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Seasonal effect on biomarker responses in sentinel species: innovation in mangrove conservation status assessment

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Abstract We evaluated the environmental quality in mangrove areas of the Western Atlantic with different levels and history of contamination, considering biomarkers for the crab *Ucides cordatus*. For this purpose, specimens were collected in two climatic seasons (rainy and dry seasons) and assays of genotoxicity (MN, micronucleus), cytotoxicity (NRRT, neutral red retention time) and biochemical (MT, metallothionein; and LPO, lipoperoxidation) were conducted. In the most impacted mangroves, there was an increase in the mean of micronucleus (frequency of MN/1000), which was associated with a shorter retention time (minutes_of NRRT). In contrast, the most pristine areas showed MN < 3 and NRRT > 100 min, with no seasonal effect, indicating a lower effect of degenerative processes by xenobiotics. The rainy season was more harmful, especially regarding cytogenotoxicity. The use of bioindicator species for environmental monitoring should be guided by an analysis of biomarkers considering the season, because during the period of highest rainfall, biomarkers values can change.

Keywords Bioindicator · Lipoperoxidation · Metallothionein · Micronucleus · Seasonality · Neutral red

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Introduction

Coastal environments suffer from several anthropic impacts, such as inappropriate land use (Petts, 1999), overexploitation of fishing resources (Kura et al., 2004; Worm et al., 2009), introduction of exotic species (Lodge et al., 2006; Silva et al., 2004), and especially discharge of untreated domestic and/or industrial effluents (Cao et al., 2018; Cesar et al., 2006; Eisler, 2010; Rainbow, 2007; Xu et al., 2019). In this scenario, mangroves are ecologically and socioeconomically relevant (Schaeffer-Novelli, 1995). They

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are in areas where human activities are intense and act on the stabilization of various contaminants, creating a biogeochemical barrier (Kulkarni et al., 2018; Luoma & Rainbow, 2008; Macfarlane et al., 2007; Prasad et al., 2019; Saifullah et al., 2002).

Due to water dynamics and physicochemical properties of mangroves, the organisms that live there have a prolonged exposure to sublethal concentrations of chemical compounds, which allow survival but promote negative changes in biological functions (Amiard-Triquet et al., 2011; McGeer et al., 2000). They can be identified and quantified by biological assays (biomarkers). Such assessments can offer quantifiable biological responses to an isolated or synergistic action of contaminants, which cause negative effects (damages) (Amiard-Triquet et al., 2011; Duarte et al., 2016, 2017, 2019, 2020; Bordon et al., 2018; Falcão et al., 2020).

Some organisms can be considered "sentinel species" because they reveal an early presence of toxicity by xenobiotics in natural environments (Ahearn et al., 2014). The 'uçá'-crab (*Ucides cordatus*) is a semi-terrestrial species endemic to mangroves. It stands out as a sentinel species for assessing environmental quality of mangroves, as it has several favorable biological characteristics (Araújo-Jr et al., 2016; Christofoletti et al., 2013; Nordhaus et al., 2009; Pinheiro & Fiscarelli, 2009; Pinheiro & Hattori, 2006; Pinheiro et al., 2017; Silva et al., 2018) and responds early to contaminants (Duarte et al., 2016, 2017, 2019; Ortega et al., 2017; Pinheiro et al., 2012, 2013).

Physiological and biochemical mechanisms of detoxification have been studied in populations of *U. cordatus* in impacted areas (Harris & Santos, 2000; Pedale et al., 2012; Ortega et al., 2016; Duarte et al., 2016, 2017, 2019, 2020). Among decapod crustaceans, there is a close relation between the species' contact routes with environmental compartments and their contamination state, although little is known about many of the contaminants' entry mechanisms, whether by eating contaminated food, breathing through gills, or just the contact of articular membranes with the sediment (Díaz-Jaramillo et al., 2013; Harris & Santos, 2000; Pinheiro et al., 2021; Vilhena et al., 2013). Biomarker responses may differ according to bioindicator species (endogenous aspect) and to variations peculiar to different climatic seasons or seasonal periods (exogenous aspect) and water variations (e.g., temperature, pH, dissolved oxygen, and chemical composition) (Azevedo et al., 2012).

According to the Köppen climate classification, two climatic seasons prevail in Brazil (Alvares et al., 2013). They are well defined by thermal and rainfall levels. In the Southeast region, the rainy (hot) season is from October to March and the dry (cold) season is from April to September. The alternation of rainfall volumes in different climatic seasons interferes with leaching of terrestrial areas, changing the composition and availability of the pollutant complex, exerting genotoxic and clastogenic effects (Polard et al., 2011). Thus, seasonality imposes pressures on the biota, changing behaviors and entry routes of xenobiotic substances into organisms.

Considering that *U. cordatus* is continuously exposed to various sources of contaminants, this study aims to (1) evaluate the environmental quality of six mangrove areas in the state of São Paulo (with different levels and history of contamination), using 'uçá'-crab as a sentinel species, utilizing exposure and effect biomarker assessments (descriptors), and (2) verify the influence of seasonality (rainy and dry seasons) on the responses of each sublethal effect.

Materials and methods

Study area

Three mangrove areas in the Central Coast of São Paulo (8858 ha) were evaluated. They were compared to three areas of the South Coast (15,193 ha), covering 99% of this ecosystem in the state of São Paulo, as follows: Central Coast (Bertioga, BET; Cubatão, CUB; and São Vicente, SAV), and South Coast (Iguape, IGU; Cananéia, CAN; and Juréia, JUR) (Fig. 1). Each sample area was represented by three subareas (replicates: $n = 18$). The location was established following a gradient of salinity (internal, intermediate, and close to estuarine mouth) aiming to include differential variations in salinity and pH, which can alter mobility/availability of pollutants, as well as their role as natural stressors of organisms (Díaz-Jaramillo et al., 2013; Gamain et al., 2016; Moreira et al., 2016; Piazza et al., 2016). Aspects about the conservation status of the areas and subareas can be found in studies by Duarte et al., (2016, 2017), which the authors

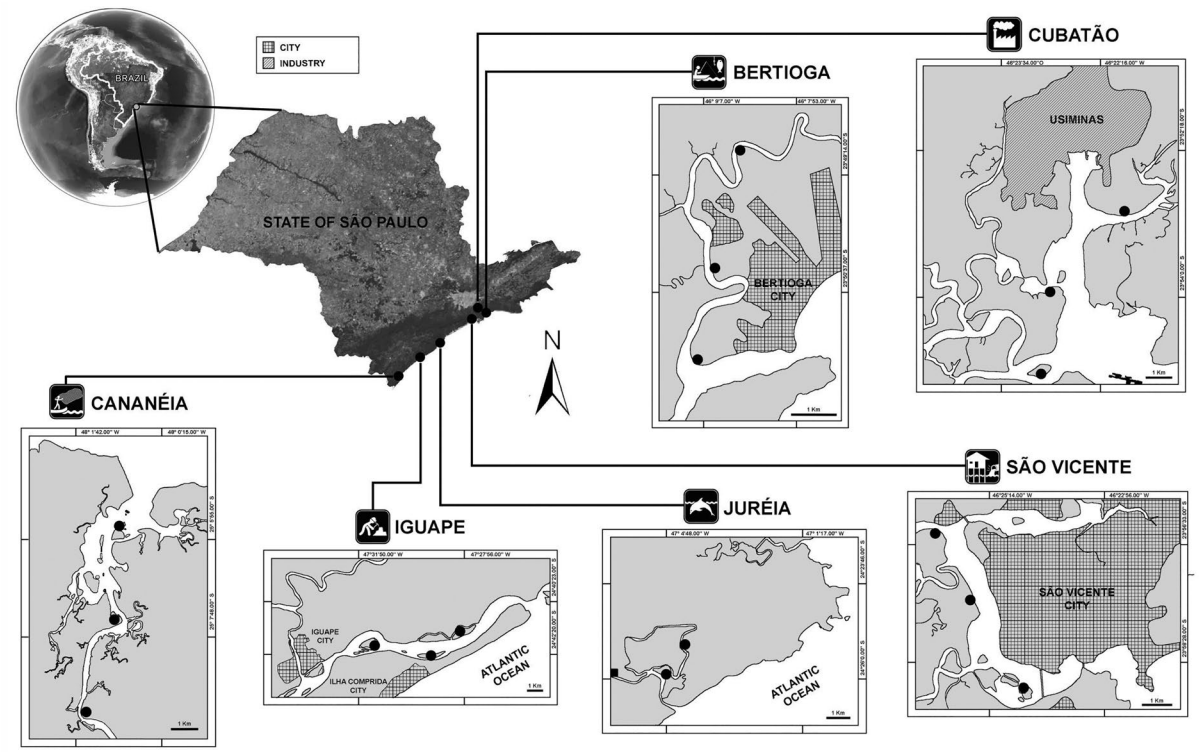

Fig. 1 Mangrove areas and their three subareas (replications) on the coast of the state of São Paulo (Brazil). Source: Satellite images from Google Earth® taken by Gustavo Pinheiro

developed at the same sample points. According to the authors, the most impacted areas follow the following hierarchical order, from the most impacted to pristine: (CUB ≅ BET ≅ SAV) > IGU > CAN > JUR. It is also important to highlight that JUR was considered a reference area, as it is a conservation unit.

Only adult animals, males in intermolt, with a carapace width ≥ 60 mm (Pinheiro & Fiscarelli, 2001) were used to assess different metabolic effects. The animals were collected by hand, totaling ten individuals/sampling subarea, on two expeditions in 2015, one during the rainy season (summer: January–March) and the other during the dry season (winter: July–August). Right after capture, the animals were taken to the laboratory, where their biometrics was performed. They were acclimatized, kept in tanks containing water from the place of origin (water column about 5 cm high), and PVC pipes for use as artificial galleries, minimizing possible stress due to behavioral interactions. The photoperiod (12:12) was constant, as well as temperature (24 ± 1 °C).

Biological responses of Ucides cordatus

Genotoxicity (MN) and cytotoxicity (NRRT)

Hemolymph samples were collected to perform a micronucleus assay (MN, frequency of micronucleated cells per thousand analyzed) and a neutral red assay (NRRT, neutral red retention time by lysosomes, in minutes), as indicated by Duarte et al. (2016).

Lipid peroxidation (LPO)

Five individuals/subarea were dissected to obtain portions of the hepatopancreas and gills (anterior and posterior). The frozen tissues were submitted to the FOX method (orange xylenol test) adapted from Hermes-Lima et al. (1995) and Jiang et al. (1991, 1992). For this, the samples were weighed, diluted with methanol (1:9), homogenized on ice, and centrifuged. Then, the supernatant was removed. For the standard assay, the following reagents were added in

sequence on an ELISA plate: 0.25 mM iron (II) sulfate + 25 mM H_2SO_4 + 0.1 mM xylenol orange, and water for a final volume of 0.9 mL. The samples were incubated for 1 h at room temperature, and later a spectrophotometer (Biotek) reading at 580 nm was performed. Cumene hydroperoxide (CHP 0.175 mM) was added and the reaction stabilized (15 min). Then, a new absorbance reading (580 nm) was performed. The absorbance difference was determined after adding CHP and dividing the initial absorbance value (sample without CHP) by the final absorbance value (sample with CHP), expressing hydroperoxides as CHP-equivalents per gram of tissue.

Metallothionein (MT)

Metallothionein is a specific indicator protein that indicates the presence of metals. It was measured according to the protocol of Viarengo et al. (1997). The gills (anterior, 2nd pair; and posterior, 6th pair) and a portion of the hepatopancreas of each crab were removed and homogenized at a 3:1 ratio (volume: tissue mass) in a pH 8.6 buffer solution (0.5 M sucrose + 20 mM Tris–HCl + 100 mM PMSF + 0.01% β-mercaptoethanol). The homogenized tissue was centrifuged (21,952 g) with 100 µL of supernatant treated with ethanol (− 20 °C) and 8 µL of chloroform. A new centrifugation was performed (7.168 g; 10 min; 4 °C), and HCl (37%) and pure ethanol were added to the supernatant. The samples were kept at − 20 °C (60 min) and centrifuged in tubes treated with an ethanol-chloroform solution. The pellets were resuspended with 30 µL NaCl (250 mM) and 30 µL EDTA solution (4 mM). The samples (20 µL) were transferred to transparent microplate wells, to which 280 µL of DTNB working solution (1200 mM) was added. The reading was performed by spectrophotometry (412 nm). The metallothionein concentration was estimated by reduced glutathione concentration (GSH) (Viarengo et al., 1997) and quantified in a spectrophotometer using Ellman's reagent at 412 nm.

Condition factor (CF)

For each animal, the individual condition factor (CF_i) was calculated using the equation $CF_i = WW_i/CW_i^b$, based on the ratio between wet weight (WW_i) and individual cephalothoracic width (CW_i). Biometric variables were recorded using a digital scale (0.01 g) and a precision caliper (0.05 mm), respectively. To estimate the growth constant by weight (constant *b*), the data of *WW vs. CW* were adjusted by the equation $WW = a \cdot CW^b$ using the individuals collected. The arithmetic mean of the condition factor (*CF* of the evaluated individuals was calculated for each climatic season. The under study where "*b*" value followed Pinheiro and Fiscarelli (2009) and the procedures adopted by these authors and by Noori et al. (2015). The values of condition factor and the calculation of average values for each area under study followed the same procedures as those indicated by Pinheiro & Fiscarelli (2009) and Noori et al. (2015).

Statistical analyses

Statistical and graphical analyses were performed using the software *RStudio v. 1.2.1335* (R Core Team, 2019) and *Past v. 4.02* (Hammer et al., 2001).

The variables were initially submitted to a normality test (*W*, Shapiro–Wilk) and homogeneity of variances (Levene). In the confirmation of normality ($P > 0.05$), the means were compared by *t*-test (*t*); and if non-parametric, the medians were compared by Mann–Whitney test (*U*). Then, the empirical points of the NRRT vs. MN relationship were submitted to regression analysis by power function ($Y = aX^b$) for the total of ordered pairs obtained ($n = 360$), as well as using the means obtained for each mangrove area by time of year ($n = 18$/each) and total data ($n = 36$). The fitting was established by the coefficient of determination (R^2). The association between the means of the variables NRRT and MN was also assessed by Spearman's correlation coefficient (*r*) with power adjustment. In all cases, a significance level of less than 5% was considered.

The data were also submitted to three-way factorial ANOVA (A, sample area; S, seasonality; and T, tissue) to assess the significance of the effects of these variables and of first- and second-order interactions. The results were represented by means (± standard deviation) for each of the six mangrove areas under study in two climatic seasons (summer and winter). In all cases, the contrast of means was confirmed by Tukey's test "a posteriori."

Relationships between metallothionein (MT) and lipidic peroxidation (LPO) responses were performed based on Spearman's correlations always applied in relation to the same tissue (hepatopancreas, anterior or posterior gills) using the "ggcorrplot" package available in R Environment (Kassambara, 2019).

Only results of the crabs from contaminated areas at the Central Coast of São Paulo state (Cubatão, São Vicente, and Bertioga—see Duarte et al., 2016) were selected to verify the role of this antioxidant defense (MT) in mitigating damage from this sublethal reversible damages (LPO).

Results

The animals used in the analyses ($n=360$) had a carapace width (CW) ranging from 62.3 to 94.2 mm (76.9 ± 5.8 mm) and a total wet weight (WW) ranging from 98.2 to 345.1 g (207.1 ± 42.8 g). Table 1 shows the values obtained for the five biomarkers used. For lipoperoxidation (LPO) and metallothionein (MT), they were higher in hepatopancreas than in gills, although they varied less. There was a greater variation in values obtained for the assays of micronucleus (MN), neutral red retention time (NRRT), and condition factor (CF).

The mean of the condition factor (CF) in the rainy season was $4.6 \cdot 10^{-4} \pm 0.18 \cdot 10^{4}$. It is similar to that of the dry period ($4.8 \cdot 10^{-4} \pm 0.15 \cdot 10^{-4}$), but differs statistically from each other ($F=33.81$, $p<0.0001$).

Genotoxicity (MN) was higher in the rainy season than in the dry season only in Cubatão mangroves. The hierarchical sequence between areas remained the same regardless of climatic season ($U=16{,}232.5$; $P=0.974$), as follows: (SAV = CUB) > (IGU = BET) > (CAN = JUR). Cytotoxicity (NRRT), represented by neutral red retention time (in minutes), presented higher medians (less physiological damage) for Juréia and Cananéia regardless of climatic season (Fig. 2). In this case, there were seasonal differences in the mangroves of Cananéia, Iguape, São Vicente, and Bertioga ($U>450$; $P<0.023$), following the hierarchical order JUR > CAN > (BET = CUB) > IGU > SAV.

There was a negative association between them ($r=-0.66$; $P<0.001$; $n=360$). The calculation of the means of each variable per mangrove area resulted in 36 ordered pairs for this relation, which was represented by the equation $NRRT = 215.16 \cdot MN^{-0.978}$ ($n=36$; $R^2=86.1\%$). The equations for this relation were similar between climatic seasons (Fig. 3) (rainy season: $NRRT = 209.1\ MN^{-0.867}$, $n=18$; $R^2=88.4\%$; and dry season: $NRRT = 228.72\ MN^{-1.11}$, $n=18$; $R^2=94.5\%$). However, in the rainy/summer period, there was a decrease of 10 min in the retention time for neutral red from four micronuclei per thousand ($MN \geq 4$).

All analyzed tissues showed a higher concentration of metallothionein (MT) in the rainy season (anterior gill: 0.183 ± 0.040 µmol GSH mg protein^{-1}; posterior gill: 0.214 ± 0.042 µmol GSH mg protein^{-1}; and hepatopancreas: 0.480 ± 0.084 µmol GSH mg protein^{-1}) compared to the dry season (anterior gill: 0.156 ± 0.033 µmol GSH mg protein^{-1}; posterior gill: 0.166 ± 0.022 µmol GSH mg protein^{-1}; and hepatopancreas: 0.333 ± 0.017 µmol GSH mg protein^{-1}).

The metallothionein (MT) concentrations recorded for each tissue were submitted to the Shapiro–Wilk test (W). They did not show a normal distribution ($0.878 \leq W \leq 0.947$; $0.0001 \leq P \leq 0.0003$). All evaluated tissues showed a significantly higher MT concentration in the rainy season than in the dry season ($U>64$; $P<0.05$) (Table 1).

The reverse was found for concentrations of lipoperoxidation (LPO). There were higher values for the dry season only for branchial tissue in only two mangrove areas (Juréia and São Vicente). In both climatic

Table 1 Values of five biomarkers (LPO, lipoperoxidation; MT, metallothionein; MN, micronucleus frequency; NRRT, neutral red retention time) in the following tissues extracted from *Ucides cordatus*: AG, anterior gill; PG, posterior gill; HP, hepatopancreas; HL, hemolymph

Biomarker	Tissue	Min	Max	$x \pm s$	CV (%)
LPO (nmol CHP/g)	AG	1.85	4.73	3.31 ± 0.69	20.8
	PG	2.14	4.53	3.35 ± 0.64	19.1
	HP	2.63	5.33	3.99 ± 0.73	18.2
MT (µmol GSH × µg protein^{-1})	AG	0.13	0.27	0.17 ± 0.04	22.2
	PG	0.14	0.31	0.19 ± 0.04	23.4
	HP	0.24	0.66	0.41 ± 0.10	24.4
MN (MN cells/1000 cells)	HL	1	7.20	4.12 ± 1.92	46.6
NRRT (minutes)	HL	24.0	143.0	69.1 ± 39.2	56.8
CF ($\times 10^3$)	-	1.67	5.80	3.63 ± 1.90	52.2

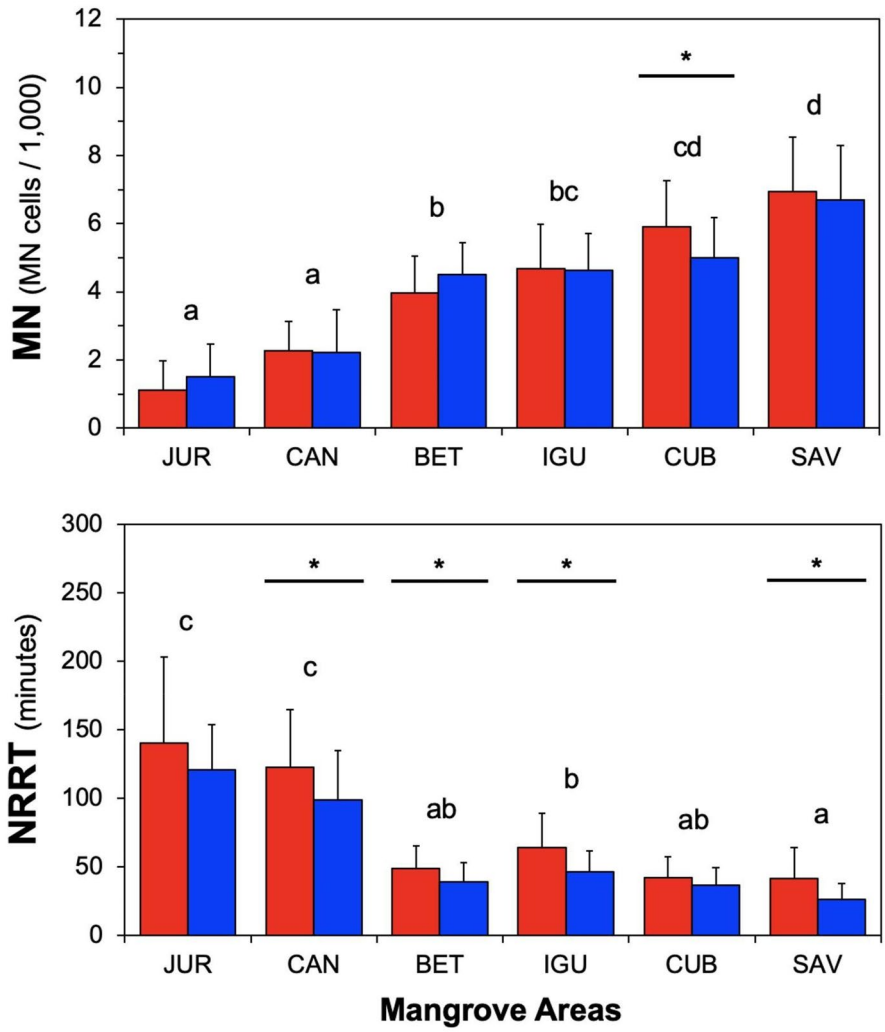

Fig. 2 Biological responses of micronucleus (MN) and neutral red (NRRT) tests of *Ucides cordatus* in mangroves on the coast of the state of São Paulo in two climatic seasons (rainy/summer, red bars; dry/winter, blue bars), where: bar, arithmetic mean; vertical line, standard deviation; lowercase letters associated with bars indicate a statistical difference between the medians recorded for mangrove areas regardless of climatic season; horizontal line with asterisk indicates a seasonal statistical difference between medians of the same mangrove area (Kruskal–Wallis, $P < 0.05$)

seasons, the hepatopancreas showed high levels of lipoperoxidation. However, values were significantly different only for Cananéia (summer > winter). The total data for each tissue were submitted to the Shapiro–Wilk test. It confirmed data normality regardless of tissue ($0.971 \leq W \leq 0.97$; $0.445 \leq P \leq 0.565$). When the means were compared seasonally for each tissue in the study areas, there was only confirmation of statistical difference for branchial tissue (anterior and posterior) of animals in Juréia ($t < -0.88$; $P < 0.013$) and São Vicente (anterior gill) ($t = -1.46$; $P < 0.032$), as well as for the hepatopancreas of the animals of Cananéia ($t = 1.15$; $P = 0.007$).

Responses between metallothionein and lipid peroxidation relationships recorded in all crab' tissues obtained in contaminated areas indicated a negative and significative Spearman's correlation, as follows: posterior gill ($r = -0.47$; $p = 0.0048$) > anterior gill ($r = -0.38$; $p = 0.0042$) > hepatopancreas ($r = -0.17$; $p = 0.024$).

The results of three-way factorial ANOVA were separated for metallothionein (MT) and lipoperoxidation (LPO). For MT, a significant variation effect was revealed for all sources of variation under study: seasonality (code *S*: $F = 202.85$; $P < 0.000$), sampling area (code *A*: $F = 28.17$; $P < 0.000$), and crab tissue (code *T*: $F = 836.45$; $P < 0.000$). This also occurred for first-order interactions (*SxA*: $F = 7.56$; $P = 0.000$; *SxT*: $F = 49.12$, $P < 0.000$, and *AxT*: $F = 2.00$; $P = 0.045$), and for second-order interactions (*SxAxT*: $F = 5.08$, $P = 0.000$) (Fig. 4). Likewise, for lipoperoxidation levels (LPO), there was also a significant

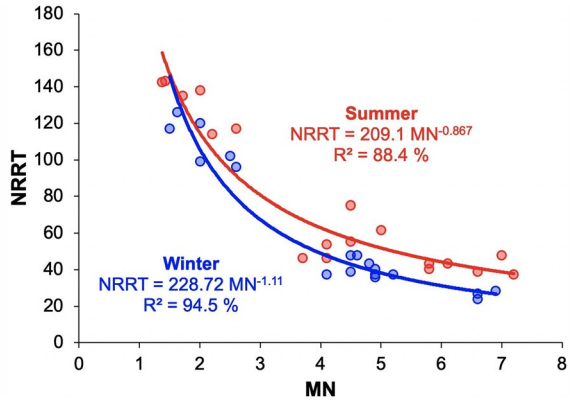

Fig. 3 Seasonality of the ratio NRRT *vs.* MN for *Ucides cordatus*. Equations were obtained by adjusting the power function to the empirical points obtained by calculating the means of neutral red retention time (NRRT, in minutes) by the means of frequency of micronucleated cells (MN, micronucleated cells/1000 analyzed). In each climatic season (summer/rainy season, in red; and winter/dry season, in blue), we used three specimens, which were collected in three subareas for each of the six mangrove areas of the state of São Paulo ($n=18$/weather station)

variation effect for all sources of variation: seasonality ($F=12.51$; $P=0.001$), sampling area ($F=10.70$; $P<0.000$), and crab tissue ($F=22.08$; $P<0.000$). As for the following first-order interactions, there was statistical significance only for interactions that involved seasonality (*SxA*: $F=7.26$; $P=0.000$; and *SxT*: $F=9.04$; $P=0.000$), but not for interactions between areas and tissues (*AxT*: $F=0.401$; $P=0.942$). This also occurred for second-order interactions (*SxAxT*: $F=0.999$; $P=0.452$) (Fig. 5).

Discussion

The most impacted mangrove areas showed a greater genotoxicity and less neutral red retention time in the rainy season (summer). The reverse occurred in pristine mangroves: there was no seasonal effect due to degenerative processes.

Animals from areas with a greater population density and urbanization and industrialization issues (Cubatão, São Vicente, and Bertioga) showed a frequency of micronucleated cells 49% higher than those from mangroves in the south of the state of São Paulo, which are in conservation units (Cananéia, Iguape, and Juréia). Although the greatest genotoxicity occurred in the rainy season (summer), there was no significant seasonal difference. This reveals that, in this case, spatial contamination (area) is possibly more determinant than the genotoxic effect. Our results corroborate several studies carried out in this same region, which have demonstrated the correlation between biological alterations in aquatic organisms and the integrated action of contaminants from different sources. Duarte et al. (2016, 2017, 2020) collected animals at the same points, and observed the responsiveness of *Ucides cordatus* as an indicator species of geno- and cytotoxic effects in the face of contamination by heavy metals, like lead, manganese, copper, mercury, and cadmium. Oliveira et al. (2020) also observed morphological changes in mollusks from sites with different degrees of diffuse contamination in the region. Pinheiro et al. (2013), Pinheiro and Toledo (2010), and Duarte et al. (2016, 2017) related the presence of local environmental contamination to an increased genotoxicity. It is important to highlight the existence of previous reports that confirm an increase in mean micronuclei during the summer for estuarine fish, particularly of the Actinopterygii class (Andrade et al., 2004; Çavas & Ergene-Gozukara, 2005; Fuzinatto et al., 2013; Kirschbaum et al., 2009).

Animals from the ecological station (Juréia), a pristine environment, showed less cytotoxic damage (130.6 ± 50.9 min) compared to areas with high anthropic pressure, as occurs with crabs from Cubatão and São Vicente, which generated the shortest neutral red retention times (39.8 ± 13.8 and 34.0 ± 19.5 min, respectively). Ortega et al. (2017) found that environmental contamination hinders the cell membranes of *U. cordatus*. Animals exposed chronically to contaminants develop defense mechanisms, ranging from an intracellular accumulation of metals, such as cadmium, to an increase in lipid oxidative stress. In this sense, Duarte et al. (2016, 2017) reported similarity in the neutral red retention times for specimens of this species from Juréia and Cananéia. They maintained their cell performance and lysosomal integrity for periods of up to 2 h. In addition, the plasticity of the neutral red assay is known to respond effectively to cytotoxic impacts by diffuse contaminants and contaminants from different sources (organic or inorganic) (Svendsen et al., 2004). Also, there was a significant seasonal difference in 67% of the mangrove areas studied (Cananéia, Bertioga, Iguape, and São Vicente). There is a trend of seasonal changes

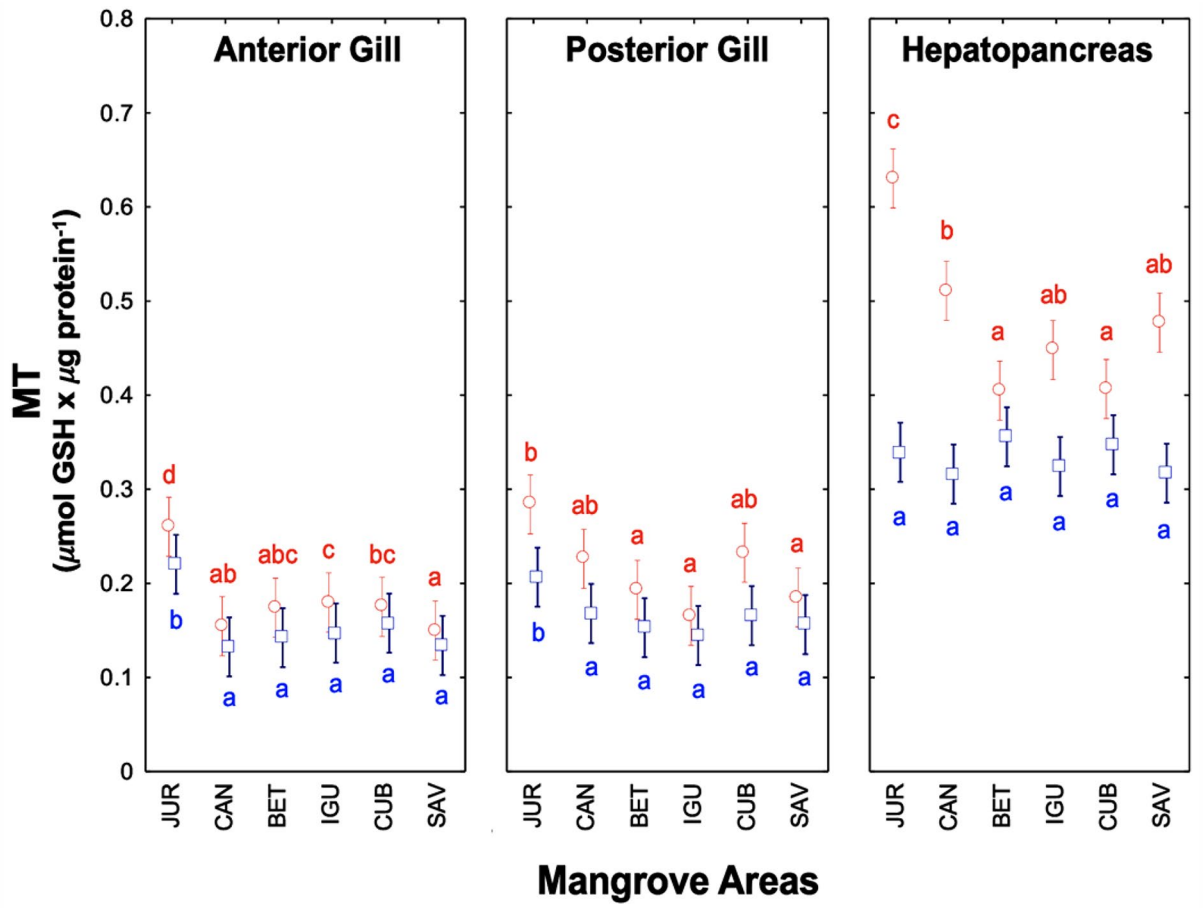

Fig. 4 SxAxT interaction for concentration of metallothioneins (MT) including the effects of seasonality (S: summer/rainy season, red color; and winter/dry season, blue color), mangrove areas (A: JUR, Juréia; CAN, Cananéia; BET, Bertioga; IGU, Iguape; CUB, Cubatão; and SAV, São Vicente), and crab tissues *Ucides cordatus* (T: anterior/posterior gills and hepatopancreas), where: markers (circle, summer; square, winter), arithmetic mean; and vertical lines, standard deviation. Means/standard deviations associated with the same letter of the same color (red/light, summer; or blue/dark, winter) did not differ statistically ($p > 0.05$) according to ANOVA with contrasts by Tukey's test

in mangrove areas belonging to large estuarine systems (Estuary of Cananéia-Iguape-Peruíbe, CIP; and Santos-São Vicente Estuary, STS-SV). This probably occurs due to the potentiation of effects of stressors of various origins, in addition to the natural stress caused by the reduction in water volume during drought. These results are consistent with the contamination history of each mangrove area. They are also evidenced by the significant negative correlation between genotoxicity and cytotoxicity. In addition, there was a reduction in neutral red retention time in the summer possibly due to the higher rainfall during this climatic season, leaching of emerged lands, and increased impacts to organisms, a fact evident in areas with a high anthropic impact, which are the same as that with MN > 4 micronucleated cells per thousand analyzed.

The values presented here for condition factors were similar to those Pinheiro and Fiscarelli (2009) found for *U. cordatus*, as well as those for the freshwater crab *Dilocarcinus pagei*, studied by Pinheiro and Taddei (2005). In the case of *U. cordatus*, Pinheiro and Fiscarelli (2009) observed a decrease in condition factor in dry months (winter) due to the proximity of the molting process, when the animal stops feeding. The opposite occurred in the rainiest months, i.e., from December to March (summer), when the species' energy reserve is invested in

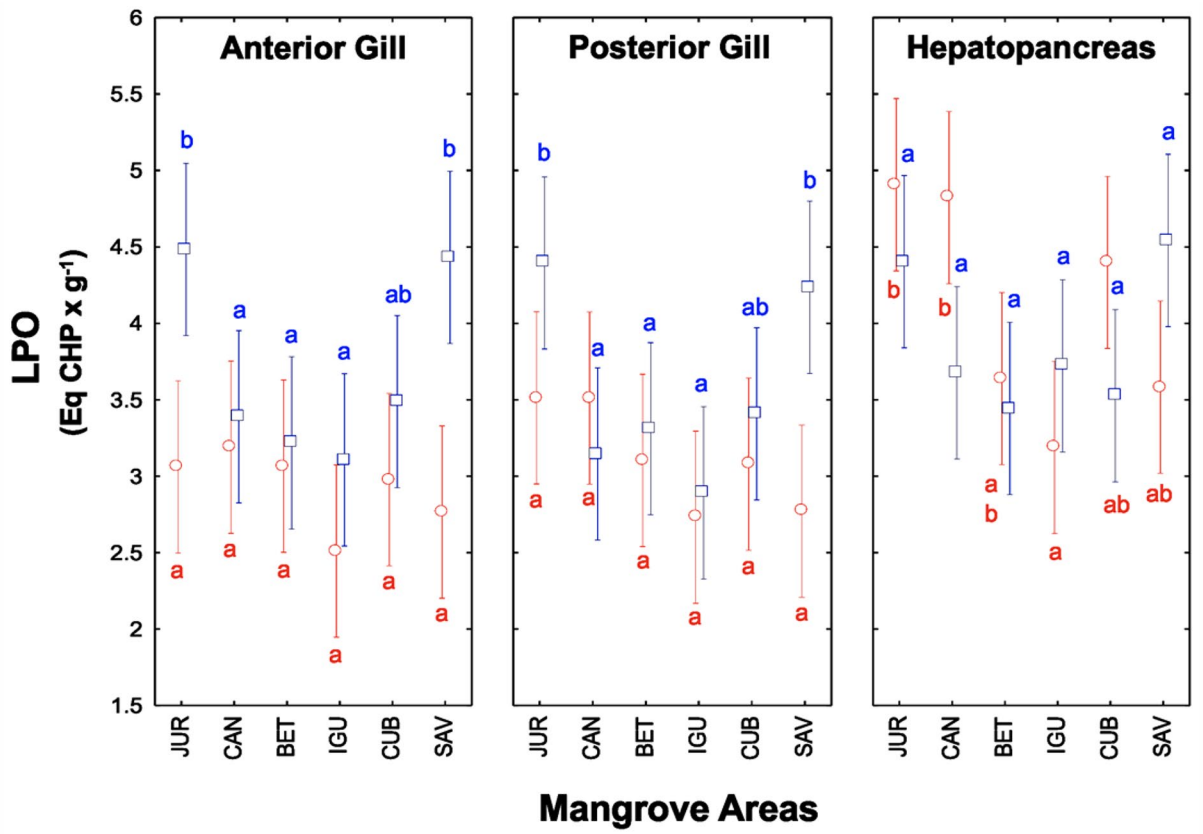

Fig. 5 SxAxT interaction for lipoperoxidation levels (LPO) including the effects of seasonality (S: summer/rainy season, red color; and winter/dry season, blue color), mangrove areas (A: JUR, Juréia; CAN, Cananéia; BET, Bertioga; IGU, Iguape; CUB, Cubatão; and SAV, São Vicente), and crab tissues *Ucides cordatus* (T: anterior/posterior gills and hepatopancreas), where: markers (circle, summer; square, winter), arithmetic mean; and vertical lines, standard deviation. Means/standard deviations associated with the same letter of the same color (red/light, summer; or blue/dark, winter) did not differ statistically ($p > 0.05$) according to ANOVA with contrasts by Tukey's test

reproduction (gonadal maturation and externalization of ovigerous mass). The data also corroborate those of Duarte et al. (2016) for the winter. The condition factor had a decreased explanatory power in determining the status of environmental quality in relation to other biomarkers (MN and NRRT) regardless of the climatic season.

Amiard et al. (2006) pointed out that, due to the variability of inductors in the synthesis of metallothionein, it is always relevant to compare populations chronically exposed to metallic contaminants. Both in this study and Ortega et al. (2017), the levels of metallothionein concentration in all tissues of *U. cordatus* were higher precisely in the ecological station (Juréia), compared to the other areas, regardless of season (rainy or dry). These results do not follow the expected pattern, which would be to record the highest concentrations of metallothionein in animals that live in more anthropized areas under the effects of a myriad of local contaminants, as already evidenced for the Grapsidae *Pachygrapsus marmoratus* and the Portunidae *Carcinus maenas* (Legras et al., 2000; Pedersen & Bjerregaard, 2000; Pedersen & Lundebye, 1996).

We agree with the hypothesis raised by Ortega et al. (2016), which is supported by the metabolic and physiological changes of this species in polluted areas, allowing its survival without requiring the synthesis of metallothionein, which would cause an increase in physiological stress. This is not expected for crabs from mangroves not impacted by metals. According to Amiard et al. (2006), animals that live in polluted

environments are exposed to various contaminants. Adaptation to this chronic exposure is a crucial characteristic for survival. Despite this fact, the concentrations of metallothioneins were significantly higher in all mangrove areas studied during the rainy season (summer). However, this seasonality in the synthesis of metallothioneins can present natural fluctuations, as Bocchetti and Regoli (2006) verified for the mussel *Mytilus galloprovincialis*. Higher concentrations of metallothioneins in the summer, mainly associated with the digestive gland, have already been described for other organisms that were used to confirm environmental quality, especially mollusks (Bocchetti & Regoli, 2006; Lavradas et al., 2016; Viarengo et al., 1997). Our results corroborate these studies, now for the 'uçá'-crab. We indicate that the temperature and the reproductive period of a species can cause seasonal fluctuations in antioxidant defenses (Lavradas et al., 2016; Orbea et al., 2002). The 'uçá'-crab has its reproductive period associated with months of increases in temperature and photoperiod, as well as in the rainiest periods (Dalabona et al., 2005; Pinheiro & Fiscarelli, 2001), which would explain the increase in this protein used as a biomarker during the summer because of increased metabolism. In addition, Engel and Brouwer (1993) suggested that natural fluctuations in the levels of metallothioneins in crustaceans may occur due to their participation in the Cu/Zn-MT regulatory system in each molting phase, decreasing in the premolting phase. The presence of pollutants, in this case, can potentiate the modulation of seasonal fluctuations in antioxidants, inducing different responses from polluted and unpolluted sites (Bocchetti & Regoli, 2006).

Another relevant aspect is the values recorded in the sampling areas of the present study. Regardless of the climatic season, they showed a decreasing order of concentration of metallothioneins in organs, namely: hepatopancreas > posterior gill > anterior gill. This confirms that tissues directly involved in uptake, storage, and excretion have a higher capacity for metallothionein synthesis (Amiard et al., 2006). Ortega et al. (2017) observed this fact regardless of the pollution status of mangroves for the Portunidae *Carcinus maenas* (Pedersen et al., 1997) and for the lobster *Nephrops norvegicus* (Canli et al., 1997). As for the difference in concentrations between gills, the results indicate the posterior gill—with an osmoregulatory function, according to Martinez et al. (1999)—as the second tissue with the highest concentration of metallothioneins and, therefore, with a relevant role in the detoxification of this hyper-hypo-regulator animal (Leite & Zanotto, 2013; Ortega et al., 2014). Analyses of organelles and vacuoles in the cytoplasm of posterior gill cells of *U. cordatus* suggest an important role in the sequestration and immobilization of toxic metals to the organism, as Ortega et al. (2014, 2017) and Duarte et al. (2019) verified for cadmium.

Biotransformation and detoxification processes generate reactive oxygen species (ROS) (Bhatti et al., 2019). Antioxidant defenses, like metallothionein, have the capacity against lipid peroxidation (LPO) damages in crustaceans (Faria et al., 2018). The present study has confirmed the role and effectiveness of metallothionein as an antioxidant defense because the results recorded indicate the significative and negative relationship with lipid peroxidation in different organs in contaminated areas. Our results are in favor of the pertinent literature.

The highest values of lipid peroxidation occurred during the winter, although with no defined pattern, compared to mangrove areas with different anthropogenic and pollutant levels. During winter, the concentration of LPO in anterior gills was frequently higher than that in posterior gills, but not than that in hepatopancreas, regardless of the climatic season. In anterior gills, whose main function is breathing (see Martinez et al., 1999), the high concentration of oxygen can produce free radicals, increasing the lipid peroxidation process (Wang et al., 2013). In addition, as it is a sensitive organ in primary contact with water, it is more susceptible to changes (Maciel et al., 2010). As one of the functions of the hepatopancreas is detoxification, marked values would be expected, a fact confirmed for *U. cordatus* (Ortega et al., 2016) and the Anomura crustacean *Lithodes santola* (Schvezov et al., 2015). Studies previously carried out on crustaceans and fish have indicated that low temperatures can also induce oxidative stress (Niyogi et al., 2001; Qiu et al., 2011; Wang et al., 2006), since the enzymatic activity suffers damage in such a situation.

Multi-level impact analyses of the 'uçá'-crab (physiological, cellular, genetic, and population), in addition to a list of tests sensitive to local contamination, are particularly relevant to understand the conservation status of the mangroves evaluated. This multivariate analysis results indicate that hepatopancreas was the best bioindicator tissue for environmental contamination due to its sensitivity to metallothioneins and

lipoperoxidation. In addition, under the same analysis, we recommend using micronucleus and neutral red biomarkers during the dry season (winter), when there is no interference from other stress sources, such as the reproductive period of this species, which occurs during the rainy season (summer). According to Lavradas et al. (2016), reproductive efforts can generate reactive oxygen species (ROS), since there is an increase in metabolic activity and, therefore, greater susceptibility to certain abiotic factors, such as hyperthermia, hypoosmotic stress, among others.

The variation in LPO and MT concentrations observed in the present study cannot be explained only by the xenobiotic substances present in mangroves, since there is a myriad of contaminants there, with different actions on organisms. This study demonstrates through the negative correlation presented by these markers, that it may represent an attempt to defend this organism. Furthermore, it is worth noting that the antioxidative system is not limited to MT, just as oxidative stress does not end with the effects of lipid peroxidation. Thus, the natural variability of these biomarkers in the species requires caution during the evaluation.

Conclusions

The approach used to establish the environmental quality of mangroves in the Western Atlantic are successful, as they relate to the history of chemical contamination in these areas. The multi-level biomarkers evaluated followed the hierarchical order of environmental quality for the six mangrove areas in the state of São Paulo: Juréia > Cananéia > (Iguape = Bertioga) > (Cubatão = São Vicente). Seasonality influences sublethal damage to which the sentinel species is subjected. The rainy season (summer) is the most harmful to the population, mainly due to cytogenotoxicity. Therefore, we recommend using the micronucleus and neutral red biomarkers together during the dry season (winter) to assess the quality of mangrove areas more effectively. Thus, environmental monitoring using biomarkers as tools should consider the time of year and season to obtain reliable results. In this sense, this information provides subsidies for environmental monitoring, refinement, and use in technological packages when classifying mangrove areas' conservation status.

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