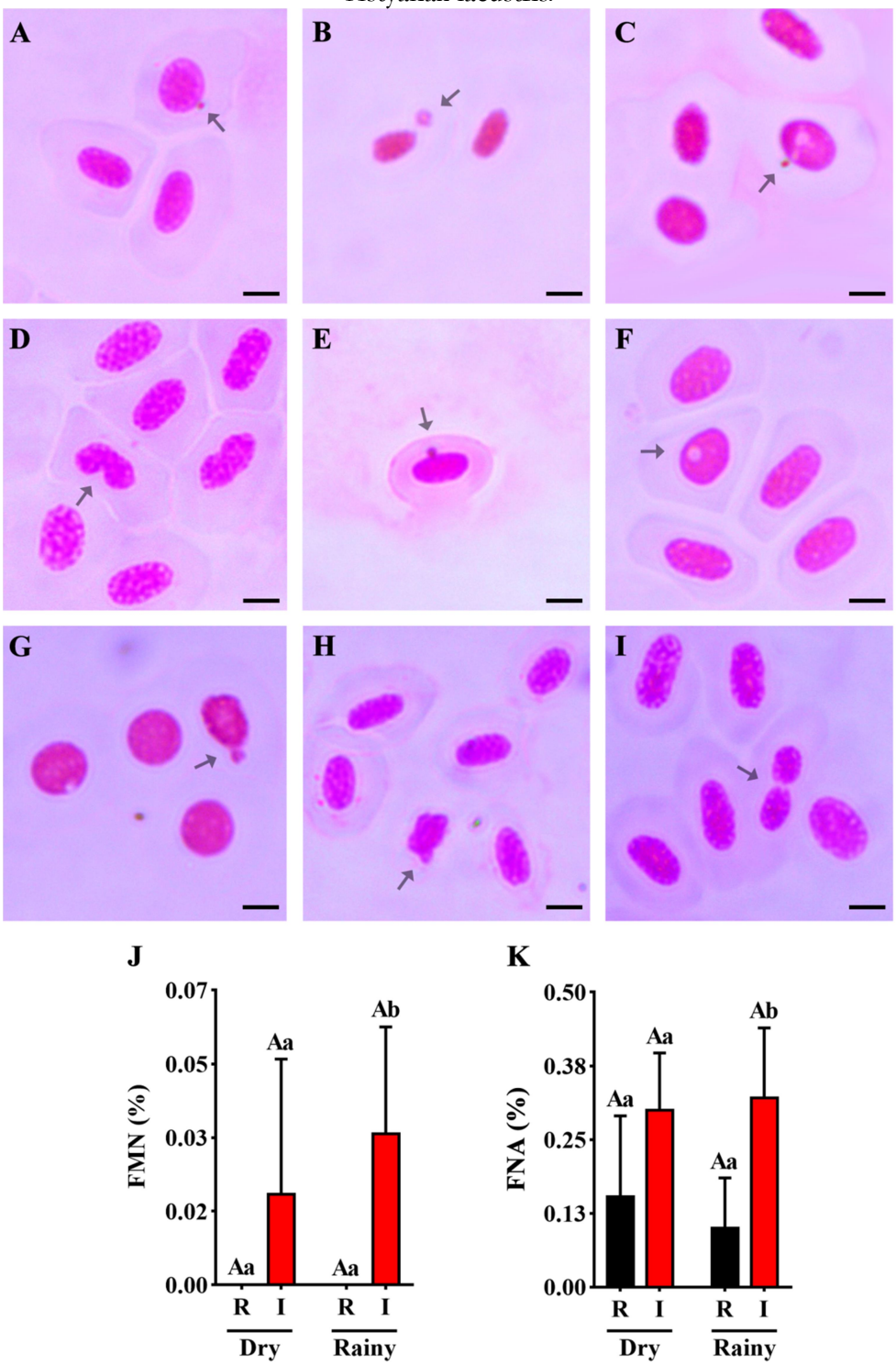
Figure 2. Enzymatic activity and lipid peroxidation of Astyanax lacustris gills and liver exposed in two different conditions during the dry and wet seasons.



R = reference - spring and I = impacted - Barreiro stream. Different capital letters indicate a significant difference between the dry and wet seasons for a given stream (Tukey's post hoc test at p <0.05). The differences are shown only between the levels of the factors that had a significant effect. Source: The Authors.

Fish exposed in the impacted stream showed differences in micronucleus frequencies and nuclear abnormalities (Figure 3A-I) during the wet season (stream effect, $F_{1.16}$ = 13.11 for FMN, $F_{1.16}$ = 13.56 for FNA, p <0.01) (Figure 3J-K). There was no seasonal or stream influence on fish mortality (p> 0.05).

Figure 3. Optical photomicrograph of micronuclei and nuclear abnormalities in erythrocytes from Astyanax lacustris.



(A-C) micronucleus; (D) notched core; (E) lobed core; (F) vacuolated; (G) broken-egg nucleus; (H) blebbed nucleus; (I) binucleated cell; in all images the magnification was 1,000x; J) Frequency of micronuclei; (K) frequency of nuclear abnormalities. Different capital letters indicate a significant difference between the dry and wet seasons for a given stream and different lower-case letters indicate a significant difference between the reference and impacted streams in a given season (Tukey's post hoc test at $p < 0.05$). The differences are shown only between the levels of the factors that had a significant effect. Scale Bar = 5 µm. Source: The Authors.

DISCUSSION

Our study is an experiment carried out in the field in order to verify the response of biochemical and genetic biomarkers of fish exposed to anthropogenic impacts compared with fish in a preserved, non-polluted environment. Our results demonstrate that antioxidant enzymes were more sensitive to seasonal changes, while micronuclei and nuclear abnormalities exhibited different results between the two environments These results show that biochemical and genetic biomarkers have different responses to temporal and spatial variations.

Although the limnological variables evaluated are within the standard parameters, it is possible to notice that the electrical conductivity in the impacted stream was greater in both dry and wet seasons. This difference in electrical conductivity is indicative of impact, since significant changes in electrical conductivity may suggest a source of contamination present in the body of water (Ghisi et al. 2017). In fact, the waters of the Barreiro stream may be contaminated by different anthropic pressures carried out along its hydrographic basin, which make its water unsuitable for human consumption (Bailão et al. 2020). The temperature variation between streams in the dry season may be due, for example, to the time of day, extent of riparian vegetation, submerged water inlet, air circulation, cloud cover, flow and depth of the stream (Fritzsons et al. 2005). The significant difference may also be due to the small variation between the measurement. Therefore, very small variations can give a significant difference in the test.

Biochemical markers showed clear seasonal variation. Various biotic factors (e.g. feeding behavior and nutritional factors) and abiotics (oxygen, temperature and the presence of xenobiotics) can influence antioxidant defenses (Martínez-Álvarez et al. 2005). The concordant responses of the antioxidant enzymes observed for both streams may be associated with temperature differences during periods of drought and rain. The dry season occurs during winter and the temperature is colder when compared to summer, which corresponds to the wet season which has warmer temperatures (Silva & Kousky 2012). As fish are ectothermic, their metabolic (Dalzochio & Gehlen 2016), physiological and behavioral processes are related to water temperature (Mininni et al. 2014). The increase in temperature is considered a natural pro-oxidant factor (Morozov & Yurchenko 2018) since oxidative stress is more evident during warming situations, due to the increase in aerobic metabolism (Mieiro et al. 2011, 2014, Feidantsis et al. 2018).

The increase in CAT activity and decrease in GPx activity during the wet season can be attributed to the similar enzymatic activity of these two enzymes (both degrade H_2O_2) that can have different effects due to the location within the cells (Weydert & Cullen 2010). CAT can be found in

peroxisomes and cytoplasm while the GPx is found in many subcellular compartments, such as mitochondria and nucleus (Ighodaro & Akinloye 2018). GPx has much greater affinity for H_2O_2 and responds to low concentrations of this molecule, while CAT has low affinity for H_2O_2 responding only to high concentrations (Halliwell & Gutteridge 2015).Therefore, the increase in temperature during the wet season may have led to the hyperthermia that caused the increase in the formation of ROS (Lushchak 2011). Although H_2O_2 is not a radical, it is considered a reactive species because it has a higher harmful activity than molecular oxygen (Lushchak 2011). The increase in the formation of ROS may also increase the expression of heat shock proteins (HSPs) that will act as sensors for redox changes that can lead to the activation of enzymes such as SOD and CAT (Madeira et al. 2013).

Lipid peroxidation did not vary according to season or flow, as shown by the results of malondialdehyde (MDA). The lack of oxidative damage can demonstrate that despite the seasonal variation of antioxidant enzymes and exposure to the impacted stream, there was no excessive production of ROS to the point of oxidizing lipids and causing the loss of biomembrane integrity (Girotti 2002, van der Oost et al. 2003).

The frequencies of micronuclei and nuclear abnormalities found in *Astyanax lacustris* erythrocytes suggest the existence of stressors in the impacted stream, especially during the wet season. The origin of these stressors may be associated with greater input of xenobiotic compounds being leached from the industrialized catchment. Similar results were found in Allium cepa treated with water from the Barreiro stream, where the genotoxic potential of the water samples was observed (Bailão et al. 2020). The authors explain the genotoxic potential as being due to the high content of iron and chromium detected in the water samples. In the literature, a considerable number of studies have also associated the frequency of micronuclei and nuclear abnormalities with exposure to genotoxic compounds present in stream water (Dalzochio et al. 2018, Lima et al. 2018, Rocha et al. 2018, Viana et al. 2018a, 2018b, Gomes et al. 2019, Sposito et al. 2019, Vieira et al. 2017, 2019). These compounds are absorbed through the gills and skin and via the intestine, and are transferred to other tissues through the blood, making blood cells the targets of their toxic effects (Oliveira et al. 2010).

CONCLUSION

Our results show that antioxidant enzymes capture seasonal changes, rather than the environmental condition of streams. This response was different from the response we expected, which was that the activity of antioxidant enzymes would be greater in the impacted stream during the dry season and that in the wet season, this effect would be minimized by increasing the flow that would

dilute the xenobiotics. Therefore, enzyme and lipid peroxidation biomarkers did not corroborate our hypothesis because they were not so sensitive to the investigated condition. The results of the analysis of micronuclei and nuclear abnormalities was also different from the response we expected as there was no seasonal response. However, in the wet season the frequency of micronuclei and nuclear abnormalities was sensitive to differences between environments. Thus, our results reinforce the potential of testing micronuclei and nuclear abnormalities as tools for biomonitoring programs, as it is a sensitive, simple, fast and inexpensive test.

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Respostas contrastantes dos biomarcadores de peixes entre riachos com diferentes condições ambientais

RESUMO:

Riachos são ambientes suscetíveis a impactos antrópicos que prejudicam os organismos aquáticos por afetarem sua homeostase. Este estudo teve como objetivo determinar variações na resposta de biomarcadores bioquímicos e genéticos de peixes sob a influência de impactos antropogênicos. Portanto, lambaris da espécie *Astyanax lacustris* foram expostos a um riacho impactado e um de seus afluentes não impactados (referência) durante as estações seca e chuvosa. Para as análises bioquímicas, avaliamos as enzimas antioxidantes catalase e glutationa peroxidase e peroxidação lipídica em amostras de tecido de brânquias e fígado. Para o teste de genotoxicidade, avaliamos micronúcleos e anomalias nucleares em amostras de sangue. As enzimas antioxidantes apresentaram variação sazonal, independente do riacho; a peroxidação lipídica não diferiu entre as estações ou entre os riachos. A frequência de micronúcleos e anormalidades nucleares foi mais frequentes no riacho impactado durante a estação chuvosa, provavelmente em resposta à lixiviação de compostos tóxicos que tende a ser aumentada nessa estação. Esses resultados apoiam o uso de biomarcadores de genotoxicidade em programas de biomonitoramento.

Palavras-chave: Ecotoxicologia; Enzimas antioxidantes; Genotoxicidade.

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