Chapter 7

Genotoxic Biomarkers in Fishes of the Chapada Das Mesas National Park, Brazil

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Abstract

Genotoxic and hematological parameters in *Hypostomus pusarum* and *Mylossoma duriventre* were used as biomarkers to assess the exposure to environmental stressors within the Chapada das Mesas National Park (PNCM). Fishes were sampled at two sites in PNCM: São Romão and Prata Waterfall. Biometric data (length and weight) were recorded, and blood was collected from all fishes for analysis. The abiotic variables were measured in each region: pH, temperature and dissolved oxygen. A drop of blood from each fish was placed on two microscope slides and smeared. The slides were left to dry at room temperature for 24 h and then fixed in absolute ethanol for 30 min. Means and standard deviations (SDs) of the biometric data of *H. pusarum* showed length and total weight bigger than *M. duriventre*. Nuclear morphological changes (NMAs) were identified in the two sampled species for the two collection points. Among the NMAs found, binucleated nucleus (BN), vacuolated nucleus (VC) and micronucleus (MN) were also found in both species; however, in *M. duriventre*, the frequency of MN and NMA was higher than *H. pusarum*. The presented data show that methodologies based on biomarkers will be used in the future park management programs.

Keywords: biomonitoring, freshwater fishes, protected area, cascudo (*Hypostomus pusarum*), pacú (*Mylossoma duriventre*)

1. Introduction

According to Ramelow et al. [1] and Schulz et al. [2], fishes are excellent tools to the aquatic environment biomonitoring because they are used for assessing a lot of environmental disturbing factors such as changes in the rate of growth and sexual maturation. Besides, changes in fish community structure, such as species abundance and diversity, may also reflect the effects of various stressors on the biotic integrity of a river [3].

In this way, erythrocytes of fish have been shown to be a safe tool for the micronucleus test [4, 5]. The micronucleus test is considered an advantageous technique whose analysis is relatively simple. In addition, the simplicity and speed of obtaining peripheral fish blood make the technique even more suitable for the evaluation of environmental contamination [6].

In this context, the selection of species that can reflect the environmental situation of PNCM becomes of great relevance to monitor the interferences that this Conservation Unit has been suffering over the years. Fishes are excellent bioindicators because they are at the top of the trophic chain and reflect the impacts in a given ecosystem through their normal and/or organic composition in the medium or long term [7].

The development and standardization of methodologies capable of predicting the effects of contamination on aquatic organisms are extremely relevant for biomonitoring studies in a Conservation Unit. Among these methodologies, the use of biomarkers of aquatic contamination in fish is particularly important because it shows initial biological responses and may be useful to subsidize monitoring and environmental management actions [8]. Biomarkers are biological responses to stress caused by pollutants and/or physical stressors and can be used to identify early signs of damage to aquatic organisms [9].

Research indicates that when aquatic ecosystems are polluted with organic and inorganic contaminants, fish will inevitably be contaminated [10, 11]. The possible effects of such contaminants on fish can be assessed by using several types of biomarkers, which are defined as the biological responses, as well as the effects caused by the pollutants and which identify signs of initial damage in organisms [12]. Livingstone [13] considers as biomarkers the bodily fluids, cells or tissues, as well as the responses of the exposed organisms, in physiological as well as behavioral or energetic levels, being, therefore, molecular biomarkers, cellular or organisms, being some of them are specific pollutants.

The genetic material of eukaryotic cells of fish species can also be altered by exposure to dissolved chemicals in the water, resulting in the formation of micronuclei, which can be used as biomarkers to assess the degree of contamination in the environment [14]. The micronuclei are derived from chromosomal fragments resulting from breaks that are not incorporated into the main nucleus of the daughter cells after mitosis due to damage introduced into the parental cells [15]. Micronuclei tests have not yet been performed in PNCM and in the water ecosystems of southern Maranhão. Thus, these data may serve to obtain more complete clues of contaminants that may be inducing.

In this way, recognizing the need to provide the sustainable use of natural resources and the environmental quality of the PNCM and to the local communities, as well as the need to know the effect of the possible impacts on the fish of the region, the aim was to contribute with the scientific knowledge related to biomarkers genotoxic in two species of sweet fish (*Hypostomus pusarum* and *Mylossoma duriventre*) in order to subsidize biomonitoring and management programs in the protected areas.

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2. Methodology

2.1. License and statement of ethics committee

Fish collection was done through the research authorization of the Chico Mendes Institute for Biodiversity Conservation—ICMBio (SISBIO, 55361/2017). The protocol of the ethics committee was approved by the State University of Maranhão (13/2017 CRMV-MA) through the Ethics and Animal Experimentation Commission (CEEA).

2.2. Study area

The Chapada das Mesas National Park (PNCM) (**Figure 1**) is a protected area, which is located in the South of Maranhão, between the following cities: Riachão, Estreito and Carolina [16].

The PNCM climate is tropical: humid and hot, characterized by having two defined seasons, one being dry and the other rainy. The rainy season is in the period from November to March, with rainfall concentrated in February [17]. This region contains an extensive and rich hydrographic network with approximately 400 springs and the main water courses that supply the city of Carolina. In addition, the PNCM protects numerous watercourses and springs from several rivers, such as the Farinha River (with numerous waterfalls), Itapecuru, Urupuchete, Corrente

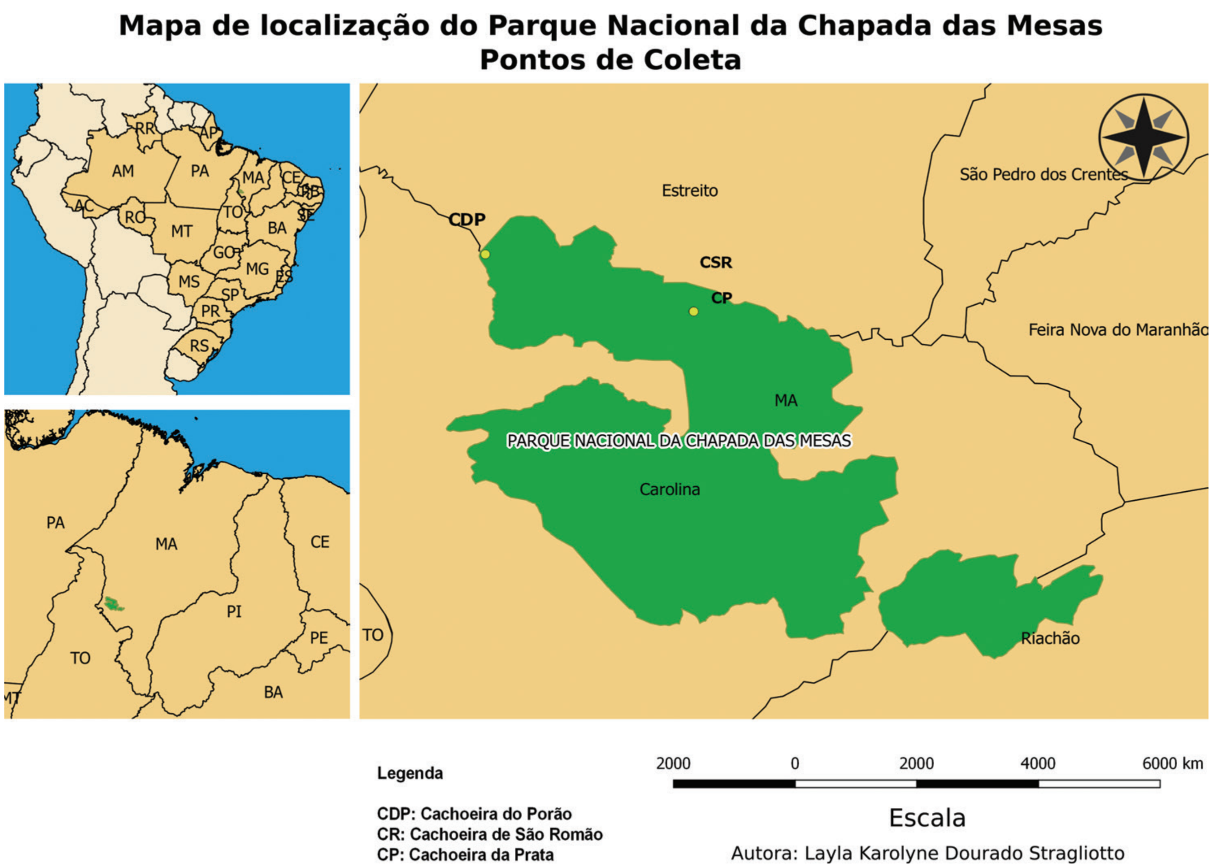

Figure 1. Map of the Chapada das Mesas National Park, Brazil showing the sampling sites. CRS = São Romão Waterfall; CP = Prata Waterfall.

and Lajinha. The hydrographic basin of the Farinha River is one of the main tributaries of the Tocantins river basin, being the most explored from the ecotourism and local point of view.

2.3. Sampling sites and fishes in the PNCM

In total, 32 fishes were sampled in PNCM: [1] São Romão Waterfall (n = 12) and [2] Prata Waterfall (n = 20). The stations were georeferenced by Global Positioning System (GPS). The fishes were collected in the rainy period (March 2017) and in the dry period (June 2017) with fixed nets 22 in the upstream and downstream of the waterfalls. The genera selected for analysis of the biomarkers were *H. pusarum* and *M. duriventre*. The selection of species is related to their habit and their frequency throughout the years in PNCM rivers and waterfalls.

2.4. Environmental parameters

Physicochemical parameters—temperature, pH and dissolved oxygen—were measured at each site during the dry and rainy season when fishes were sampled. The parameters were analyzed using the ASKO multiparameter.

2.5. Micronuclei, morphological nuclear abnormalities and biometric data

Specimens of *H. pusarum* and *M. duriventre* were sampled, transferred to a plastic vat with water and then anesthetized for 5 min in clove solution. Blood was collected from the gills of individual *H. pusarum* and *M. duriventre* (n = 32 from the two sampling sites) using heparinized syringes. A drop of blood from each fish was placed on two microscope slides and smeared. The slides were left to dry at room temperature for 24 h and then fixed in absolute ethanol for 30 min. One set of slides (n = 32) was stained with 10% Giemsa diluted in phosphate buffer (pH 6.8) and analyzed using a light microscope. A total of 2000 cells per slide were analyzed. Micronuclei and morphological nuclear abnormalities in the erythrocytes were deemed indicative of genotoxicity [18].

For each fish specimen, biometric data—total length (TL), fork length (FL), standard length (SL) and total weight (TW)—were recorded.

2.6. Statistical analysis of data

The obtained data were submitted to the normality test, and the obtained results were compared by Student's t-test. For the differences in location between the means obtained for the two collection sites and the biometric data, the multiple comparison test ($P < 0.05$) was used.

3. Results and discussion

3.1. Environmental parameters

The mean values of the abiotic variables recorded in the PNCM throughout the sampled period (rainy and dry season) were measured and are shown in **Table 1**. Temperature and pH remained practically constant in both areas during rainy and dry periods.

Parameters.	São Romão Waterfall	Prata Waterfall	Recommended values
Dissolved O_2 (ppm)[a]	11	12.5	>5 mg/L[b]
pH[a]	7.32	7.45	6.5–8.0[b]
Temperature[a] (°C)	28.0	28.5	28–32°C[b]

[a]Mean value during the dry and rainy seasons.
[b]Resolution No. 357, CONAMA (Brazilian Legislation) 15 March 2005.

Table 1. Environmental parameters analyzed at each sampling location in Chapada das Mesas National Park, Brazil.

These data indicate that all the abiotic factors of PNCM waterfalls are within the values accepted by the National Environmental Council [19]. CONAMA Resolution No. of 17 May 2011, which complements and amends Resolution No. 357/2005 of 17 March 2005, presents specific values that classify freshwater bodies (lentic and lotic) and shows that below recommended levels, these values may cause adaptive changes in the morphology of erythrocytes of bioindicator species (such as fish) and, consequently, a decrease in hematocrit values [16, 20].

3.2. Biometric data

3.2.1. *M. duriventre*

The results from statistical analysis of the *M. duriventre* biometric in São Romão and Prata Waterfall from PCNM can be observed in **Table 2**.

The biometric data submitted to the normality test for *M. duriventre* indicated that there is not a significant difference between the treatments for the São Romão and Prata Waterfall. According to Pinheiro-Sousa [8], the statistical difference can be related to the environmental conditions of the available resources in two distinct points of a protected area.

In addition, the biometrics data were higher for fish in the dry period in the São Romão Waterfall and in the rainy period for Cachoeira da Prata. This difference between the size

Parameter (mean ± SD)	Means ± Standard deviations (SD)			
	São Romão Waterfall		Prata Waterfall	
	Rainy season	Dry season	Rainy season	Dry season
TL (cm)	7.63 ± 1.02	14.06 ± 5.25	16.5 ± 4.02	13.1 ± 4.62
FL (cm)	6.9 ± 1.08	13.14 ± 5.17	12.25 ± 5.58	13.12 ± 4.78
SL (cm)	6.16 ± 0.90	11.4 ± 4.67	14.2 ± 3.46	11.16 ± 4.01
TW (g)	6.66 ± 4.61	36.4 ± 37.40	93.66 ± 58.73	61.6 ± 59.21

Total number of species sampled = 19; number of species in São Romão Waterfall = 8 and number of species in Prata Waterfall = 11. Biometric data: TL (total length); FL (fork length); SL (standard length) and TW (total weight).

Table 2. Biometric data of *M. duriventre* sampled in the São Romão and Prata Waterfall, Chapada das Mesas National Park, Brazil.

of the individuals shows that the reproductive and growth behavior of the pacú is different for the two areas sampled. Individuals of *M. duriventre* have diurnal habits, are migratory, and in the ebb form schools and migrate upstream to spawn in the confluences of rivers and waterfalls in the reproductive periods. The reproduction is long, covering drought (November) and flood (May), being the most intense spawning between December and February [21] for the Amazonian regions.

Thus, for the PNCM, where two seasons are defined, one of which is dry (May/October) and the other rainy (November/April), the growth and reproduction relationship of *M. duriventre* has a differentiated structure, although it did not indicate a statistical difference between the means and standard deviation analyzed.

3.2.2. H. pusarum

The results from statistical analysis of the *H. pusarum* (cascudo) biometric in São Romão and Prata Waterfall, from PCNM can be observed in **Table 3**.

The biometric data submitted to the normality test for *H. pusarum* also indicated that there is no significant difference between treatments for São Romão and Prata Waterfall. However, taxa of *H. pusarum* captured in the dry season presented higher values of total length (CT) and weight (PT) for the two sampling areas.

In addition, the biometric data were higher for the cascudo than for the pacú. In this case, spawning in females of *H. pusarum* occurs at distinct periods for the different species of the Loricariidae family, offering an adaptive advantage since it reduces the intraspecific competition [22]. Thus, it is probable that the cascudo was captured in all the reproductive cycles for the sampling points of the PNCM, which conferred a greater biometry than the pacú. As *M. duriventre* migrate upstream to reproduce, it was probably not observed in minor individuals (in growth and feeding period) in the São Romão and Prata Waterfall.

Parameter (mean ± SD)	Means ± Standard deviations (SD)			
	São Romão Waterfall		São Romão Waterfall	
	Rainy season	Dry season	Rainy season	Dry season
TL (cm)	16.95 ± .0.63	15.75 ± 0.77	14.2 ± 6.42	19.99 ± 6.16
FL (cm)	14.1 ± 0.10	12.65 ± 0.77	12.83 ± 5.39	18.86 ± 6.79
SL (cm)	13.05 ± 0.35	11 ± 0.70	11.76 ± 4.57	17.34 ± 6.38
TW (g)	32 ± 28.28	26 ± 2.82	13.66 ± 12.66	128.33 ± 97.22

Total number of species sampled = 13; number of species in São Romão Waterfall = 4 and number of species in Prata Waterfall = 9. Biometric data: TL (total length); FL (fork length); SL (standard length) and TW (total weight).

Table 3. Biometric data of *H. pusarum* sampled in the São Romão and Prata Waterfall, Chapada das Mesas National Park, Brazil.

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3.2.3. Micronucleus (MN) and nuclear morphological changes (NMC) in M. duriventre and H. pusarum

Table 4 shows the incidence of micronucleus (MN) and nuclear morphological changes (NMC) in erythrocytes of *M. duriventre* and *H. pusarum* collected at the different sampling points in the PNCM.

Studies applied to resistant aquatic species are considered of great relevance, since the alterations found in any level of biological organization (molecular, biochemical and cellular) can indicate the degree of impact of a given ecosystem [23]. In relation to the genotoxic changes found, a low incidence of MN and NMA was observed for the two species sampled at the PNCM collection points. However, the genotoxic changes found were greater for pacú (*M. duriventre*) than the cascudo (*H. pusarum*).

According to the bioecology of the taxa, the cascudo presents a dermis/benthic habit and, probably, the environmental conditions and the possible environmental impacts of the PNCM in relation to the pacú are probably more resistant. These data are important and highlight cascudo as a bioindicator species more appropriate for biomarker studies in the PNCM. In addition, these data corroborate the general theory of the biomarkers of aquatic contamination that states that benthic species are more appropriate for studies of biomonitoring in relation to species potential sources of pollution in aquatic ecosystems [8].

The **Figure 2** shows a photomicrograph of the changes found in *M. duriventre* and *H. pusarum* for the two areas sampled at different points in the PNCM. Micronucleus (MN), vacuolated nuclei (VN) and binucleated nuclei (BN) were found. In addition, most cells were found in the defense system such as lymphocytes and eosinophils.

The use of hematological and genotoxic parameters in model organisms (such as fish) has allowed to evaluate the quality of aquatic ecosystems and the effect of pollutants as well as changes in their toxic potential after exposure to the environment [24]. According to these

		Rain							
		São Romão Waterfall				Prata Waterfall			
Species	**N**	**MN**	**NV**	**NB**	**NE**	**MN**	**NV**	**NB**	**NE**
Mylossoma sp.	9	0	0	50	33	0	8	123	0
Hypostomus sp.	5	0	1	5	0	2	0	7	0
		Dry							
		São Romão Waterfall				**Prata Waterfall**			
Species	**N**	**MN**	**NV**	**NB**	**NE**	**MN**	**NV**	**NB**	**NE**
Mylossoma sp.	10	18	0	75	0	22	15	69	0
Hypostomus sp.	7	4	0	45	0	18	0	38	0

n = total number of fishes sampled; MN = micronucleus; NV = vacuolated nucleus; NB = binucleated nucleus.

Table 4. Frequency of micronucleus and morphologic changes in ***M. pusarum*** and ***M. duriventre*** from the Chapada das Mesas National Park, Brazil.

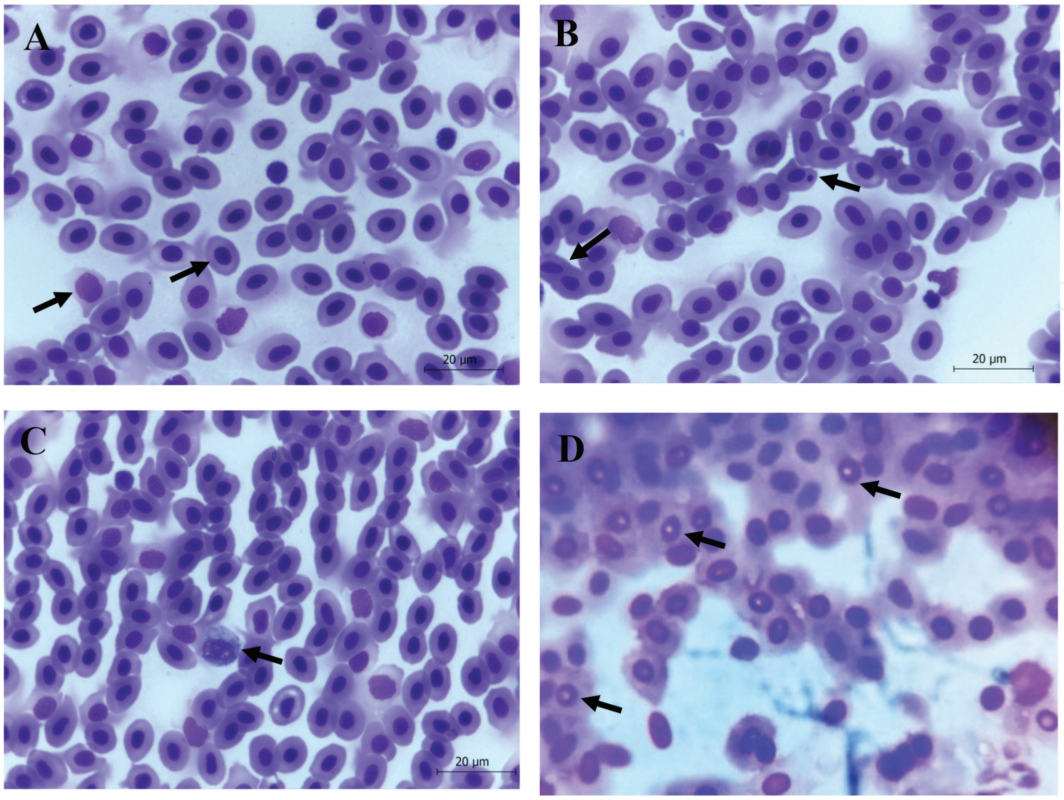

Figure 2. Photomicrograph (×1000) of erythrocytes of *H. pusarum* and *M. duriventre* stained with Giemsa from the São Romão and Prata Waterfall, Chapada das Mesas National Park, Brazil, showing (A) lymphocytes and normal cells—erythrocytes (arrow), (B) binucleated nucleus (arrow) and micronucleus (arrow), (C) eosinophils (arrow) and (D) vacuolated nucleus (arrows).

authors, the biological parameters discussed are verified at the cellular level and provide two types of analyses that reveal damage to the genetic material: the micronucleus test. The increase in the frequency of micronucleated cells is a marker of genotoxic effect that may reflect a exposure to agents with clastogenic mode of action (chromosome breakdown [25]. In the present study, the effect of the antigen on the chromosome number was not significant.

The incidence of NM to the PNCM sampling points was lower than NMA. These data differ from studies performed by Pinheiro-Sousa [8] and Carvalho-Neta et al. [26] who found a higher incidence of MN for the Environmental Protection Area of Maracanã. Thus, despite the low frequency of NMA and MN, especially of micronuclei, it is suggested that the Waterfalls of São Romão and Prata still do not suffer from point sources of pollution.

The presence of nuclear morphological changes (NMA) should be considered as complementary data to micronucleus records and as changes resulting from the induction by cytogenotoxic agents [18, 27] or by induction of pollutants. In fish, several types of nuclear anomalies do not yet have their origin completely understood. However, Carrasco et al. [28] and Galvan [29] have described and photographed some morphological changes found in fish erythrocyte nuclei. These alterations were classified as follows: [1] binucleate nuclei: nuclei that present cuts of two nuclei and nuclear membrane bounded and [2] nucleus vacuolizados: these nuclei present a region that resembles the vacuoles inside. These vacuoles are devoid of any visible material along the nuclear structure [29].

In addition, a large number of defense cells were found in the material analyzed. These include eosinophils and lymphocytes. According to Ranzani-Paiva and Silva-Souza [30], the eosinophils present diverse sizes, relatively small, and can vary according to the quantity or size of granules contained in the cytoplasm. The nucleus is rounded and eccentric with compact chromatin. This type of cell is distributed throughout the connective tissue, especially in the gastrointestinal tract and gills. One of the eosinophils functions is to intervene in the chronic inflammation processes, mainly in cellular defense, as how the fish was analyzed for PNCM.

In contrast, the lymphocytes are predominantly rounded cells, varying in size with the basophilic cytoplasm and without visible granulations, the nucleus has a rounded form, dense chromatin, and its relation with the cytoplasm is elevated [31]. Lymphocytes prevail in the body's defense reaction, but in stress situations, the number of circulating lymphocytes decreases [32]. Pickering [33] reported that lymphocyte decline may be related to. This is an important step in reducing the fish's ability to defend against pathogens. These data indicate that the degree of pacú and cascudo stress is probably low when compared to other studies in Maranhão Conservation Units [8, 26, 34].

Thus, the evaluation of hematology is an important tool for understanding fish sanity of the resources available in PNCM. For *M. duriventre* and *H. pusarum*, these results should be supported by a chemical analysis of the São Romão and Prata Waterfalls to evaluate the degree of impact that this region has been suffering along the process of ecotourism expansion and, of possible, indirectly influenced ventures of the park.

4. Conclusions

Means and standard deviations (SDs) of the biometric data of *H. pusarum* showed length and total weight are greater than *M. duriventre*. Nuclear morphological changes (NMAs) were identified in the two sampled species for the two collection points. Among the NMAs found, binucleated nucleus (BN), vacuolated nucleus (VC) and micronucleus (MN) were also found in both species; however, in *M. duriventre*, the frequency of MN and NMA was higher than *H. pusarum*. Probably, the cascudo (*H. pusarum*), considered a benthic species and resistant to environmental conditions, presented a lower frequency of genotoxic alterations than the Pacú (*M. duriventre*), that is, a species that presents a migratory habit and sensitive to environmental variables. Besides, the frequency of MN and NMA was not significant to indicate possible environmental impacts in the two sampled areas. The presented data show that methodologies based on biomarkers and bioindicator species can be used in future biomonitoring and park management programs.

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