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ZOOLOGY

Comparison of nuclear abnormalities in *Astyanax bifasciatus* Cuvier, 1819 (Teleostei: Characidae) of two sections of rivers from the middle Iguaçu

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ABSTRACT. Most changes in water bodies are result of human activities that have the potential to undermine the environmental integrity of aquatic ecosystems. Changes in genetic material can be evidenced by the frequency of nuclear abnormalities in fish blood cells, in response to genotoxic agents even at low concentrations. Thus, we aimed at comparing the frequencies of nuclear abnormalities of fish kept in acclimation, with fish collected in Timbó river (Santa Cruz do Timbó, Santa Catarina State), under low anthropogenic interference, and fish collected in Iguaçu river (União da Vitória, Paraná State), a polluted river. The highest frequency of changes in nuclear morphology of fish erythrocytes was found in the urbanized area around the Iguaçu river, while, the fish collected in areas with preserved riparian forest surrounding the Timbó river and acclimated fish showed no morphological changes. Our results suggest that genotoxic compounds in the Iguaçu river are acting as stressors to aquatic communities, especially fish. In addition, the absence of nuclear abnormalities in fish from the Timbó river suggests that rivers without significant environmental changes can serve as reference point for comparative studies of genetic modifications for the species studied.

Keywords: micronucleus; ecotoxicology; mutagenesis; pollution.

Comparação de anormalidades nucleares em *Astyanax bifasciatus* Cuvier, 1819 (*Teleostei*: *Characidae*) de dois trechos de rios do médio Iguaçu

RESUMO. A maioria das alterações dos corpos hídricos é resultante das atividades antrópicas que são prejudiciais à integridade ambiental dos ecossistemas aquáticos. Alterações no material genético podem ser evidenciadas pela frequência de anormalidades nucleares em peixes, em resposta a agentes genotóxicos, mesmo em baixas concentrações. Dessa forma, o estudo consistiu em comparar as frequências de anormalidades nucleares de peixes mantidos em aclimatação, com peixes coletados no rio Timbó (Santa Cruz do Timbó, Estado de Santa Catarina), local com pouca interferência antropogênica e, peixes coletados no rio Iguaçu (União da Vitória, Estado do Paraná), um rio poluído. As maiores frequências de alterações na morfologia nuclear dos eritrócitos dos peixes foram visualizadas na área urbanizada ao redor do rio Iguaçu, quando comparados com os peixes coletados em área com mata ciliar preservada, no rio Timbó, e os peixes aclimatados, que não apresentaram alterações morfológicas. Esses resultados sugerem que há compostos genotóxicos no trecho médio do rio Iguaçu que são estressores para os peixes. Além disso, a não observação de anormalidades nucleares no rio Timbó sugere que rios sem alterações ambientais podem servir como ponto de referência para estudos comparativos de modificações genéticas para a espécie estudada.

Palavras-chave: micronúcleo; ecotoxicologia; mutagênese; poluição.

Introduction

Like any aquatic environment, the basin of the Iguaçu river is exposed to pollution processes caused by the huge variety and quantity of chemicals (Livingstone, 1998), and presents pollution evidence from industrial waste from the manufacture of paper, fertilizers and waste from agricultural practice (Bueno-Krawczyk et al., 2015). These toxic substances released into the environment may interact with biota, causing changes (Arias et al., 2007) that can have a genotoxic potential and/or mutagenic and clastogenic effects (Zenkner, Soares, Prá, Köhler, & Rieger, 2011), favoring the formation of micronucleus and nuclear abnormalities as they can be absorbed and stored in the animal body (Benites, Doncato, Minho, & Perazzo, 2014).

Different types of nuclear lesions have been described and classified as “Blebbed”, “Notched”, “Lobed”, “Vacuolated” and “Micronucleus” (Carrasco, Tilbury, & Myers, 1990). These abnormalities are used as indicators of genotoxic damage (Pachecco & Santos, 1998; Aylon & Garcia-Vazquez, 2000).

Therefore, environmental stress can be detected early using bio-indicators (Brito & Luz, 2015), among which fish species are recommended (Cotelle & Ferrard, 1999) since they play an important ecological role in the food chains, transferring energy to other trophic levels (Cort & Ghisi, 2014). Moreover, these organisms have genetic structure similar to mammals (Barbazuk et al., 2000; Lieschke & Currie, 2007) responding similarly to environmental contamination.

Species of the genus *Astyanax* have been used as biological models for different studies because they have dynamic life cycles with a high reproductive potential (Zenkner et al., 2011), having capacity of absorbing quickly compounds which are directly added into the water and accumulate them in different tissues (Silva, 2014), as well as endemic species to the Iguaçu river basin, showing thus high abundance (Garavello & Sampaio, 2010). Studies using this genus as bioindicators in the environmental integrity analysis have been widely spread (Zenkner et al., 2011).

A tool used for genotoxicity check on a species is the piscine micronucleus (Hooftman & Raat, 1982). Micronuclei are whole chromosomes or partials that are not incorporated into the nucleus of the daughter cell during cell division and which appear as a small round dark structure, identical in appearance to the cell nucleus (Al-Sabti & Metcalfe, 1995); they may reflect exposure to agents with clastogenic (chromosome breakage, when DNA is the target of the chemical agent) or aneugenic (effect on the number of chromosomes in most cases, the chemical agent does not target the DNA) action mode (Albertini et al., 2000).

Studies correlating the alterations in fish health due to water pollution in the Iguaçu river basin are incipient and the existing ones demonstrate the presence of drugs, such as naproxen, salicylic acid, acetylsalicylic acid (Ide, Osawa, Marcante, Pereira, & Azevedo, 2017), phosphorus (Sodré, Schnitzler, Scheffer, & Grassi, 2012) and caffeine in the Middle Iguaçu region (Bueno-Krawczyk et al., 2015), commonly used as indicator of anthropic activity in natural environments, related to the discharge of untreated domestic sewage (Seiler, Zaugg, Thomas, & Howcroft, 1999).

Therefore, in order to verify the response pattern generated by the analysis of micronucleus in fish erythrocytes and the response of this biomarker to water pollution, we compared the frequencies of nuclear abnormalities in fish of the species *Astyanax bifasciatus* collected in the Timbó river with fish collected in the Iguaçu river, and fish kept in acclimation for 60 days.

Material and methods

Study area and fish sampling

We defined two sampling sites. In the Iguaçu river (26°13'41.7"S and 051°05'59.6"W), fish (n=15) (IBAMA license - SISBIO 40229-1) were collected in a point of the river localized in União da Vitória, Paraná State, under strong anthropogenic interference, with many surrounding houses and absence of riparian vegetation (Figure 1 - P1). In the Timbó river (26º25’45”S e 50º 50’ 46.1” W), fish were collected in a point of the river localized in Santa Cruz do Timbó, Santa Catarina State, under little anthropic interference and preserved riparian vegetation (Figure 1 - P2). In this sampling, 20 fish were collected (IBAMA license - SISBIO 42950-1), of which 10 were analyzed soon after collection and, the remainder (n = 10) were transported to the Pisciculture of the *Universidade Estadual do Paraná* (UNESPAR), where they remained in acclimation conditions with filtered water, available oxygen and daily feeding for 60 days.

Water chemical analysis

For metal analysis, we collected surface water of the Iguaçu river and stored in 500 mL polyethylene bottles. The sample was analyzed, after centrifuging, by inductively coupled plasma optical emission (ICP OES) for large and selected oligoelements. The spectrophotometry was done by atomic absorption according to American Public Health Association (APHA, 2012). This analysis of heavy metals was performed only for the sample from the Iguaçu river due to its known history of pollution (*Instituto Brasileiro de Geografia e Estatística* [IBGE], 2012).

Samples

In laboratory, fish were anesthetized with clove oil diluted in water (1.5 μL L^{-1}). For the piscine micronucleus test, blood samples were obtained by caudal puncture with heparinized syringes. For each specimen, blood was drawn and one thin-layer slide was prepared using a drop of blood. Blood smears were air-dried for 24 hours, fixed in absolute ethanol for 10 min., and then stained with 10% Giemsa solution for 10 min. Afterwards, they were washed with running water. A total of 2000 cells per slide were analyzed at 1000× magnification on an optical microscope. The Micronucleus test was performed according to Hooftman and Raat (1982). For the analysis of erythrocyte nuclear abnormalities (ENA), we used the classifications proposed by Carrasco et al. (1990).

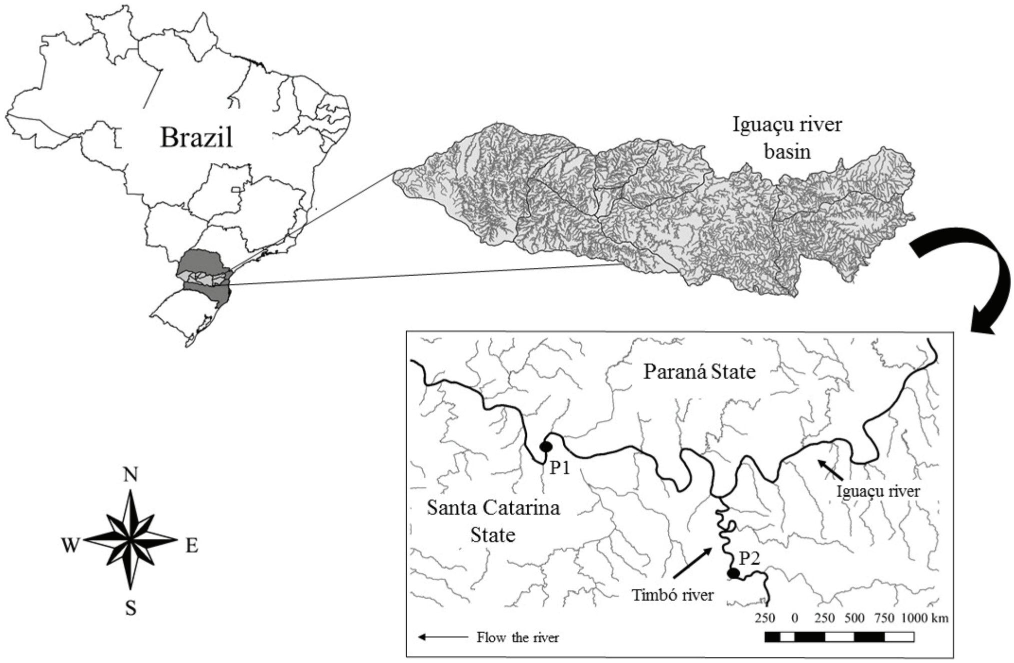

Figure 1. Geographical localization of sampling sites: Iguaçu river, Paraná (P1) and Timbó river, Santa Catarina (P2).

Data analysis

Since our data were independent samples and did not reach normality and homoscedasticity assumptions for analysis of variance, we used the Kruskal-Wallis non-parametric equivalent, followed by Dunn's post-hoc test, to identify significant differences between the analyzed rivers and the acclimation group. We used Vegan (Oksanen et al., 2016) and the Dunn Test (Dinno & Dinno, 2017) statistical packages in software R (Core Team 2018). Statistical significance was accepted at $p < 0.05$.

Results

We did not observe the occurrence of cell and micronucleus abnormalities in the fish collected in the Timbó river and the fish left in acclimation. In Iguaçu river, fish showed high frequencies of nuclear abnormalities and frequency (1.15%) of micronucleus (0.35%).

In addition to micronucleus, we recorded three types of alterations in the nuclear morphology of blood cells of *Astyanax bifasciatus*: Lobed, Blebbed and Notched (Figure 2).

When we compared the nuclear abnormalities between the fish collected in the Iguaçu river, Timbó and in acclimation (Table 1), we observed that the fish of the Iguaçu river presented significant differences in the frequency of nuclear abnormalities for Notched, Blebbed and micronucleus when compared with the Timbó river and Acclimation group, whereas for Lobed, no significant differences were detected between these groups (Table 2 and Figure 3).

Table 1. Kruskal-Wallis test for differences in the frequency of Lobed, Blebbed, Notched and Micronucleus between Iguaçu river, Timbó river and acclimation group. Bold p-values indicate significance at $p < 0.05$ (df = degrees of freedom; X^2=Chi-Squared).

Kruskal –Wallis test	df	X^2	p-value
Lobed	2	5.833	0.054
Blebbed	2	7.504	**0.023**
Notched	2	9.302	**0.009**
Micronucleus	2	7.512	**0.009**

Table 2. Dunn's post-hoc test for differences in the frequency of Blebbed, Notched and Micronucleus between Iguaçu river, Timbó river and acclimation group. Bold p-values indicate significance at $p < 0.05$.

Dunn's test	p-value
Blebbled	
Iguaçu x Timbó	**0.032**
Iguaçu x Acclimation	**0.032**
Timbó x Acclimation	1.000
Notched	
Iguaçu x Timbó	**0.016**
Iguaçu x Acclimation	**0.016**
Timbó x Acclimation	1.000
Micronucleus	
Iguaçu x Timbó	**0.032**
Iguaçu x Acclimation	**0.032**
Timbó x Acclimation	1.000

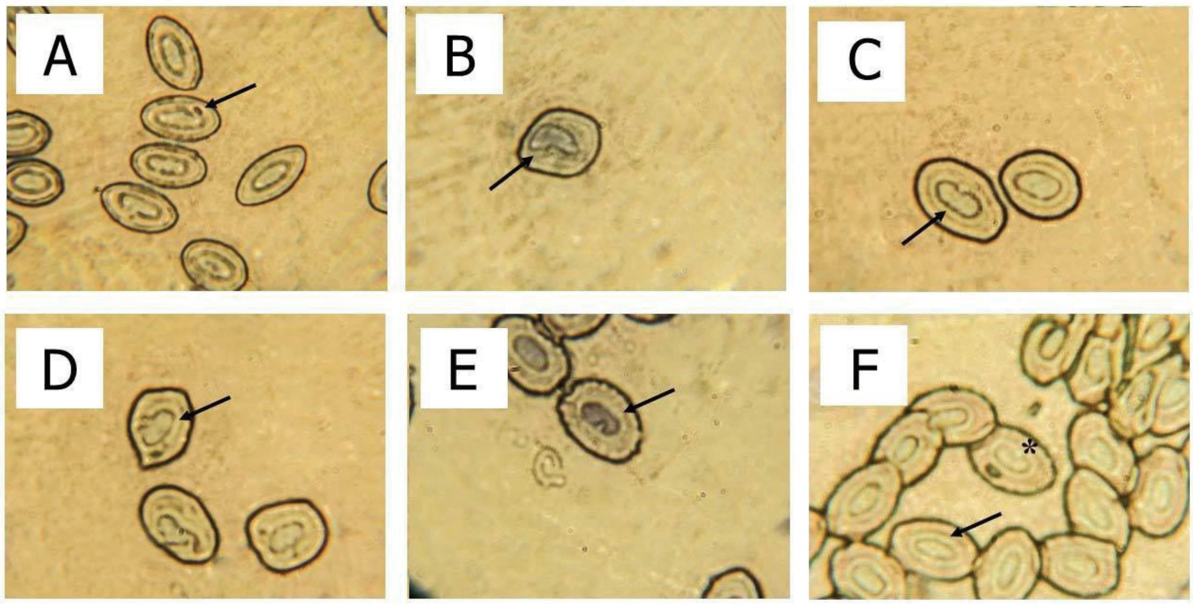

Figure 2. Micronucleus and nuclear abnormalities (stained with Giemsa) found in erythrocytes of *Astyanax bifasciatus* from Iguaçu river. (A) Micronucleus (40X); (B and C) Lobed (40X); (D) Blebbed (40X) (E) Notched (40X) (F) normal cell (arrow) (40X) and Notched (*).

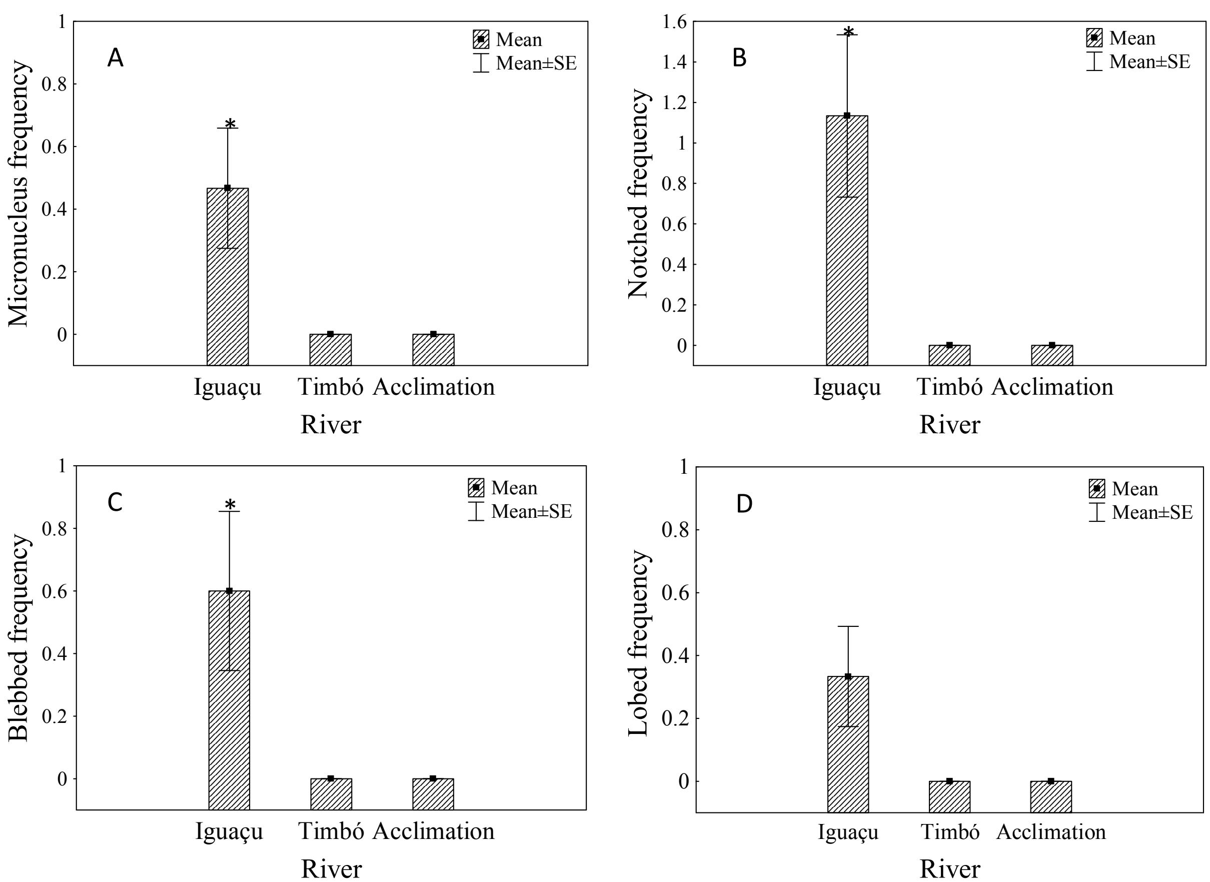

Figure 3. Frequency of micronucleus nuclear abnormalities in *Astyanax bifasciatus* from Iguaçu river, Timbó river and in acclimation. * Significant difference ($p < 0.05$). (A) Micronucleus; (B) Notched; (C) Blebbed; (D) Lobed.

Of the metals tested in the Iguaçu river, the concentrations of aluminum (0.3 mg L^{-1}) and of lead (0.02 mg L^{-1}) exceeded the maximum value (0.1 and 0.01 mg L^{-1} respectively) allowed by *Conselho Nacional do Meio Ambiente* (CONAMA, 2005) resolution 357/05 for class 3 waters (Table 3). Other metals did not show values above the established by the Resolution.

Table 3. Values found in the characterization of heavy metals in the sampling site in the Iguaçu river. Bold numbers represent the values above the acceptable.

Analyzed metals	Maximum values allowed (mg L^{-1})	Values found in the Iguaçu river (mg L^{-1})
Aluminum	0.1	**0.369**
Barium	0.7	0.030
Cadmium	0.001	-0.002
Cobalt	0.05	0.035
Chrome	0.05	0.137
Copper	0.009	0.002
Iron	0.3	-0.042
Magnesium	0.1	-0.000
Manganese	0.1	-0.000
Nickel	0.025	-0.005
Lead	0.01	**0.028**
Vanadium	0.1	0.050
Zinc	0.18	-0.002

Discussion

Fish of the Timbó river showed no cell nucleus morphological change, which may be due to the riparian forest of their surroundings that has important role in preservation of aquatic ecosystems, since it retains residues provided by leaching, controls fluvial dynamics besides absorbing pollutants reducing the ecotoxicological effects on aquatic biota, including fish (Peterjohn & Correl, 1984; Tabacchi et al., 1998; Lobón-Cerviá, Hess, Melack, & Araújo-Lima, 2015).

The opposite was observed in the Iguacu river, where fish collected presented high frequencies of nuclear abnormalities, including the occurrence of micronucleus. This may be due to the absence of riparian vegetation and the high degree of human disturbance, mining, dumping of waste from the pulp industry and disposal of waste from sewage treatment. These abnormalities and the presence of micronucleus may be related to a chronic exposure of the water ecosystem, since these activities generally cause degradation of water resources, affect the aquatic biota in addition to altering the physical and chemical characteristics of the water (Helena, Vega, Barrado, Pardo, & Fernández, 1999) causing impacts on the water body (Silva & Nepomuceno, 2010; Ribeiro et al., 2013; Cort & Ghisi, 2014). The effects of pollution cause impacts on the water body (Silva & Nepomuceno, 2010; Ribeiro et al., 2013; Cort & Ghisi, 2014), which may affect the structure of fish populations (Bifi, Baumgartner, Baumgartner, Frana, & Debona, 2006) and, cause long-term, genetic changes in species.

Hemachandra and Pathiratne (2016) warn of the effects of industrialization on the ecological integrity of aquatic ecosystems. Indeed, the Iguaçu river receives large discharges of domestic and industrial effluents without treatment and, some authors observed an increase in the number of nuclear alterations of the Lobed and Blebbed types and micronucleus formation in fish exposed to concentrations of industrial effluents from textile factories (Çavas & Ergene-Gozukara, 2003). Besides that, exposure to drugs can cause negative effects on aquatic organisms (Ribas, Zampronio, & Assis, 2015), even at low concentrations (Guiloski, Ribas, Pereira, Perbiche, & Assis, 2015). For example, Ragugnetti et al. (2011) observed that the frequency of micronucleus for the species *Oreochromis niloticus* increased proportionally with time of exposure to ibuprofen.

Furthermore, in our study, the presence of aluminum three times above that allowed by Brazilian legislation, found in the Iguaçu river, is worrisome since this metal causes toxic effects to biota, especially when exposure occurs over a prolonged period (Gensemer & Playle, 1999). Some studies have shown the aluminum ability to promote the formation of micronucleus and nuclear morphological changes in fish. García-Medina et al. (2013), when determining the cytotoxic and genotoxic effects of common carp (*Cyprinus carpio*) erythrocyte exposure to aluminum, found that the aluminum concentrations tested produced higher oxidative stress and induced higher frequencies of micronucleus in the species. Likewise, Galindo, Troilo, Cólus, Martinez, and Sofia, (2010), observed the formation of nuclear abnormalities in erythrocytes of *Prochilodus lineatus*, when exposed to different concentrations of aluminum. Similarly, lead also causes changes once it is a quite toxic metal (Türkmen, Türkmen, Tepe, Töre, & Ates, 2008) capable of altering vital fish structures, causing edema and cerebral hemorrhages (Adeyemo, 2008), hepatic and hematological diseases in the organisms (Ates, Orun, Talas, Durmaz, & Yilmaz, 2008), and morphologically alter the cellular nucleus of fish, as reported by Ferraro, Fenocchio, Mantovani, Ribeiro, and Cestari (2004). Moreover, fish are bio-concentrator organisms and many contaminants, even that at low concentrations, can affect their physiology and survivability (Grisolia et al., 2009).

We verified that *Astyanax bifasciatus* is sensitive to pollution. The same species was used as a chemical and genetic bioindicator in the middle section of the Iguaçu river by Bueno-Krawczyk et al. (2015), who observed changes in fish health conditions probably related to the presence of heavy metals and caffeine in the environment. The Astyanax genus has been used as a bioindicator because of its sensitivity to anthropogenic actions, such as agricultural conditions and wastewater, presenting cellular stress (Costa-Silva et al., 2015; Matozo, Turek, & Noleto, 2015) and higher frequencies of nuclear abnormalities, including the micronucleus formation (Dalzochio et al., 2018).

Conclusion

We concluded that Iguaçu river contains contaminants that cause genotoxicity effects in fish. Fish exposed to water of this river showed nuclear abnormalities and micronuclei in the blood cells while fish from Timbó river and in the acclimation group did not. The results emphasized the need for discussion about Iguaçu river conservation and regulation of the inputs of contaminants into water bodies. Timbó river is a water body without significant environmental changes and can serve as reference point for comparative studies of genetic modifications for the studied species.

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