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Effects of blood metal(loid) concentrations on genomic damages in sharks^{\star}

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ABSTRACT

The use of effect biomarkers has contributed to the understanding of the sublethal effects of contaminants on different organisms. However, the analysis of genotoxic markers as an indicator of organism and environmental health in sharks is underexplored. Thus, the present study investigated the relationship between the genomic damage frequency in erythrocytes and metal(loid) concentrations in whole blood of three shark species (Galeocerdo cuvier, Negaprion brevirostris and Ginglymostoma cirratum), taking into account climatic seasonality. The results showed that G. cuvier, an apex predator, presented the highest total erythrocyte genomic damage frequencies together with the highest mean whole blood concentrations of Al, Cd, Cr, Fe, Mn, Ni, Pb and Zn. The shark N. brevirostris also presented high levels of metal(loid), indicating a greater susceptibility to these contaminants in species that preferentially feed on fish. In contrast, G. cirratum, a mesopredator, presented the lowest erythrocyte damage frequencies and whole blood metal(loid) concentrations. The presence of micronuclei was the most responsive biomarker, and Al, As and Zn had an important effect on the genomic damage frequencies for all species evaluated. Zn concentration influenced the binucleated cells frequencies and Al concentration had an effect on the total damage and micronuclei frequencies in G. cuvier and N. brevirostris. Binucleated cells and blebbed nuclei frequencies were affected by As concentration, especially in G. cirratum, while showing a strong and positive correlation with most of the metals analyzed. Nonetheless, baseline levels of metal(loid) blood concentrations and erythrocyte genomic damage frequencies in sharks have not yet been established. Therefore, minimum risk levels of blood contaminants concentrations on the health of these animals have also not been determined. However, the high genomic instability observed in sharks is of concern considering the current health status of these animals, as well as the quality of the environment studied.

1. Introduction

Determining the sublethal effects of contaminants is relevant from an ecological point of view (Hauser-Davis and Wosnick, 2021), since they can be early detected at the individual and basal level (Van der Oost

et al., 2003; Adam et al., 2023). This approach provides an early warning signal of significant biological effects of great importance for biomonitoring (Monserrat et al., 2007; Lam, 2009; Lionetto et al., 2021). This allows the proposition of strategies to mitigate the potential negative effects arising from the exposure to pollutants, which might, in

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a long term, contribute to the reduction of vulnerable populations.

The analysis of damage to genetic material has been frequently used in diagnosing the environmental health of different ecosystems, due to its simplicity and low cost. Furthermore, these biomarkers are sensitive to contact, concentration and time of exposure to pollutants, thus contributing to the understanding of the sublethal effects of contaminants on different organisms (Adam et al., 2023). Biomarkers of effects, such as genotoxic markers, have notable applicability in environmental quality assessment procedures and are being frequently used in Brazilian Conservation Units monitoring using sentinel species (Adam et al., 2023). Among these, the micronucleus assay (macrolesions in DNA) and nuclear abnormalities are widely used to evaluate the genotoxicity potential, both in tests (in vivo and in vitro) and ex situ experiments (Stankevičiūtė et al., 2017; Shah et al., 2021). Also, they are employed in in situ monitoring, due to their high sensitivity in detecting damage caused by environmental stressors (Sommer et al., 2020; Adam et al., 2023).

In the last years, different studies have highlighted the use of sharks as sentinel species due to their susceptibility to bioaccumulate different types of contaminants, such as metals and Persistent Organic Pollutants (POPs) (Torres et al., 2017; Menezes-Sousa et al., 2021; Alves et al., 2022; Hauser-Davis and Wosnick, 2021). Indeed, many shark species occupy elevated trophic levels in food webs among different ecosystems (Cortés, 1999). Furthermore, characteristics such as longevity, slow growth and low metabolism contribute to the bioaccumulation of contaminants in sharks (Gelsleichter and Walker, 2010). Metal(loid) are the most studied contaminants in sharks and other cartilaginous fish (Tiktak et al., 2020). However, the effects of these elements on the health of these organisms are less investigated (Wosnick et al., 2023).

Recently, high frequencies of genotoxic markers (micronuclei and nuclear abnormalities) were observed in shark species from the South Atlantic associated with exposure to pollutants, especially surfactants and metals such as Cd and Pb. This emphasizes the importance and relevance of these animals as biomonitors of environmental health (Araújo et al., 2023). However, elements contributing to the increased frequency of genomic damage in sharks are still not investigated. Therefore, some questions may be raised: (1) is there a relationship between whole blood metal(loid) concentrations and the frequency of genomic damages in erythrocytes of sharks? and (2) is there any seasonal variation in these frequencies?

Therefore, the present study investigates the relationship between whole blood metal(loid) concentrations and the frequency of genomic damages in erythrocytes in three species of sharks, *Galeocerdo cuvier*, *Negaprion brevirostris* and *Ginglymostoma cirratum*, that inhabit an island environmental protection area in the South Atlantic Ocean, taking into account the climatic seasonality of the study area. As sharks are among the most threatened taxa nowadays (IUCN, 2024), this information is relevant to understand the ecological risks associated with chemical contamination, which may involve a decrease in the fitness of individuals, an increase in the risk of extinction of the local population and a decrease in species diversity.

2. Methodology

2.1. Study area

The Fernando de Noronha Archipelago $(03^{\circ}51'S - 32^{\circ}25'W)$ is a priority Marine Protected Area for the conservation of marine communities and endemic species in the South Atlantic Ocean. Also, it hosts the only oceanic mangrove forest in the region. Located 350 km off the coast of Rio Grande do Norte State (Northeastern Brazil), the Fernando de Noronha Archipelago is formed by 21 volcanic islands and covers a total area of 26 km² (Fig. 1). Seventy percent of its area comprises the Fernando de Noronha National Marine Park – PARNAMAR, which is under



Fig. 1. Location of the Fernando de Noronha Archipelago (Pernambuco State, Northeastern Brazil).

an integral protection regime. The other 30% of the territory is under a protection regime of sustainable use, where the urbanized portion of the territory is located. The archipelago has around 3167 inhabitants (IBGE, 2022) and has an infrastructure that includes airport, roads, housing and local commerce. Despite efforts towards a sustainable management, the region has serious socio-environmental conflicts, characterized by typically urban environmental problems related to housing, as well as dumping and treatment of effluents (Fioravanso & Nicolodi, 2021). The region has also received a continuous annual increase in the number of tourists, with an influx of 149,839 tourists in 2022 (ATDEF, 2022). Although the archipelago is an environmental protection area with seawater in better conditions than other continental areas with large human settlements, the region has been suffering negative impacts associated with a lack of waste and sewage treatment, associated with the intense and increasing flow of tourists. Indeed, we reported in a very recent study low concentrations of metals (Cd, Cr, Cu, Hg, Mn, Pb, and Zn) and high concentrations of surfactants such as linear alkylbenzene sulfonate (LAS) in the seawater of the archipelago (Araújo et al., 2023).

2.2. Shark blood sampling

Thirty sharks of the species Ginglymostoma cirratum (n = 3), Galeocerdo cuvier (n = 9) and Negaprion brevirostris (n = 18) were captured during the months of July 2019, January-February 2020 and February 2022. They were captured with longlines and drum lines launched around the Fernando de Noronha Archipelago. Total length (TL, cm) and sex of each individual captured were determined, and a blood sample was collected by puncture of the caudal vein. Two drops of each sample were used to prepare blood smears for the analysis of erythrocyte genomic damage. In addition, 3 mL of sample were stored in vacuumsealed tubes containing ethylenediaminetetraacetic acid (EDTA) for the analysis of blood metal(loid) concentrations. All individuals were marked with numbered plastic tags for identification in case of recapture. Individuals evaluated were released immediately after data collection. All procedures were conducted in accordance with the Ethics Committee on Research with Animals of the Federal Rural University of Pernambuco (license #059/2019) and Brazilian Ministry of the Environment (IBAMA/ICMBio - SISBIO # 67989-6).

2.3. Genomic damage analysis

In the laboratory, blood smears were fixed in absolute methanol for approximately 5 min, washed in distilled water and stained with absolute Giemsa for 3 min. After drying at room temperature, the blood smears were analyzed under optical microscopy at $100 \times$ magnification. Occurrence of genomic damage was evaluated in 3000 erythrocytes per individual, following the micronucleus assay procedures described by Heddle (1973) and Schmid (1975). The methodology to quantify nuclear morphological changes followed the procedures described by Çavas and Ergene-Gozukara (2005), in accordance with the standardization protocol proposed by Adam et al. (2023). For each individual, total genomic damage was calculated based on the sum of micronuclei and other nuclear abnormalities observed. For each shark species, mean values for each genomic damage category (micronuclei, blebbed nuclei, lobed nuclei, notched nuclei and binucleated cells), as well as the total genomic damage, were calculated.

2.4. Blood metal(loid) concentrations analysis

The determination and quantification of aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb) and zinc (Zn) was carried out at the Institute of Biological Sciences of the Federal University of Rio Grande – FURG. Subsamples of approximately 1 mL of whole blood were dried in oven (60° C for 24 h) and acid digested with the addition of 500 µL of 65% nitric acid (HNO₃) (Suprapur, Merck, Darmstadt, Germany) in

oven (60 °C for 24 h). The final volume was adjusted to 10 mL with ultrapure (18.2 MΩ/cm) water –(MasterSystem MS-2000, Gehaka, São Paulo, Brazil). Dilutions of 10× and 5× were used for the analysis of Hg and the other elements, respectively. Metal(loid) quantification was performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, PlasmaQuant MS Q, Analytik Jena, Jena, Germany). ICP-MS parameters were adjusted as follows: radio frequency (RF) power: 1300 W; plasma (argon) flow: 15 L min⁻¹; auxiliary flow: 1.2 L min⁻¹; argon flow in the nebulizer: 0.42 L min⁻¹; replicate readings: 5; dwell time: 50 ms; and detector operation mode: dual mode. Yttrim-89 (⁸⁹Y⁺) was used as internal standard. The following isotopes and respective gases were employed for metal(loid) quantification: ²⁷Al[He], ⁷⁵As[H2], ¹¹⁴Cd [H2], ⁵²Cr[He], ⁶⁵Cu[He], ⁵⁷Fe[He], ²⁰²Hg[H2], ⁵⁵Mn[He], ⁶⁰Ni [He], ^{206,207,208}Pb[He], and ⁶⁶Zn [He].

Metal(loid) concentrations were determined based on calibration curves built for each element analyzed, using a serial dilution of a multielement standard (1000 mg L^{-1}) solution (Merck, Darmstadt, Germany). Coefficients of determination (R^2) of the calibration curves were 0.999556, 0.999989, 0.999605, 0.999940, 0.999971, 0.999870, 0.999996, 0.999964, 0.999986, 0.999984 and 0.999967 for Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn, respectively. Quality control and assurance procedures for metal(loid) quantification in shark blood samples were based on the regular analysis of blanks and spiked matrices, as well as of a certified reference material (CRM) for fish protein (DORM-5) from the National Research Council Canada (Ottawa, Canada). Mean recovery rates for metal(loid) analyzed in the CRM corresponded to 92.1, 81.4, 84.4, 89.2, 87.3, 88.4, 81.5, 83.5, 90.3, 83.4 and 89.5% for Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn, respectively. The limit of detection (LOD) was three times the standard deviation (SD) value of the blank signals (3xSD; n = 10). They were 0.2555, 0.0371, 0.0014, 0.0321, 0.0556, 0.7595, 0.0017, 0.0120, 0.0178, 0.1912 and $0.2147~\mu g~L^{-1}$ for Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn, respectively. The limit of quantification (LOQ) corresponded to ten times the SD value of the blank signals (10xSD; n = 10). They were 0.8516, 0.1237, 0.0045, 0.1071, 0.1854, 2.5316, 0.0055, 0.3990, 0.0593, 0.6373 and 0.7157 $\mu g \ L^{-1}$ for Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn, respectively.

2.5. Data analysis

Data homogeneity and the normality of the residuals were tested using the Levene Test (Car Package, R Core Team, 2020) and the Shapiro-Wilk Test (Stankevičiūtė et al., 2017), respectively. The Kruskal-Wallis Test was used to identify differences between species for metal(loid) concentrations and genomic damages. Pair-wise comparisons were performed using the Nemenyi Test to identify difference among groups (PMCMRplus Package, R Core Team, 2020). The Wilcoxon/Mann-Whitney Test was used to compare metal(loid) concentrations and genomic damages between female and male sharks, as well as between dry and rainy seasons.

The relationship between genomic damage frequency and blood metal(loid) concentrations was verified using the Spearman correlation analysis and the Principal Component Analysis (PCA) based on a correlation matrix with standardized variables (Stats Package, R Core Team, 2020).

Generalized Additive Models (GAM) (Wood et al., 2016) were also generated seeking to highlight the variables or set of variables that most explain the variations observed in the genomic damage frequency. Results of the Spearman correlation analysis were considered to generate the GAM models, with the choice of only one of the variables in case of the presence of correlations with r > 0.8. The selected variables were modeled using the Poisson distribution and a smoothing function (*splines*). A step-by-step procedure was conducted in order to test the inclusion of new explanatory variables (*forward*) based on the lowest values of the Criteria of Akaike information (AIC), the highest values of the pseudo Coefficient of Determination (R2) and the highest values of the model explanation percentage (*Deviance explained*) (Mgcv Package, R Core Team, 2020). From the third variable, analysis of variance (ANOVA) using the Chi-square test was used to determine whether the new model was significantly different from the previous one, justifying the inclusion of this new variable at the 0.05% significance level.

3. Results

3.1. Shark length, sex, and genomic damages frequencies

Among the 30 sharks analyzed, the highest average total length was observed for *G. cuvier* (251 cm) followed by *N. brevirostris* (244 cm) and *G. cirratum* (177 cm). Significant differences among species were observed for total genomic damage (p = 0.010), with the highest average value being observed for *G. cuvier* (167) and *N. brevirostris* (138), whereas *G. cirratum* presented the lowest value (15) among the shark species evaluated (Table 1; Fig. 2; Table S1.1). Similarly, the highest average values of micronuclei frequency were observed for *G. cuvier* (110) and *N. brevirostris* (89), in contrast with *G. cirratum* (6) (p = 0.015, Fig. 2; Table S1.1). In turn, blebbed nuclei frequencies were significantly higher in *N. brevirostris* compared to the other two shark species (p = 0.016; Fig. 2; Table S1.1). Lobed nuclei (p = 0.030) and notched nuclei (p = 0.019) frequencies were also different between *G. cuvier* and *G. cirratum* (p < 0.05). No significant difference was observed regarding the sex of individuals analyzed (p > 0.05).

Regarding climatic seasonality (dry and rainy seasons), significant differences were observed only for total damage (p = 0.006) and micronuclei frequency (p = 0.004). However, these results were possibly biased due to the high damage frequencies observed in *G. cuvier* in the dry season (p = 0.016) (Table 2; Fig. 3).

3.2. Blood metal(loid) concentrations

Nineteen blood samples of *G*. *cirratum* (n = 3), *N*. *brevirostris* (n = 7)and G. cuvier (n = 9) were collected and analyzed for metal(loid) paralleled by the analysis of genomic damages (Table S1.2a). Metal(loid) evaluated were detected in all samples analyzed, with G. cuvier showing the highest mean concentration values of Al (41.34 μ g L⁻¹), Cd (0.15 μ g L^{-1}), Cr (1.38 µg L^{-1}), Fe (4435.49 µg L^{-1}), Mn (16.70 µg L^{-1}), Ni (61.05 $\mu g \ L^{-1}), \ Pb$ (1.06 $\mu g \ L^{-1}) \ and \ Zn$ (297.83 $\mu g \ L^{-1}) \ while$ N. brevirostris presented the highest mean concentration values of As (174.90 μ g L⁻¹), Cu (172.80 μ g L⁻¹) and Hg (2.42 μ g L⁻¹) (Fig. 4; Table S1.2a). Significant difference among shark species was observed only for As (p = 0.026), Fe (p = 0.006) and Zn (p = 0.016), with G. cuvier always showing the highest concentrations. The sharks G. cuvier and N. brevirostris showed higher concentrations of As and Fe than G. cirratum. The mean value of Zn concentration was higher in G. cuvier and lower in G. cirratum (Fig. 4; Table S1.2b). No significant difference was observed between the sexes of sharks (p > 0.05).

Regarding the climatic seasonality, Al was the only element analyzed presenting significant different mean concentration values between the dry and rainy seasons (p = 0.012), with the higher value being observed during the dry season. This was observed for all shark species evaluated. However, this significant difference was not observed for each species

(Fig. 5; Table S1.2b).

3.3. Relationship between frequencies of genomic damages and blood metal(loid) concentrations

Total genomic damage and micronuclei frequency presented the highest positive correlation value observed between variables ($r_s = 0.942$), followed by lobed nuclei and binucleated cells ($r_s = 0.736$), and lobed nuclei and notched nuclei ($r_s = 0.625$) (Table S1.3a). Regarding the correlation between metal(loid), Mn and Zn showed the highest positive correlation values ($r_s = 0.905$), followed by Cu and Hg ($r_s = 0.8434$), Cu and Mn ($r_s = 0.844$), Cu and Zn (p = 0.824) and Cr and Ni ($r_s = 0.823$) (Table S1.3b).

The lobed nuclei frequency and the blood Fe concentration showed the highest positive correlation value between response and predictor variables ($r_s = 0.701$), followed by micronuclei frequency and blood Al concentration ($r_s = 0.665$) and total genomic damage and blood Al concentration ($r_s = 0.656$). Evidently, the relationship between total genomic damage and micronuclei frequency influenced this result, since, in general, micronuclei frequency showed the highest value among the genomic damages analyzed.

Negative and weak relationships were also observed between blebbed nuclei frequency and blood Pb ($r_s = -0.111$), Ni ($r_s = -0.049$) and Cr ($r_s = -0.025$) concentrations, as well as between binucleated cells frequency and blood Al concentration ($r_s = -0.054$) (Table 2).

The results of PCA for all species showed a close relationship between blood Al, As and Zn concentrations with the different categories of genomic damages. As and Zn concentrations showed a relationship with the binucleated cells frequency in relation to axis 1, which explained 44.51% of data variability. In turn, blood Al concentration showed a relationship with total genomic damage, as well as the frequency of micronuclei, blebbed nuclei, lobed nuclei and notched nuclei in relation to axis 2, which explained 20.01% of data variability (Fig. 6 A).

Analyzing by shark species, PCA results for N. brevirostris showed a close relationship between micronuclei frequency and blood Al concentration in relation to axis 1, which explained 34.13% of data variability. In turn, blebbed nuclei frequency showed a relationship with blood Hg concentration while binucleate cells frequency showed a relationship with blood Zn concentration in relation to axis 2, thus explaining 29.63% of data variability (Fig. 6 B). The results of PCA for G. cirratum showed a relationship between total genomic damage, blebbed nuclei and micronuclei frequencies, as well as blood As and Cu concentrations. Also, a relationship between lobed nuclei frequency and blood Fe concentration was observed in relation to axis 1, which explained 63.78% of data variability. The results of PCA for G. cirratum also showed a close relationship between notched nuclei, binucleated cells and blood Hg concentration in relation to axis 2, thus explaining 36.22% of data variability (Fig. 6 C). The results of PCA for G. cuvier showed that total damage, blebbed nuclei frequency and blood Al concentration are strictly related and presented the smallest variations between the data analyzed in relation to the 2 axes of the PCA, with the first and second axes explaining 45.87 and 18.93% of data variability, respectively. In addition, a weak relationship was observed between lobed nuclei and binucleated cells frequencies and blood Zn

Table 1

Number of individuals sampled per dry and rainy season, sex, total body length and average values of genomic damages observed in different species of sharks captured in the Fernando de Noronha Archipelago.

Species	NR	ND	F	М	Ι	TL	TD	MN	BN	LN	NN	BC
Ginglymostoma cirratum	3		2	1		177	15	6	3	2	4	1
Galeocerdo cuvier	4	5	7	2		251	167	110	14	16	16	11
Negaprion brevirostris	2	16	11	6	1	244	138	89	20	9	10	11

Caption: NR = total number of individuals captured in the rainy season; ND = total number of individuals captured in the dry season; F = Females; M = Males; I = Unidentified sex; TL = mean total length; TD = mean total damage; MN = mean micronuclei frequency; BN = mean blebbed nuclei frequency; LN = mean lobed nuclei frequency; NN = mean notched nuclei frequency; BC = mean binucleated cells frequency.



Fig. 2. Frequency of different categories of genomic damage in the different shark species evaluated. Dots represent outliers, * indicates significant different mean values among shark species (p < 0.05; Kruskal-Wallis Test), and different lowercase letters indicate significant differences between groups (p < 0.05; Nemenyi test).

Table 2

Results of the Spearman's rank correlation test (rho) between blood metal(loid) concentrations and total genomic damage and frequencies of the different genomic damage categories in the shark species evaluated.

	Total damage	Micronuclei	Blebbed nuclei	Lobed nuclei	Notched nuclei	Binucleated cells
Al	0.6564283 (p = 0.0023)*	0.6649123 (p = 0.0025)*	$0.549847 \ (p = 0.01473)^*$	0.292643 (p = 0.224)	0.203436 (p = 0.4035)	-0.053860 (p = 0.8266)
As	0.3756034 (p = 0.113)	0.3789474 (p = 0.1104)	0.570049 (p = 0.01083)*	0.233586 (p = 0.3358)	0.036108 (p = 0.8833)	0.395594 (p = 0.0936)
Cd	0.0957821 (p = 0.6965)	0.0562144 (p = 0.8192)	0.178980 (p = 0.4635)	0.252429 (p = 0.2971)	0.186950 (p = 0.4435)	0.339085 (p = 0.1556)
Cr	0.1325143 (p = 0.5886)	0.1666667 (p = 0.4937)	-0.025470 (p = 0.9176)	0.267081 (p = 0.269)	0.143550 (p = 0.5577)	0.012362 (p = 0.9599)
Cu	0.1860465 (p = 0.4457)	0.1385965 (p = 0.5703)	0.231006 (p = 0.3413)	0.310273 (p = 0.1961)	-0.009690 (p = 0.9686)	0.398243 (p = 0.0913)
Fe	0.2158842 (p = 0.3747)	0.1192982 (p = 0.6258)	0.010540 (p = 0.9658)	0.700758 (p = 0.0008)*	0.293265 (p = 0.223)	0.482130 (p = 0.0367)*
Hg	0.2632734 (p = 0.2761)	0.1842105 (p = 0.4486)	0.388231 (p = 0.1005)	0.312917 (p = 0.1921)	0.164687 (p = 0.5005)	0.340846 (p = 0.1533)
Mn	0.2817025 (p = 0.2426)	0.2140351 (p = 0.3773)	0.331138 (p = 0.1661)	0.485682 (p = 0.03502)*	0.157641 (p = 0.5192)	0.498024 (p = 0.03)*
Ni	0.1878017 (p = 0.4413)	0.2087719 (p = 0.3894)	-0.049190 (p = 0.8415)	0.231823 (p = 0.3396)	0.044034 (p = 0.8579)	0.037087 (p = 0.8802)
Pb	0.1254936 (p = 0.6087)	0.0842105 (p = 0.7318)	-0.111550 (p = 0.6494)	0.301458 (p = 0.2098)	0.054602 (p = 0.8243)	0.470651 (p = 0.0419)*
Zn	0.4695042 (p = 0.04255)	0.3771930 (p = 0.1122)	$0.361002 \ (p = 0.1289)$	$0.579998 \ (p = 0.00924)^*$	0.205198 (p = 0.3994)	$0.556304 \ (p = 0.0134)^*$

Caption: p in parentheses indicates the p-value of the Spearman's rank correlation test (rho); * indicates significant p-values (p < 0.05).

concentration in relation to axis 2 (Fig. 6 D).

To create the generalized additive models (GAM), blood Mn, Cu and Ni concentrations were excluded from the data group due to their positive and strong correlations with other metal(loid) (rho >0.8). Micronuclei frequency was also excluded from the analysis due to their strong and positive correlation with the total genomic damage.

Modeling the total genomic damage and other variables, without considering the differences among species, showed that all metal(loid) analyzed have a positive effect on the genomic damage frequencies observed. The model with blood As concentration alone presented the highest explanation percentage (Dev.exp %), associated with the lowest values of the Akaike Information Criterion (AIC) (Dev.exp % = 85.2; AIC = 273), followed by the blood concentrations of Zn (Dev.exp % = 78.1; AIC = 336) and Al (Dev.exp % = 53.9; AIC = 550). Thus, the blood As, Zn and Al concentrations were evaluated in a single model that presented an explanation percentage of 99.9% and an AIC equal to 159, indicating that among the models evaluated this can be considered as the best one describing the effect of blood metal(loid) concentrations on



🖨 G.cirratum 🛱 G. cuvier 🛱 N. brevirostris

Fig. 3. Seasonal variation (dry and rainy season) in the frequencies of genomic damage categories in the different shark species evaluated. Dots represent outliers, * indicates significant different mean values between dry and rainy seasons for all shark species, and different lowercase letters indicate significant differences between dry and rainy seasons for each shark species (p < 0.05; Wilcoxon\Mann-Whitney test). More information in Table S1.1 of the Supplementary Material.

the total genomic damage in the sharks evaluated, without taking into account the difference among species (Table 3).

When models are generated by shark species, the total genomic damage in *G. cuvier* suffer a positive and significant effect when considering the blood Al concentration (Dev.exp % = 99.8; AIC = 79.5), followed by blood concentrations of Hg (Dev. exp % = 99.9; AIC = 79.6), Pb (Dev.exp % = 99.9; AIC = 79.7) and Zn (Dev.exp % = 100; AIC = 79.9). When these variables are modeled together, the best fit was observed for the interaction among blood concentrations of Al, Hg, Pb and Zn (Dev.exp % = 99.8; AIC = 79.7). However, despite the negligible difference among AIC values, blood Al concentration appears to have a significant effect on the genomic damage frequency in sharks (Table 3).

For the shark *N. brevirostris*, blood Cr concentration showed the most significant effect on the frequency of total genomic damage among all the metal(loid) evaluated (Dev.exp % = 98.5; AIC = 59.8), followed by blood concentrations of Al (Dev.exp % = 96.8; AIC = 61.5) and Hg (Dev. exp % = 98.3; AIC = 61.6). These variables were modeled together, however the model generated with the blood concentrations of Cr and Al (Dev.exp % = 98.4; AIC = 61.3) showed the best fit in relation to the model generated with the blood concentrations of Cr, Al and Hg (Dev. exp % = 99.7; AIC = 62.4), which did not present a significant difference in relation to the previous model generated (p = 0.242). Therefore, the inclusion of this third variable was discarded (Table 3).

For the shark *G. cirratum*, it was only possible to generate the models individually, due to the low number of specimens sampled. In this case,

the blood concentrations of Al (Dev.exp % = 99.1; AIC = 17.7), As (Dev. exp % = 94.6; AIC = 17.7) and Fe (Dev.exp % = 86.4; AIC = 17.8) presented the most significant effects in relation to the total genomic damage frequency for this shark species (Table 3).

4. Discussion

Among the species analyzed, the shark G. cuvier presented the highest averages of total genomic damage together with the highest average blood concentrations of eight out of the eleven metal(loid) analyzed (Al, Cd, Cr, Fe, Mn, Ni, Pb and Zn), followed by the shark N. brevirostris and G. cirratum. These results corroborate that G. cuvier, followed by N. brevirostris, might be more susceptibility to environmental exposure to contaminants, as previously observed in the same study area (Araújo et al., 2023). The susceptibility of G. cuvier may be, at some extent, related to the intrinsic characteristics of its life cycle, such as high trophic position (Dicken et al., 2017; Ferreira et al., 2017). For instance, feeding habits may influence the metal exposure in sharks (Le Bourg et al., 2019; Boldrocchi et al., 2021) and, consequently, the occurrence of genomic damages (Grisolia et al., 2009), suggesting that the genomic damages expression can also occur through biomagnification. Furthermore, it is possible that the generalist and necrophagous eating habit, life expectancy of up to 50 years, and displacement in urbanized coastal areas makes G. cuvier more vulnerable to anthropogenic impacts (Wosnick et al., 2021a; Shipley et al., 2021).



Fig. 4. Blood metal(loid) concentrations in the different shark species evaluated. Dots represent outliers, * indicates significant different mean values among shark species (p < 0.05; Kruskal-Wallis Test), and different lowercase letters indicate significant differences between groups (p < 0.05; Nemenyi test). More information in Tables S1.2a and b of the Supplementary Material.

The low genomic damage frequencies and blood metal(loid) concentrations observed in *G. cirratum* may also reflect the trophic position of this shark species, but it may also indicate a potential resistance or greater resilience of this shark compared to the other two species evaluated. This greater resistance or resilience may be a response to prolonged exposure to contaminants. Indeed, as suggested in previous studies, the benthic habit of *G. cirratum*, associated with its site fidelity and resident behavior, may favor this exposure, thus resulting in the use of metal detoxification alternative mechanisms by this shark species, which have not yet been well elucidated (Wosnick et al. et al., 2021a, Wosnick et al. et al., 2021b, Araújo et al., 2023). However, the low number of individuals sampled in the present study reduces the accuracy and precision of the results obtained, thus requiring further sampling and data analysis efforts to better elucidate this point.

In some cases, the shark *N. brevirostris* presented high average blood concentrations of As, Cu and Hg compared to the other two shark species. Interestingly, the average blood concentration of Zn observed in *N. brevirostris* in the present study was higher than that recorded from muscle tissue analysis in a study on the epigenetic effects of a dredging event on the control population of *N. brevirostris* of South Bimini, Bahamas, located 48 miles from Miami, Florida (Beal et al., 2021). This same study showed that the concentrations of Mn, Zn, Se, Cd and Pb in the muscle of *N. brevirostris* significantly explained variations observed in DNA methylation patterns in this shark species, a biomarker that, like the genomic damage frequencies, can vary in response to stress situations and environmental disturbances. Considering that the genotoxic effect of Zn has already been reported in other studies with fish (Abdel-Khalek et al., 2020b; Ajitha et al., 2021; Mahjoubian et al.,

2023), the high genomic damage frequencies observed in *N. brevirostris* evaluated in the present study were expected.

In general, the blood metal(loid) concentrations observed in the present study can be considered low when compared to what was observed for these same species through muscle tissue samples in other regions of the Atlantic. Comparatively, the blood concentrations of Cd, Pb, Cr, Mn, Cu, Zn and As observed in G. cirratum, N. brevirostris and G. cuvier were evidently lower in the present study than those observed in the muscle of the same species from the Bahamas (Shipley et al., 2021). Contrary to our results, this study showed higher concentrations of several metals in muscles of N. brevirostris and G. cirratum compared to those found in G. cuvier. The blood concentrations of Cd, Hg and Pb observed for G. cirratum and G. cuvier in the present study were also much lower than that observed for these same species in specimens from the Maranhão State, Northeastern Brazil (Wosnick et al., 2021a), and for an individual of G. cuvier collected in the Pará State, Northern Brazil (Souza-Araujo et al., 2021). It is important to highlight that the biological matrices evaluated in these studies differ, mainly because metal (loid) is transported through the blood circulation to various organs, where they are then accumulated (Authman et al., 2015). Therefore, metal(loid) concentrations in muscle tissue indicate old contamination, while metals concentrations present in blood indicate recent contamination, corresponding to the time of blood cell turnover (hours to days). Consequently, lower blood metal(loid) concentrations are expected when compared to the muscles and organs metal(loid) concentrations, such as the liver and gills. Thus, the lower concentrations in shark specimens evaluated in the present study demonstrate that the metal (loid) effects can be observed even at low concentrations, and of early



Fig. 5. Seasonal variation (dry and rainy seasons) in blood metal(loid) concentrations in the different shark species evaluated. Dots represent outliers, * indicates significant different mean values between dry and rainy seasons for all shark species (p < 0.05; Wilcoxon\Mann-Whitney test). Despite the significant difference between the dry and rainy seasons for all species in relation to Al concentrations, this difference was not observed within the groups (p > 0.05; Wilcoxon\Mann-Whitney test). More information in Table S1.2b of the Supplementary Material.

form, depending on the tissue evaluated.

Metal concentrations in sharks from Fernando de Noronha are also lower than those observed in the plasma of white sharks (*Carcharodon carcharias*) from South Africa, except for Cd, Fe and Ni (Merly et al., 2019). This difference may reflect different impacts in different environments, in addition to differences among species in the bioaccumulation process of these metals.

The average concentrations of Al, As, Cd, Cu, Mn and Pb observed in the blood of sharks evaluated in the present study were also considerably lower compared to those reported for blood samples of green turtles (Chelonia mydas) captured in the Fernando de Noronha Archipelago, except for Zn (Prioste et al., 2015). As mentioned before, trophic factors might influence exposure and consequently metal(loid) concentrations in fish tissues (Lacerda et al., 2020). For instance, dietary and direct exposure are important routes of exposure to Zn, with its concentration increasing rapidly after absorption (ATSDR and Roney, 2005). Therefore, the high Zn concentrations observed in sharks may be related to the predominance of the type of protein in the diet of these organisms. Indeed, G. cirratum has a dietary preference for crustaceans, fish and cephalopods, whereas N. brevirostris has a dietary preference for fish, especially sardines and other large fish associated with the reef, such as groupers. As previously mentioned, G. cuvier is an apex predator having the greatest variety of food items among the three shark species evaluated, including teleosts, reptiles, birds, marine mammals and other

elasmobranchs (Cortés, 1999; Shipley et al., 2021). In contrast, green turtles are omnivorous during their first years of life and herbivorous during their adult phase, helping these animals to maintain intimate contact with the substrate, which in turn can favor exposure to contaminants adsorbed to the substrate, thus explaining the higher concentrations of other elements found in this species at the same study area (Prioste et al., 2015). Not surprisingly, the Zn concentration observed in the present study obey the order of trophic positions, with the highest average concentrations observed for *G. cuvier* (apex predator), followed by *N. brevirostris* (mesopredator) and *G. cirratum* (mesopredator).

Blood Zn concentrations also show positive relationships with most other blood metal(loid), especially As, Mn and Cu. Generally, in natural environments, metals, whether coming from natural sources or anthropogenic activities, interact with aquatic organisms as a mixture, commonly containing more than one element and in combination with other pollutants. Thus, although Al, As and Zn were associated to the measured genomic damage metrics, indicating a greater sensitivity of the shark species analyzed to these contaminants, we cannot rule out the effects of biochemical interactions among all metal(loid) observed and even other substances not quantified herein. In particular, Hg, Fe, Cr, Cu and Pb, which, despite not having comparatively strong relationship as Al, As and Zn, these elements may also influence the frequency of genomic damage in sharks, considering that its genotoxic effects on fish are well known. Therefore, the mixture of metal(loid) presents in the A) All species

B) Negaprion brevirostris



Fig. 6. Results of the Principal Component Analysis (PCA) for the shark species evaluated. A) All shark species together; B) Negaprion brevirostris; C) Ginglymostoma cirratum, and D) Galeocerdo cuvier.

blood of sharks, as well as the presence of other pollutants or stressors not identified in the present study, probably contribute to the genotoxic responses observed.

The Fernando de Noronha Archipelago is a tropical oceanic island of volcanic origin, with soil naturally rich in metal(loid), since pedogenesis in volcanic rocks is characterized by the neoformation of non-crystalline phases known to have strong metal retention (Oliveira et al., 2011). This characteristic of the region may contribute to the high concentrations of some metals such as Al and Fe (>10 μ g L⁻¹) observed in the blood of the sharks analyzed in the present study, apart from *G. cirratum*. However, different sources of contaminants in this region may have contributed to the high rate of genomic damage observed in the sharks, since the archipelago has been suffering from environmental constraints related to inadequate management of solid waste, as well as effluents and energy demand based on diesel oil burning (Fioravanso and Nicolodi, 2021).

Seasonal variations in metal(loid) concentrations are common in environmental matrices. In the present study, the significant difference observed in blood Al concentration between dry and rainy seasons may be related to the smaller number of individuals collected during the rainy season. Wosnick et al. (2021b) observed influence of rainy and dry seasons on concentrations of Pb and other elements in *G. cirratum*. Physiological responses of various marine fish to metal(loid) exposure also were shown to be influenced by climatic seasonality in the Brazilian coast, including genomic damage (Bevitório et al., 2022). In this context, although the present study lacks a systematic seasonal sampling over some years, the seasonal influence observed should not be overlooked.

The genomic damage observed even at low blood concentrations of metal(loid) in sharks evaluated in the present study flags the potential deleterious effects of these chemical contaminants at the molecular level. Genomic damage was mostly influenced by blood concentrations of Al, As and essential metals, thus proving, once again, to be a suitable biomarker for biomonitoring studies in sharks (Araújo et al., 2023), as observed in finfish (Omar et al., 2012; Gusso-Choueri et al., 2016). In fact, all metal(loid) analyzed in shark blood have the ability to cause DNA damage (Nirchio et al., 2019; Singh et al., 2019; Xavier et al., 2019; Abdel-Khalek et al., 2020a; Flores-Galván et al., 2020; Gökalp et al.,

Table 3

Generalized Additive Models (GAM) for the effects of blood metal(loid) concentrations on the total genomic damage frequencies observed for each shark species analyzed, as well as for all shark species together, without taking into account differences among species.

GAM - all shark species							
Formula	Estimate	Dev. exp %	AIC	wiAIC	R-sq. (adj)	p ANOVA	
Total damage ~ s (Al)	4.86442	53.9	550	1	0.302*		
Total damage ~ s (As)	4.71725	85.2	273	1	0.652*		
Total damage ~ s (Cd)	4.94237	12.4	908	1	-0.174		
Total damage ~ s (Cr)	4.90559	31.1	750	1	-0.163		
Total damage ~ s (Fe)	4.8775	36.3	705	1	-0.195		
Total damage ~ s (Hg)	4.83603	49.1	592	1	-0.0841		
Total damage ~ s (Pb)	4.9359	16.8	876	1	-0.485		
Total damage \sim s (Zn)	4.7352	78.1	336	1	0.453*		
Total damage \sim s (As) + s(Zn)	4.6903	99.8	159	1	0.988		
Total damage $\sim s$ (As) + s(Zn) + s (Al)	4.69166	99.9	159	1	0.993	0.07852	

GAM - Galeocerdo cuvier

Formula	Estimate	Dev. exp %	AIC	wiAIC	R-sq. (adj)	p ANOVA
Total damage ~ s (Al)	5.0384	99.8	79.5	1	0.982*	
Total damage ~ s (As)	5.03613	100	79.8	1	0.972	
Total damage ~ s (Cd)	5.03768	99.9	79.7	1	0.956	
Total damage ~ s (Cr)	5.03561	100	79.8	1	0.96	
Total damage ~ s (Fe)	5.03585	100	79.9	1	0.976	
Total damage ~ s (Hg)	5.03774	99.9	79.6	1	0.969*	
Total damage ~ s (Pb)	5.03734	99.9	79.7	1	0.98*	
Total damage ~ s (Zn)	5.03583	100	79.9	1	0.978*	
Total damage \sim s (Al) + s(Hg)	5.08264	55.8	171	1	-0.163	
Total damage \sim s (Al) + s(Hg) + s (Pb)	5.05105	87.3	103	1	0.665	2.20E- 16
Total damage \sim s (Al) + s(Hg) + s (Pb) + s(Zn)	5.03632	99.9	79.7	1	0.969	1.04E- 06

GAM - Negaprion brevirostris

Formula	Estimate	Dev. exp %	AIC	wiAIC	R-sq. (adj)	p ANOVA
Total damage ~ s (Al)	5.09228	96.8	61.5	1	0.82*	
Total damage ~ s (As)	5.09228	99.5	62.3	1	0.858	
Total damage ~ s (Cd)	5.10031	43.9	77.5	1	0.186	
Total damage ~ s (Cr)	5.09277	98.5	59.8	1	0.944*	
Total damage ~ s (Fe)	5.09132	98.7	62	1	0.868	
Total damage ~ s	5.09195	98.3	61.6	1	0.866*	
Total damage ~ s (Pb)	5.09947	45.3	75.6	1	0.31	

Table 3	(continued)
Table 5	

GAM - Negaprion brevirostris								
Formula Estimat		Dev. exp %	AIC	wiAIC	R-sq. (adj)	p ANOVA		
Total damage ~ s (Zn)	5.0911	99.8	62.4	1	0.866			
Total damage \sim s (Cr) + s(Al)	1age ~ s 5.09118 s(Al)		61.3	1	0.891	*		
Total damage \sim s (Cr) + s(Al) + s (Hg)	5.09056	99.7	62.4	1	0.851	0.242		
GAM - Ginglymost	oma cirratum							
Formula	Estimate	Dev. exp %	AIC	wiAIC	R-sq. (adj)	p ANOVA		
Total damage \sim s(Al)	2.7268	99.1	17.7*	1	0.983			
Total damage ~ s(As)	2727	94.6	17.7	1	0.899			
Total damage \sim s(Cd)	2.7279	65.3	17.8	1	0.339			
Total damage \sim s(Cr)	2.7291	28.5	17.9	1	-0.414			
Total damage \sim s(Fe)	2.7272	86.4	17.8	1	0.744			
Total damage ~ s(Hg)	2.7284	49.4	17.9	1	-0.045			
Total damage ~ s(Pb)	2.7275	78.9	17.8	1	0.603			
Total damage $\sim s(Zn)$	2.7299	3.52	18	1	-0.931			

Caption: * = Models selected based on the lowest values of the Akaike Information Criteria (AIC), the highest values of the pseudo coefficient of determination (R²adj) and the highest percentage of model explanation (Dev. exp %).

2022; Mandal, 2020; Rodrigues et al., 2020; Abdullah et al., 2021; Castro et al., 2021; Passos et al., 2021; Córdoba-Tovar et al., 2023; Da Silva et al., 2023; Kumar et al., 2023a,b). However, there is a gap in assessing the safety tolerance of metal(loid) concentrations for sharks worldwide. In present study, blood concentrations of Al, Cd, Cr, Cu, Mn, Pb, Hg and Zn are below the maximum tolerance limits of inorganic contaminants in food, according to Brazilian and international legislation, while blood concentrations of As, Fe and Ni (the latter only for G. cuvier) were observed above the maximum limits established as safe for human consumption (Joint et al., 2000; 2011, 2012; ANVISA, 2022). It is important to notice that not only non-essential elements, which are usually toxic at lower levels, were associated to genomic damage, but more interestingly, essential elements such as Zn. Essential elements are vital for physiological functions, but at supraphysiological levels might exert toxicity, potentially jeopardizing organism functions and, eventually survival (Lima and Merçon, 2011; Slobodian et al., 2021).

As demonstrated by GAM analysis, the statistical effects of metal (loid) concentrations, as explanatory variables, were species-specific. Generalized additive models combining the three shark species showed different results than those modeled separately to each species. Still, blood Al concentration was the predominant metal contributing to the observed increase in total genomic damage frequency. Furthermore, due to the lack of correlation with other metal(loid), blood Al concentration might present a greater capacity to induce changes in the genetic material in sharks. However, further studies are needed to confirm this hypothesis. Previous studies reported a frequency-dependent increase in genomic damage associated with Al concentration in fish (Lacerda et al., 2020; Kousar et al., 2022), with this genotoxic potential of Al being attributed to its ability to oxidize purines and pyrimidines (Niu et al., 2005; Jackson and Bartek, 2009). However, higher Al concentrations have also been linked to higher genomic damage frequencies during the dry period for Geophagus brasiliensis, an omnivorous and benthopelagic cichlid fish species found in Brazilian river basins that is considered a good sentinel species for studies on environmental monitoring (Lacerda

et al., 2020).

Genomic damage observed in the present study may have also been influenced by As, as previously reported for teleost fish after exposure to low (10 and 50 μ g L ⁻¹) and high (500 μ g L ⁻¹) concentrations of this metalloid (Jha et al., 2019). Although Marchiset-Ferlay et al. (2012) suggested micronucleus induction as a suitable biomarker to assess As genotoxicity, the effects observed herein concern increase in binucleated cells and blebbed nuclei frequencies. Generally, As is a metalloid with a high toxicity level, as they are easily absorbed, especially by marine organisms, which accumulate substantial amounts of As more efficiently than terrestrial organisms due to their similarity to phosphate essential as a modular building block for various substances used by cells (Barra et al., 2000).

The essential metal Zn flood concentration also statistically influenced the genomic damage frequencies in the sharks analyzed in Fernando de Noronha. Genotoxic effects of Zn and Cu alone or in combination have also been reported for bony fish species, with the micronuclei frequencies increasing significantly towards the interaction of these elements (Bagdonas and Vosylienė, 2006; Obiakor et al., 2010). In this context, although Zn is an important metal for physiological functions, imbalances in its homeostasis can trigger deleterious effects (Hogstrand, 2011), such as those observed in the present study. Alone, Zn concentration was shown to have a positive effect on the micronuclei frequency in the teleost *Channa punctata* (Ratn et al., 2018). In other tissues, such as kidney, Zn concentrations have also been correlated with genomic damage in the Atlantic salmon, *Salmo salar* (Stankevičiūtė et al., 2017).

From a physiological perspective, genomic damage can lead to teratogenicity, disturbances in embryonic development and reproduction (Ramsdorf et al., 2012). Also, genomic damages were negatively correlated with fish growth (Mustafa et al., 2011), suggesting cascading effects that could compromise the individual and its fitness. Repair mechanisms are present and responsible for the reduction in genomic damage in fish, especially after exposure to contaminants (Marques et al., 2014). However, metal(loid) themselves, such as Al and As, for example, can act inhibiting the repair system in fish (Yadav and Trivedi, 2009; Paul et al., 2022). Therefore, the changes observed in the present study suggest that, if the environmental exposure of sharks to metal (loid) assessed here continues, effects at the population level in the long term should be considered (Nacci et al., 1996), especially in the shark *G. cuvier*.

Finally, it is worth highlighting that high genomic instability for a prolonged period can irreversibly affect individuals. Despite the abovementioned issues, genomic damage might affect the progeny through effects on development, growth and survival (Fenech, 2000; Bolognesi and Cirillo, 2014). Therefore, the relationship existing between genomic damage and metal(loid), as demonstrated in the present study, can directly and indirectly contribute to the increased vulnerability of shark populations, which are inherently already susceptible to anthropogenic actions (Dulvy et al., 2014). Furthermore, it can contribute in the long term to other ecological and social issues resulting from the decline of these populations.

5. Conclusion

This study reports, for the first time, the genomic damage associated to blood metal(loid) concentrations in sharks. The results reported herein show that environmental exposure of sharks to metal(loid) concentration might elicit effects at the genetic level, especially in species at higher trophic position, such as *G. cuvier* followed by *N. brevirostris*. This is concerning, since, the basal levels of metal(loid) concentrations in the blood of sharks have not yet been established, the minimum levels of risk to the health of sharks consequently cannot yet be determined. None-theless, the high genomic damages frequencies, remarkably for micronuclei, observed in blood cells point to a great risk to the health of sharks. It also reflects the environmental impact to which the Fernando

de Noronha Archipelago, a considerably remote region, has been subjected. In this context, it is worth noting that sharks from this archipelago showed the lowest concentrations of metal(loid) when compared to sharks from other study areas that also used *G. cuvier*, *N. brevirostris* and *G. cirratum* as bioindicators. Such comparisons suggest that in areas with greater human density and diverse polluting sources, these organisms may be at greater risk of genetic instability than that observed in the sharks evaluated in the present study.

The results from the present study also reinforce the susceptibility and genotoxic sensitivity of sharks to metal(loid), especially the tiger shark *G. cuvier*, as well as the effectiveness of using these animals as sentinel species in diagnoses and environmental monitoring studies. Furthermore, the results presented here highlight the importance of using parameters of genomic damage in sharks from different species in the scope of environmental monitoring programs in areas with different levels of anthropization, seeking to highlight, in a comparative way, the effects of shark exposure to contaminants on the response of this biomarker of effect.

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CRediT authorship contribution statement

Camila Brasilino Botêlho de Araújo: Writing - original draft, Methodology, Investigation, Formal analysis, Conceptualization. Sibele Alves de Mendonça: Writing - review & editing, Methodology, Formal analysis. Danielle de Lima Viana: Writing - review & editing, Supervision, Project administration. Mariana da Fontoura Martins: Writing review & editing, Methodology, Formal analysis. Patrícia Gomes Costa: Writing - review & editing, Methodology, Formal analysis. Adalto Bianchini: Writing - review & editing, Methodology, Formal analysis. Paulo Guilherme Vasconcelos de Oliveira: Writing - review & editing, Supervision, Project administration. Rodrigo Augusto Torres: Writing - review & editing, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. Fábio Hissa Vieira Hazin: Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. Monica Lúcia Adam: Writing - review & editing, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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