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Chronic toxicity after the oil spill on the Brazilian coast based on ecotoxicological biomarkers in the reef fish *Stegastes fuscus* (Cuvier, 1830)

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ABSTRACT

The Brazilian coast was affected by crude oil in 2019 that contaminated coral reef areas. Oil Polycyclic aromatic hydrocarbons (PAHs) are responsible for triggering sublethal toxicity in fish. This study aimed to evaluate chronic effects and recovery of the keystone damselfish *Stegastes fuscus* after exposure to crude oil in October 2019. Fish were sampled from seven oil affected reef areas and one non-affected, 17, 24 and 34 months after the oil spill. Analysis of biliary PAHs, biochemical and genotoxic biomarkers were carried out. Biliary PAHs in fish from Paiva and Suape areas were significantly reduced 17 months after the oil spill. Biliary PAHs and ethoxyresorufin-O-deethylase (EROD) activity in fish sampled from Janga, Paiva, Suape, Serrambi, Carneiros and Mamucabas reefs were higher than in specimens from Cupe reference area. Male and immature individuals presented higher activities than females for EROD, Glutathione S-transferase and catalase and lower values for superoxide dismutase and glutathione. Increased micronuclei frequencies were observed in Paiva, Suape, Serrambi and Carneiros samples. The results indicate that these reef areas are not being influenced by the 2019 oil spill. Contamination by PAHs occurs chronically in these locations such as the urbanized Janga reef area, where the highest bile PAH concentrations and EROD induction were detected. The integrated index of biomarker responses indicated changes in biochemical biomarkers in all reef areas related to Cupe, possibly reflecting exposure to other unmeasured contaminants. Continued monitoring with the species *Stegastes fuscus* is necessary to obtain information on the contamination status of these environments over the years.

1. Introduction

The northeastern Brazilian coast was affected by oil in late August 2019, and in the months following the first event approximately 5379 tons of oil residue were removed from these locations (Soares et al., 2023). This oil spill event was considered the most extensive and severe ever recorded on the Brazilian coast (Lira et al., 2021) and reached several coastal marine habitats, such as: coral reefs, tidal flats, seagrass meadows and mangroves (Magris and Giarrizzo, 2020; Nunes et al., 2023).

The oil traveled thousands of kilometers along the Brazilian continental margin, carried by the North Brazil Current and the Brazil Current, until it reached the coast, through maritime drift, waves and tidal currents (Müller et al., 2021). Brazil is considered susceptible to oil spills due to some factors in the region where it is located, such as oil production, ocean circulation, high vessel traffic between the Caribbean, Africa and Europe, and lack of an ocean surveillance system (Magalhães et al., 2022; Soares et al., 2023; Soares et al., 2022).

The oil masses that reached the Brazilian coast had a viscous appearance, denser than seawater, and weathering processes may have

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affected the original composition of the oil (Lourenço et al., 2020). Oil appearance was very similar to typical oil mousse formed after emulsification, which is also typical to occur with Venezuelan oil (Melo Alves et al., 2024). The oil reached Costa dos Corais Marine Protected Area, the first federal conservation unit established to protect Brazilian reefs in the Northeast, characterized by high rates of endemism, biological richness, provision of ecosystem services, but vulnerable to local and global impacts (Soares et al., 2020).

After the extensive removal of oil from the Brazilian coast between October 2019 and early 2020, sporadic resurgence of oil was observed on beaches in the States of Sergipe and Pernambuco in 2021 and 2022, both in northeast region of Brazil. Studies suggest that the oil found in both locations was linked to the original spill in 2019 (Lima et al., 2023; Lourenço et al., 2023), which may indicate that some of the oil was trapped in subtidal sediments or in hard structures such as reefs and was later transported to the coast by ocean currents (Lourenço et al., 2023).

Among the compounds that constitute crude oil, polycyclic aromatic hydrocarbons (PAHs) are those that cause the most environmental concern due to their ubiquity, toxicity and persistence in aquatic environments, presenting low solubility in water and high lipophilicity (Costa et al., 2023; Khan et al., 2023). The United States Environmental Protection Agency identifies 16 parent PAHs as priority pollutants due to their toxicity. Furthermore, derivatives of PAHs, including nitro-PAHs, oxy-PAHs, halogenated and alkylated PAHs, are gaining notoriety for being potentially more toxic and bioavailable than their parent compounds (Peng et al., 2023; Yuan et al., 2015). Water samples from oil-affected sites in the Northeast region of Brazil showed an abundance of low molecular weight and alkylated parent PAHs (Melo Alves et al., 2024), a typical characteristic of petrogenic contamination (Wang et al., 1999).

Fishes are used in monitoring studies that evaluate biological responses resulting from exposure to environmental contaminants such as PAHs (Dearnley et al., 2022). Sublethal effects caused by exposure to PAHs at various levels of biological organization in fish have already been documented, including delays in embryolarval development, biochemical, histopathological, cardiac, genetic, reproductive and behavioral changes (Alves et al., 2017; Collier et al., 2013; Incardona et al., 2015; Johansen et al., 2017; Mariz et al., 2024; Martínez-Gómez et al., 2006; Payne et al., 2003; Torreiro-Melo et al., 2015).

The metabolites of PAHs are temporarily stored in the gallbladder until they are directed along with the bile to the digestive tract after eating (Silva et al., 2021). Quantification of PAH metabolites stored in the gallbladder is an important biomarker of exposure and potential deleterious effects on fish health (Dearnley et al., 2022).

The activity of ethoxyresorufin-O-deethylase (EROD) is considered sensitive for determining the inductive response of cytochrome P4501A1 (CYP1A1) and several field studies have demonstrated the suitability of analyzing the enzymatic kinetics of EROD in episodes of contamination by PAHs and oil spills (Martínez-Gómez et al., 2006). Glutathione S-transferase (GST) is responsible for catalyzing the conjugation of glutathione (GSH) with hydroxylated metabolites in phase I (Santana et al., 2018). Analysis of GST activity is also suggested as a sensitive biomarker for petroleum contamination (Kerambrun et al., 2012).

Phase 1 and 2 biotransformation of PAHs has the potential to generate reactive oxygen species (ROS) (Ballesteros et al., 2017), and antioxidant defense enzymes have the function of neutralizing or degrading ROS, such as catalase (CAT) and superoxide dismutase (SOD), which are used as biomarkers of exposure to PAHs (Santana et al., 2018). When ROS are not neutralized, oxidative stress occurs in cells and results in oxidative damage such as DNA strand breakage, lipid peroxidation, and enzyme protein inactivation (Pan et al., 2022). Inhibition of brain acetylcholinesterase (AChE), an enzyme important for neural transmission, is also used as a biomarker of neurotoxicity in fish exposed to PAHs (Briaudeau et al., 2021).

The incidence of micronuclei in fish erythrocytes has been used as a tool to monitor genotoxic effects in organisms exposed to PAHs (Shirmohammadi et al., 2018). The micronucleus assay is one of the most widely used methods to assess genotoxicity, due to its speed, sensitivity and low cost (Araújo et al., 2023).

Stegastes fuscus (Cuvier,1830) is an endemic fish occurring along the entire Brazilian tropical coast and is considered a key species in reef environments, as it controls algae biomass, increasing productivity in these areas (Araújo et al., 2020; Feitosa et al., 2012). Furthermore, it is not a migratory species and has a long life and slow growth, reaching around 15 years of age (Daros et al., 2016). The species was considered suitable for studies on biological effects caused by exposure to crude oil, as it is a reef resident and is in constant interaction with reef environments. In a previous study, PAHs in water samples from 2019 oil-hit sites, biliary PAHs, and biochemical biomarker responses in liver and brain tissue in the reef fish species *S. fuscus* analyzed shortly after the 2019 oil hit the coast indicated that contamination by PAHs occurred in the areas affected by the oil spill, together with effects on fish exposed to the oil with significant biliary bioconcentration of PAHs and changes in biochemical biomarkers (Melo Alves et al., 2024). Long-term monitoring studies of affected areas are essential to understanding the chronic effects caused by oil contamination and the state of conservation of these environments.

Therefore, this study aims to evaluate chronic effects and recovery of the keystone fish species *Stegastes fuscus* after exposure to crude oil in October 2019. Resident individuals from seven oil affected coral reef areas were sampled 17, 24 and 34 months after the oil spill, followed by analysis of biliary PAHs, biochemical and genotoxic biomarkers.

2. Materials and methods

2.1. Study areas

The study was carried out in seven reef areas in the state of Pernambuco that were hit by crude oil in October 2019 and one reef area that was not hit. The areas affected by the oil and monitored were located in site 1: Janga Beach; site 2: Paiva Beach; site 3: Suape Port; site 4: Muro Alto Beach; site 5: Cupe Beach; site 6: Carneiros Beach, and Site 7: Mamucabas reef area. Site 8 is in Serrambi beach, a region where oil was not seen (Fig. 1). Further details of the sites are provided in Table 1.

2.2. Fish sampling

Stegastes fuscus individuals were sampled in the eight reef areas from all sampling sites during three distinct periods, named T1, T2, and T3 (Table 1). The first one occurred between March 10 and 17, 2021 (T1, 17 months after the peak of the oil spill), when precipitation ranged from 220 to 278 mm (APAC, 2022), characterized as a rainy season. The second campaign took place between October 5 and 19, 2021 (T2, 24 months after the oil spill), when precipitation ranged from 10.5 to 34.5 mm (APAC, 2022), considered a dry season. The third campaign took place between August 11 and 29, 2022 (T3, 34 months after the oil spill), when precipitation ranged from 174 to 241.5 mm (APAC, 2022), considered a rainy season (Table 1).

Individuals of *S. fuscus* were captured at low tide using fish rods with small hooks and shrimp as fresh bait. Fish were collected from tide pools during low tide at similar depths between sites. The depth of the sampling areas ranged from 1.0 to 1.5 m. Captured fish were kept alive in floating cages until the end of sampling, typically less than two hours. The fish were then transported alive to the laboratory in water from the collection site with aerators and at a low density (1 fish/8 L). In the laboratory, the fish were sacrificed by individually immersing them in ice-cold seawater at a temperature between 2 and 4 °C. The fish remained submerged until opercular movements ceased, followed by quick spinal cord sectioning with sharp scissors. Blood was collected, followed by the preparation of blood smears. Subsequently, the total weight, standard and total length and sex of the individuals were

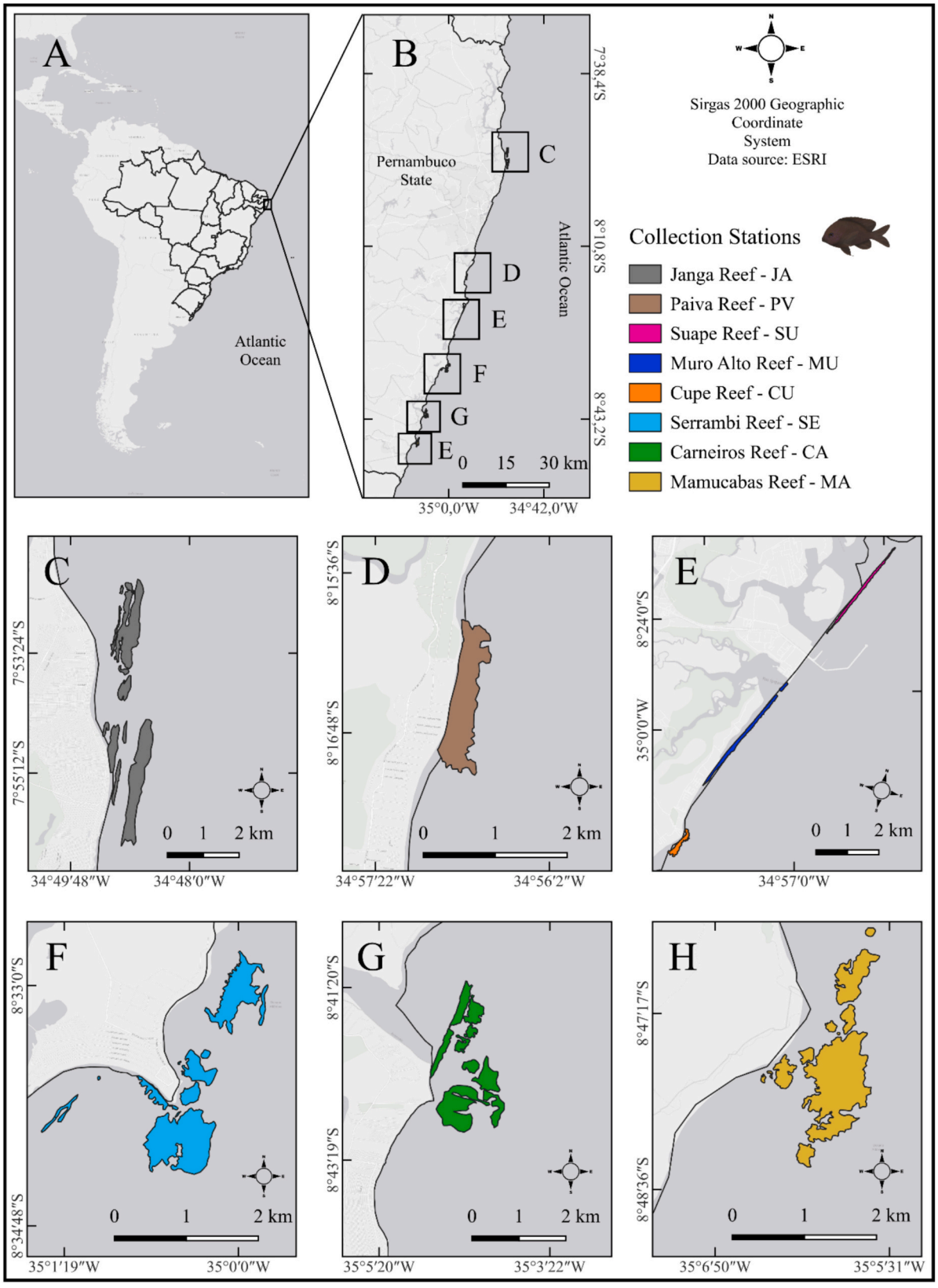

Fig. 1. Map showing the *Stegastes fuscus* collection stations across eight reef areas along the coast of Pernambuco State, from March 2021 to August 2022. (A) South America with Brazil outlined in black; (B) Coast of Pernambuco State; (C) Janga Reef (JA); (D) Paiva Reef (PV); (E) Suape Reef (SU, pink), Muro Alto Reef (MU, dark blue), and Cupe Reef (CU, orange); (F) Serrambi Reef (SE); (G) Carneiros Reef (CA); (H) Mamucabas Reef (MA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Information on sampling sites, number of samples and morphometric data (mean ± standard deviation) of *Stegastes fuscus*.

Coral reef area	Coordinates	Dates of collection	Months since the 2019 oil spill	Season	Anthropic influence	Total number of samples	Number of females	Number of males	Number of immature	Total weight	Standard length
Janga (JA-T1)	7°55'11.09"S, 34°49'8.41"W	March 2021	17	Rainy	Urban	20	8	12	0	36.7 ± 6.9	8.9 ± 0.5
Janga (JA-T2)		October 2021	24	Dry		20	14	6	0	39.4 ± 6.9	8.9 ± 0.6
Janga (JA-T3)		August 2022	34	Rainy		17	8	9	0	34.7 ± 8.3	8.7 ± 0.5
Paiva (PV-T1)	8°16'56.7"S,34°56'48.6"W	March 2021	17	Rainy	Urban river effluents	20	2	7	11	23.8 ± 5.9	7.8 ± 0.6
Paiva (PV-T2)		October 2021	24	Dry		20	10	8	2	25.2 ± 3.2	7.8 ± 0.4
Paiva (PV-T3)		August 2022	34	Rainy		15	2	0	13	30.6 ± 4.1	9.2 ± 0.5
Suape (SU-T1)	8°22'51.4"S, 34°57'14.30"W	March 2021	17	Rainy	Port Industrial Complex	20	3	16	1	51.1 ± 9.8	9.8 ± 0.7
Suape (SU-T2)		October 2021	24	Dry		20	7	13	0	43.4 ± 6.6	9.4 ± 0.6
Suape (SU-T3)		August 2022	34	Rainy		15	5	10	0	53.7 ± 12.9	10.9 ± 1.0
Muro Alto (MU-T1)	8°25'58.89"S, 34°58'42.83"W	March 2021	17	Rainy	Tourist	20	2	14	4	35.1 ± 7.2	8.8 ± 0.5
Muro Alto (MU-T2)		October 2021	24	Dry		20	10	10	0	32.1 ± 5.5	8.6 ± 0.5
Muro Alto (MU-T3)		August 2022	34	Rainy		15	9	6	0	41.6 ± 7.7	10.4 ± 0.8
Cupe (CU-T1)	8°27'21.64"S, 34°58'57.60"W	March 2021	17	Rainy	Tourist	20	5	8	7	36.7 ± 8.3	9.1 ± 0.5
Cupe (CU-T2)		October 2021	24	Dry		20	12	8	0	33.6 ± 3.6	8.7 ± 0.3
Cupe (CU-T3)		August 2022	34	Rainy		20	7	2	1	31.6 ± 4.1	8.7 ± 0.4
Serrambi (SE-T1)	8°33'47.0"S,35°0'25.0"W	March 2021	17	Rainy	Tourist	20	7	7	6	26.6 ± 6.3	8.0 ± 0.7
Serrambi (SE-T2)		October 2021	24	Dry		20	6	14	0	34.2 ± 5.3	8.7 ± 0.3
Serrambi (SE-T3)		August 2022	34	Rainy		15	5	8	2	33.9 ± 8.0	9.4 ± 0.7
Carneiros (CA-T1)	8°42'7.53"S, 35° 4'40.15"W	March 2021	17	Rainy	Tourist	20	5	12	3	32.7 ± 6.2	8.5 ± 0.5
Carneiros (CA-T2)		October 2021	24	Dry		20	8	12	0	46.0 ± 8.4	9.6 ± 0.7
Carneiros (CA-T3)		August 2022	34	Rainy		15	8	7	0	43.6 ± 8.2	10.8 ± 0.6
Mamucabas (MA-T1)	8°47'3.77"S, 35° 6'8.12"W	March 2021	17	Rainy	Tourist and urban river effluents	20	6	14	0	36.9 ± 5.3	9.1 ± 0.6
Mamucabas (MA-T2)		October 2021	24	Dry		20	8	10	2	37.7 ± 6.1	9.0 ± 0.5
Mamucabas (MA-T3)		August 2022	34	Rainy		15	7	8	0	36.6 ± 4.6	10.6 ± 0.5

recorded. Sampled individuals were within similar size ranges to avoid sampling individuals that were not yet at reproductive stages. Sex can be determined in *S. fuscus* only after dissection, and stages of reproduction were classified based on the development of their gonads, when they were identified as females or males when respective gonads were visible, or immature when individuals that did not have developed gonads. The gallbladder, liver and brain were removed, stored in microtubes and kept in a -80 °C freezer until the analyses were performed. Sampling was authorized by SISBIO 73228-2 (Biodiversity Authorization and Information System). The procedures performed were approved by the Animal Experimentation Ethics Committee of the Federal University of Pernambuco and in accordance with international guidelines guidelines (AVMA, 2020; NRC, 2011; Percie du Sert et al., 2020).

2.3. Fish densities based on visual census

The density of *S. fuscus* in the sampled areas was assessed by the visual census method by strip transect adapted from (Brock, 1954; Samoilys and Carlos, 2000). Fish were counted in a specific area using transects 10 m long by 2 m wide, whose length was measured with a tape measure. The species was recorded during 6 visual censuses in each sampling area, divided into two distant sectors to increase the spatiality of the sampling. The transects were oriented parallel to the coastline, except when carried out in natural pools, where they followed the edge of the pools. Among all the sample areas of the study, only Suape, Muro Alto and Carneiros did not have the necessary visibility to carry out fish quantification methods by visual census, due to the local turbidity of the water.

2.4. Fluorimetric quantification of polycyclic aromatic hydrocarbons in bile

PAH metabolites in bile were analyzed using the fixed wavelength fluorescence (FF) method (Aas et al., 2000). Bile analyses were performed after diluting the samples at a ratio of 1:1000 (v/v) in HPLC-grade methanol and 50 % (v/v) ultrapure water. Fluorescence readings in bile were analyzed in duplicate in 1 cm path length quartz cuvettes (Hellma, Germany) on a Spectramax M3 spectrofluorometer

(Molecular Devices). The excitation/emission pairs for the PAHs analyzed were 290/335 nm for naphthalene (NAP_{bile}), 340/382 nm for pyrene (PYR_{bile}) and 380/430 nm for benzo(*a*)pyrene (BAP_{bile}) (Aas et al., 2000), 260/380 nm for phenanthrene (PHE_{bile}) (Krahn et al., 1984; Krahn et al., 1993), and 310/360 nm for chrysene (CHR_{bile}) (IOC, 1984). Mean fluorescence of two replicates of 500 μL per bile sample was expressed as fluorescence units relative to HPLC grade methanol/ultrapure water 50 % (v/v) blanks, or relative fluorescence units (RFU). The tests for the use of bile dilution at a ratio of 1:1000 and analysis of the linearity of increasing fluorescence signals ($R^2 > 0.98$) were performed in our previous study (Melo Alves et al., 2024).

2.5. Biochemical biomarkers

The liver and brain tissues of the fish were homogenized in a phosphate buffer solution (pH 7.4) in a 1:5 ratio (mass/volume). The samples were then centrifuged for 30 min at 9000 xg at 4 °C using a refrigerated centrifuge (model 5415R, Eppendorf). The supernatant (fraction S9) was transferred to microtubes and stored at −80 °C for later analysis of biochemical biomarkers. Biochemical assays were performed using a microplate reader (model Spectramax M3, Molecular Devices) set at 25 °C.

Quantification of total proteins was performed using the method proposed by Bradford (1976), based on a calibration curve using bovine serum albumin (BSA), in concentrations between 0 and 5 μg of BSA/well ($R^2 > 0.99$).

Enzyme kinetics analysis was performed using methods adapted for 96-well microplates. Ethoxyresorufin-O-deethylase (EROD) activity was measured by the method of Hahn et al. (1993) adapted for fluorescence microplate as described in Silva et al. (2021). Glutathione S-transferase (GST) was analyzed by the method of Habig et al. (1974), and acetylcholinesterase (AChE) by the method of Ellman et al. (1961). Lipid peroxidation (LPO) was quantified by the TBARS (thiobarbituric acid reactive substances) assay Utley et al. (1967), adapted by Adeyemi et al. (2014). The tests of the mentioned biochemical biomarkers were performed and calculated according to the detailed methodology described in Melo Alves et al. (2024).

Superoxide dismutase (SOD) activity was measured as proposed by Marklund and Marklund (1974). The reading was performed by adding to each well 50 μL of the liver S9 fraction, 55 μL of ultrapure water, 140 μL of 0.05 M tris HCl/1 mM EDTA assay buffer at pH 8.5 and 6 mM pyrogallol in 0.05 M HCl. The activity was expressed in U SOD min^{-1} mg $protein^{-1}$.

Quantification of reduced glutathione (GSH) was performed according to the method proposed by Beutler et al. (1963). The reading was performed after adding to each well 25 μL of fraction S9, 50 μL of 2.5 Mm 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 175 μL of 0.1 M potassium phosphate buffer, pH 7.4. The reaction was measured by absorbance at 412 nm and applied to an analytical curve ranging from 5 to 400 μM GSH ($R^2 > 0.99$). The GSH concentration was calculated as μg GSH mg $protein^{-1}$.

2.6. Genotoxic biomarkers

Blood smears were prepared immediately after the fish were euthanized, followed by a small incision in the dorsal region of each individual, removing approximately 0.5 mL of peripheral blood with the aid of a pipette containing heparin solution (Hemofol®, Cristália, Itapira, SP, Brazil). Two slides with blood smears were prepared for each individual to ensure that analysis could be performed on at least one of them. After drying for five minutes at room temperature, the slides were fixed for approximately ten minutes in absolute methanol (purity ≥99.9 %, Merck, n° 1.06007.4000, Darmstadt, Germany), then washed in distilled water and stained with pure Giemsa (Sigma-Aldrich, n° 1.09204, St. Louis, MO, EUA) for five minutes, followed by a new wash to remove excess dye and grouped again for drying. Blood smears were analyzed under optical microscopy (Labomed, LX 400), using a 100× objective and the occurrence of cells presenting extra-nuclear bodies (micronuclei) and nuclear morphological alterations called "blebbed" nuclei, "notched" nuclei, "vacuolated" nuclei and binucleated cell, as described by Carrasco et al. (1990) and Cavaş and Ergene-Gözükara (2005). Genomic damage was analyzed in 3000 erythrocytes per individual. Medians of nuclear morphological alterations were calculated for each site. Total genomic damage was measured from the sum of micronuclei and nuclear anomalies counted per individual (Araújo et al., 2023).

2.7. Integrated Biomarker Response Index

The "Integrated Biomarker Response Index version 2 individual (IBRv2i)", proposed by Mattos et al. (2024), was used to combine the responses of the analyzed biochemical biomarkers while preserving the variability of the generated data. In order to calculate the IBRv2i, the log2 of the ratio between the response of each biomarker of the samples from the different areas studied and the average response of the samples from the reference site was obtained. After this, the scale was standardized (*Z*-scoring), obtaining the individual variation of the biomarkers and the values for each biochemical endpoint were returned to the absolute value. Then, the sum of the biomarkers was performed (IBRv2i) (Mattos et al., 2024). An IBRv2i was calculated for each sampling period (T1, T2, and T3) at the eight study sites, using samples where all biochemical biomarkers were evaluated. The biochemical biomarkers used for the calculation were: EROD, GST, CAT, SOD, GSH, AChE, and LPO.

2.8. Statistical analysis

Statistical comparisons were assessed using permutational multivariate analysis of variance (PERMANOVA). Comparisons were made between samples from the same area collected at different times and between samples from reef areas in relation to the reference site. The analyses of data related to biliary PAHs, biochemical biomarkers and genotoxicity took into account the fixed factors 'area', 'time' and 'sex', based on a Euclidean distance similarity matrix using 9999 permutations, and also Monte Carlo tests. Significant differences were based on an alpha level of 0.05. When differences between the sexes were identified in the evaluated parameters, the statistical analysis was performed separately for females and males.

Comparative analyses of the integrated response index were performed using the fixed factor 'area'. Pair-wise comparisons were performed when significant differences were detected in the biomarkers in relation to the sampling sites and times. The analyses were performed using PRIMER 6.1 + PERMANOVA software. Pearson correlation coefficients were calculated between the parameters weight, standard length, relative fluorescence units, biochemical biomarkers, micronuclei, nuclear abnormalities and sum of nuclear abnormalities.

3. Results

3.1. Quantification of PAH equivalents in bile

Significant sex differences were not found between samples for biliary PAHs. Analysis of PAHs accumulated in bile indicated that among the 8 sampled areas during the study, the lowest medians for NAP_{bile}, PHE_{bile}, CHR_{bile}, PYR_{bile} and BaP_{bile} were verified in the samples from the Cupe Beach Reef Area, when compared to what was quantified in the other locations. For this reason, Cupe reef area was chosen as the regional reference, and the median of biliary PAHs of the samples from the three sampling collections at this site was used for statistical comparisons (green line in Fig. 2).

Comparison between areas indicated higher NAP_{bile} (Fig. 2A) in reef areas of Janga (T1 and T3), Paiva (T1, T2 and T3), Suape (T1), Muro Alto

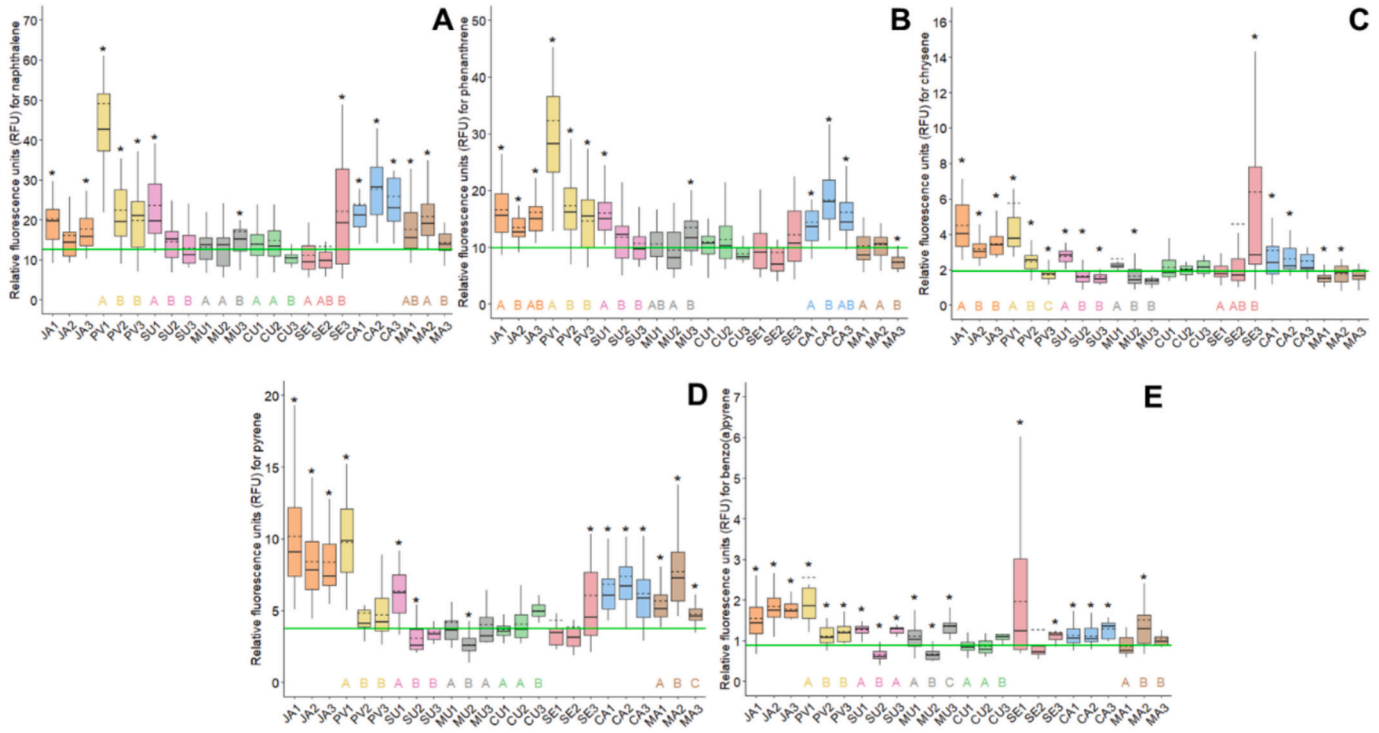

Fig. 2. Relative fluorescence units (RFU) for naphthalene, phenanthrene, chrysene, pyrene, and benzo(*a*)pyrene in *Stegastes fuscus* bile samples in the reef areas of Janga (JA), Paiva (PV), Suape (SU), Muro Alto (MU), Cupe (CU), Serrambi (SE), Carneiros (CA), and Mamucabas (MA). Numbers 1, 2 and 3 after the reef area code indicate samples from March 2021, October 2021 and August 2022, respectively. The green line indicates the median for the data from the Cupe regional reference reef area.
*: significant difference in relation to Cupe regional reference area (Permanova, fixed factor area, $p < 0.05$). Significant temporal differences within each site are indicated by capital letters below the box plot; a different letter colour was used for each site (Permanova, fixed factor time, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(T3), Serrambi (T3), Carneiros (T1, T2 and T3), and Mamucabas (T1 and T2), compared to Cupe. Furthermore, comparison of samples from the same location at different sampling times indicated higher NAP_{bile} in samples from Paiva and Suape in T1. Samples from Muro Alto and Serrambi indicated higher NAP_{bile} in T3. Samples from Cupe and Mamucabas indicated a reduction in NAP_{bile} in T3.

Comparison between areas indicated higher PHE_{bile} (Fig. 2B) in reef areas of Janga (T1, T2 and T3), Paiva (T1, T2 and T3), Suape (T1), Muro Alto (T3), and Carneiros (T1, T2 and T3), compared to Cupe (Fig. 2B). A comparison of samples from the same location at different sampling times indicated higher PHE_{bile} in samples from Janga, Paiva and Suape in T1, Muro Alto in T3 and Carneiros in T2. Samples from Mamucabas indicated a reduction in NAP_{bile} in T3.

Comparison between areas indicated higher CHR_{bile} (Fig. 2C) in reef areas of Janga (T1, T2 and T3), Paiva (T1 and T2), Suape (T1), Serrambi (T3), and Carneiros (T1 and T2), compared to Cupe (Fig. 2C). A comparison of samples from the same location at different sampling times indicated higher CHR_{bile} in samples from Janga, Paiva, Suape and Muro Alto in T1 and Serrambi in T3.

Comparison between areas indicated higher PYR_{bile} (Fig. 2D) in reef areas of Janga (T1, T2 and T3), Paiva (T1), Suape (T1), Serrambi (T3), Carneiros (T1, T2 and T3), and Mamucabas (T1, T2 and T3) compared to Cupe (Fig. 2D). A comparison of samples from the same location at different sampling times indicated higher PYR_{bile} in samples from Paiva and Suape in T1, Muro Alto in T2, Cupe in T3 and Mamucabas in T2.

Comparison between areas indicated higher BAP_{bile} (Fig. 2E) in reef areas of Janga (T1, T2 and T3), Paiva (T1, T2 and T3), Suape (T1 and T3), Muro Alto (T1 and T3), Serrambi (T1 and T3), Carneiros (T1, T2 and T3) and Mamucabas (T2) compared to Cupe (Fig. 2E). A comparison of samples from the same location at different sampling times indicated higher PYR_{bile} in samples from Paiva and Suape in T1, Muro Alto in T3, Cupe in T3, and Mamucabas in T2.

3.2. Biochemical biomarkers

Biochemical parameters measured in fish from Cupe were considered regional references, in accordance with the fact that these fish also indicated lowest bile PAH fluorescence. Medians for all fish samples from Cupe reef were used as reference for each biochemical parameter for statistical comparisons. Furthermore, significant differences were observed between the sexes of the individuals, where males and immature individuals did not differ significantly from each other but were significantly different from reproductively active females. Differences between the sexes were found for EROD, GST, CAT, SOD and glutathione. EROD activity was higher in males and immature individuals compared to females in fish sampled from JA-T1, MU-T3, CU-T2, CU-T3, CA-T2 and MA-T2 (Permanova, $p < 0.05$). Within females, EROD was higher in fish sampled from JA-T3 and CA-T3 compared to females from Cupe reference area (Fig. 3A). Within males and immature, EROD was higher in fish sampled from JA-T1, JA-T3, PV-T1, SU-T3, MU-T2, SE-T2, CA-T3, and MA-T2 compared to males from Cupe reference area (Fig. 3B). A comparison of female and male and immature individuals samples from the same location at different sampling times indicated higher EROD in JA-T3 and CA-T3.

GST activity was higher in males and immature individuals from JA-T1, JA-T2, PV-T2, SU-T1, CU-T1 and MA1, (Permanova, $p < 0.05$). Within females, GST activity was lower in fish sampled from JA-T2, CA-T2, CA-T3 and MA-T3 when compared to females from Cupe reference area (Fig. 3C). Differently, GST activity was higher in females from SE-T1 compared to females from Cupe. Within males and immature individuals, GST activity was lower in fish sampled from JA-T2, JA-T3, PV-T3, SU-T3, MU-T3, SE-T3, CA-T2, CA-T3, MA-T2, and MA-T3 when

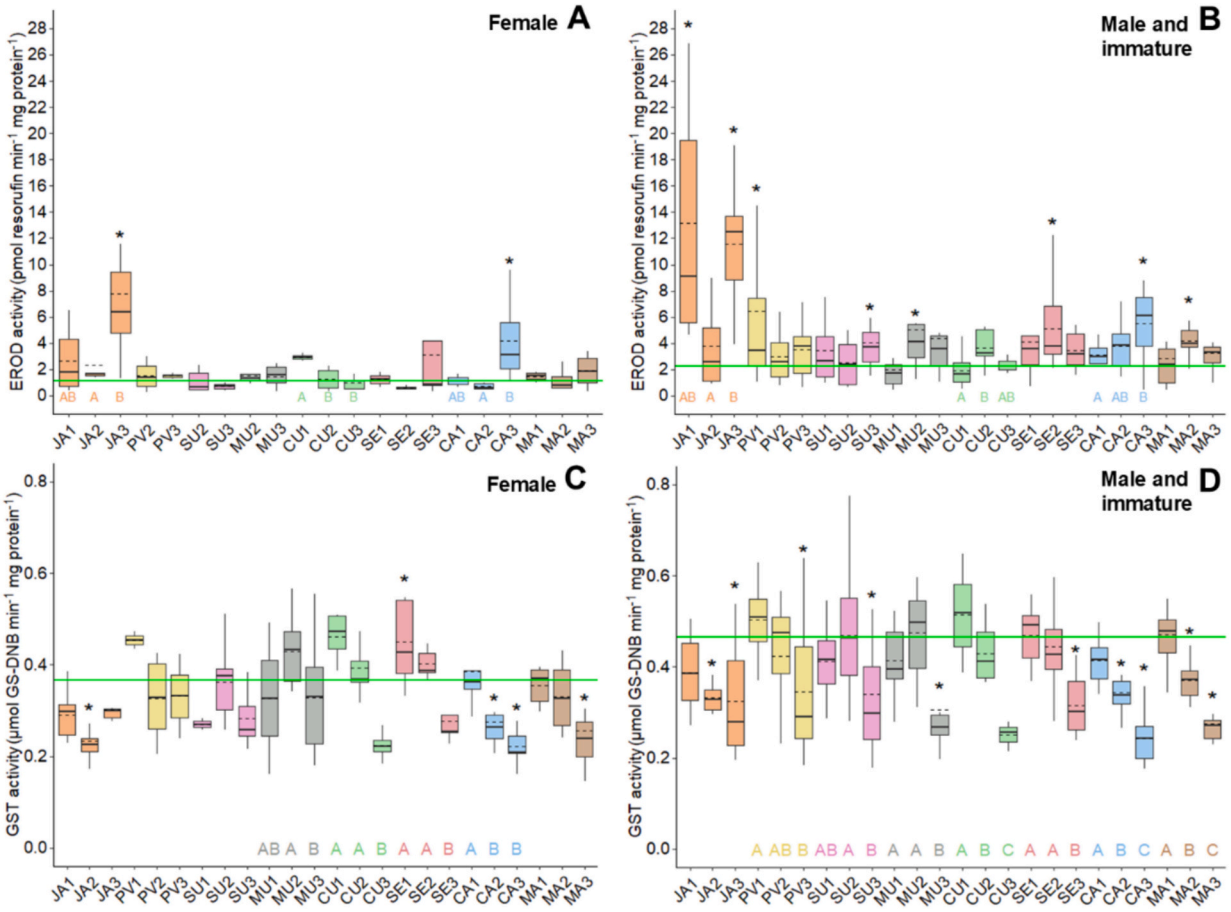

Fig. 3. Ethoxyresorufin-O-deethylase (EROD) and Glutathione S-transferase (GST) activities in the liver of female and male *Stegastes fuscus* sampled in the reef areas of Janga (JA), Paiva (PV), Suape (SU), Muro Alto (MU), Cupe (CU), Serrambi (SE), Carneiros (CA), and Mamucabas (MA). Numbers 1, 2 and 3 after the reef area code indicate samples from March 2021, October 2021 and August 2022, respectively. The green line indicates the median for the data from the Cupe regional reference reef area.

*: significant difference in relation to Cupe regional reference area (Permanova, fixed factor area, $p < 0.05$). Significant temporal differences within each site are indicated by capital letters below the box plot; a different letter colour was used for each site (Permanova, fixed factor time, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared to males and immature individuals from Cupe reference area (Fig. 3D). A comparison of female samples from the same locations at different sampling times indicated lower GST activity at CU-T3, SE-T3 and CA-T3 while male and immature individuals samples indicated lower GST activity at PV-T3, SU-T3, MU-T3, CU-T3, SE-T3, CA-T3, and MA-T3.

CAT activity was higher in males and immature individuals from JA-T1, JA-T2, SU-T2, SU-T3, CA-T2, CA-T3 and MA-T2 (Permanova, $p < 0.05$). Within females, CAT activity was lower in fish sampled from JA-T1, JA-T2, SU-T1, SU-T2, MU-T2, SE-T2, CA-T2 and MA-T2 when compared to females from Cupe reference area (Fig. 4A). Differently, CAT activity was higher in females from JA-T3, PV-T3, MU-T3, SE-T1, SE-T3 and CA-T3. Within males and immature individuals, CAT activity was lower in fish sampled from JA-T1, SU-T1, CA-T2 and MA-T2 when compared to males and immature individuals from Cupe reference area (Fig. 4B). Differently, CAT activity was higher in males and immature individuals from JA-T3, PV-T3, MU-T3, SE-T3, CA-T3 and MA-T3.

SOD activity was lower in males and immature individuals from JA-T1, PV-T2, CU-T3, SE-T3, CA-T2 and MA-T2 (Permanova, $p < 0.05$). Within females, SOD activity was lower in fish sampled from PV-T1, PV-T3, MU-T3 and CA-T3 when compared to females from Cupe reference area (Fig. 4C). Differently, SOD activity was higher in females from JA-T1, SE-T1, SE-T2 and MA-T1. Within males and immature individuals, SOD activity was lower in fish sampled from PV-T3 when compared to males and immature individuals from Cupe reference area (Fig. 4D). Differently, SOD activity was higher in males and immature individuals from PV-T1, SU-T1, SE-T2, CA-T1, and MA-T1.

GSH levels decreased in males and immature individuals from JA-T1, SE-T1 and CA-T1 (Permanova, $p < 0.05$). Within females, GSH levels decreased in fish sampled from PV-T2, CA-T2 and CA-T3 when compared to females from Cupe reference area (Fig. 4E). Differently, GSH levels increased in females from JA-T3, SE-T1, CA-T1, and MA-T1. Within males and immature individuals, GSH levels decreased in fish sampled from CA-T2 when compared to males and immature individuals from Cupe reference area (Fig. 4F). Differently, GSH levels increased in males and immature individuals from PV-T1, PV-T3, SU-T1, SU-T3 and MA-T1.

Lipid peroxidation (LPO) did not differ between sexes (Permanova, $p > 0.05$) and was higher in fish from JA-T1, SU-T1, SU-T2, CA-T2, and CA-T3 when compared to Cupe reference area (Fig. 5A).

Acetylcholinesterase activity did not differ between sexes (Permanova, $p > 0.05$) and was higher in fish from PV-T1 when compared to Cupe reference area (Fig. 5B). Differently, AChE was inhibited in fish from SE-T2 and CA-T2 when compared to Cupe reference area (Fig. 5B).

3.3. Genotoxic biomarkers

Frequency of micronuclei was higher in samples from PV-T1, SU-T1,

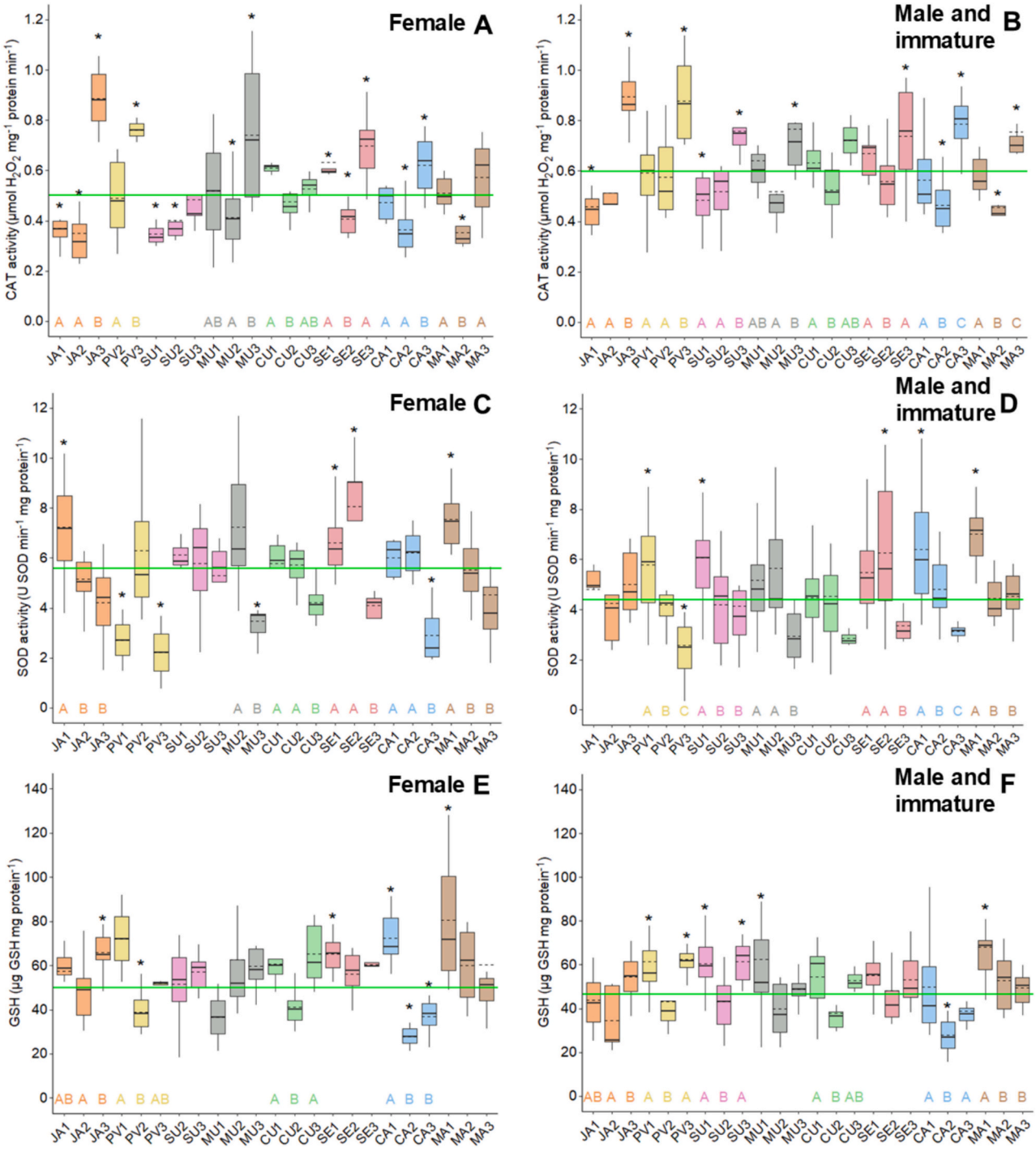

Fig. 4. Catalase (CAT) and superoxide dismutase (SOD) activities, and glutathione (GSH) concentration in the liver of females and males *Stegastes fuscus* sampled in the reef areas of Janga (JA), Paiva (PV), Suape (SU), Muro Alto (MU), Cupe (CU), Serrambi (SE), Carneiros (CA), and Mamucabas (MA). Numbers 1, 2 and 3 after the reef area code indicate samples from March 2021, October 2021 and August 2022, respectively. The green line indicates the median for the data from the Cupe regional reference reef area. *: significant difference in relation to Cupe regional reference area (Permanova, fixed factor area, $p < 0.05$). Significant temporal differences within each site are indicated by capital letters below the box plot; a different letter colour was used for each site (Permanova, fixed factor time, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SU-T2, SU-T3, SE-T1, and CA-T2, compared to the samples from Cupe reference area. A comparison of samples from the same location at different sampling times indicated higher frequency of micronuclei in SE-T1 and CA-T2 (Fig. 6A). Frequency of cells with notched nuclei was higher in samples from SU-T3, SE-T3, MA-T1, and MA-T3, compared to the samples from Cupe reference area (Fig. 6B). Frequency of cells with bebbled nuclei was higher in samples from SU-T1, SU-T2, SE-T3, MA-T1 and MA-T3, compared to the samples from Cupe reference area (Fig. 6C). Total genomic damage was significantly higher in samples from SU-T1, SU-T2, SU-T3, SE-T3, and MA-T1, compared to samples from the Cupe reference area (Fig. 6D).

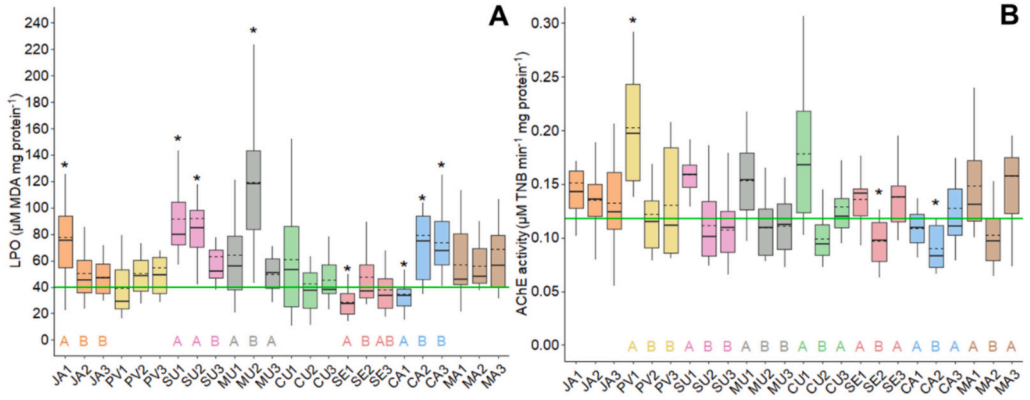

Fig. 5. Lipid peroxidation and AChE activity in the liver of *Stegastes fuscus* sampled in the reef areas of Janga (JA), Paiva (PV), Suape (SU), Muro Alto (MU), Cupe (CU), Serrambi (SE), Carneiros (CA), and Mamucabas (MA). Numbers 1, 2 and 3 after the reef area code indicate samples from March 2021, October 2021 and August 2022, respectively. The green line indicates the median for the data from the Cupe regional reference reef area. *: significant difference in relation to Cupe regional reference area (Permanova, fixed factor area, $p < 0.05$). Significant temporal differences within each site are indicated by capital letters below the box plot; a different letter colour was used for each site (Permanova, fixed factor time, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

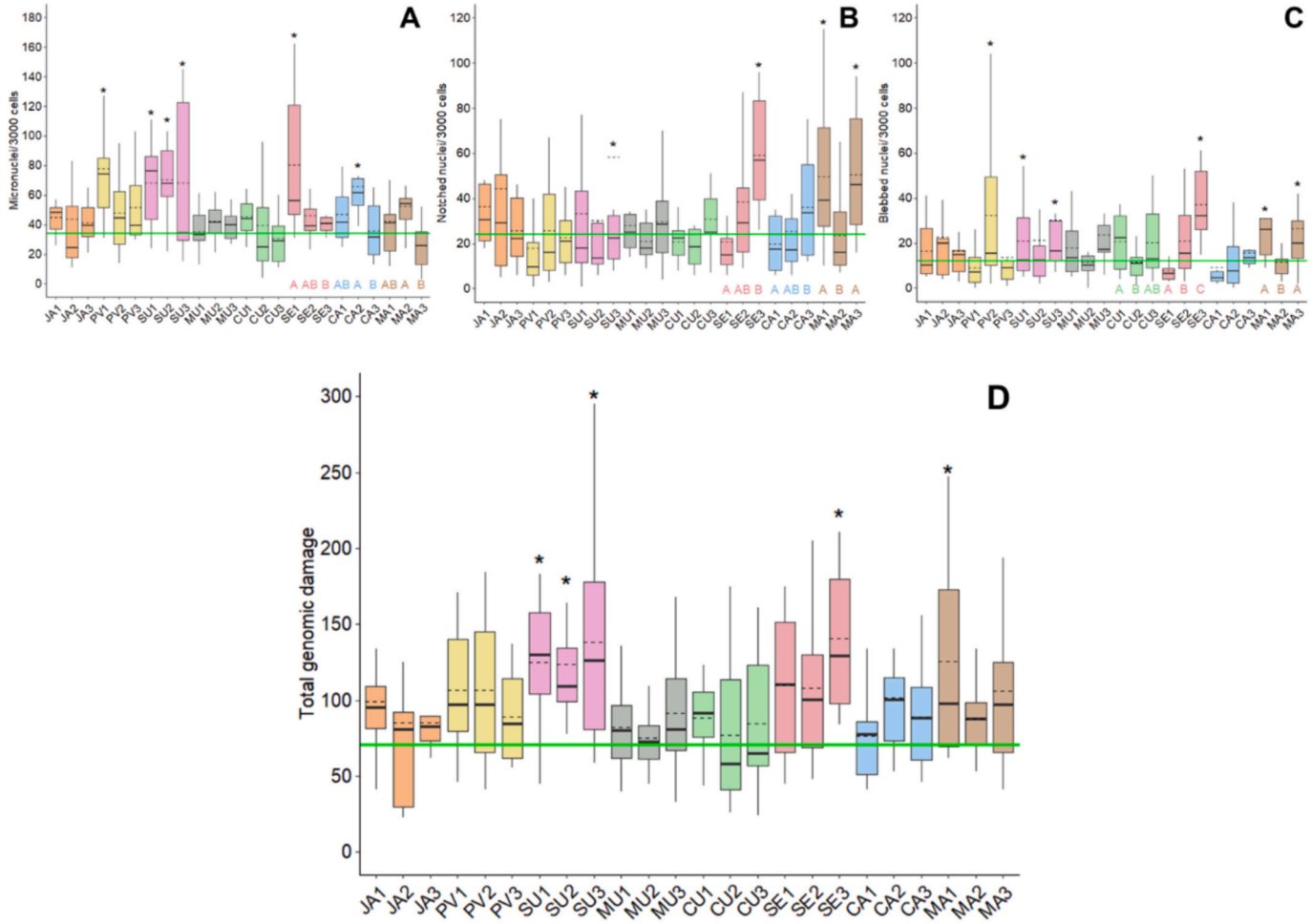

Fig. 6. Occurrence of total genomic damage in erythrocytes of *S. fuscus* collected in March (1) and October (2) of 2021, and August 2022 (3) in the reef areas of the beaches of Janga (JA), Paiva (PV), Suape (SU), Muro Alto (MU), Cupe (CU), Serrambi (SE), Carneiros (CA) and Mamucabas (MA). Numbers 1, 2 and 3 after the reef area code indicate samples from March 2021, October 2021 and August 2022, respectively. The green line indicates the median for the data from the Cupe regional reference reef area. *: significant difference in relation to Cupe regional reference area (Permanova, fixed factor area, $p < 0.05$). Significant temporal differences within each site are indicated by capital letters below the box plot; a different letter colour was used for each site (Permanova, fixed factor time, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Spearman correlation coefficients

Positive Spearman correlation coefficients were found between the biliary PAHs PHE, CHR, PYR, and BaP and EROD (Table 2). AChE correlated positively with the bile PAHs CHR, PYR, and BAP. Negative associations were found between GST and the PAHs PYR and BAP. GSH concentration was negatively correlated with NAP and PHE, and LPO was negatively correlated with CHR and BaP.

EROD correlated positively with CAT activity and negatively with SOD, GSH and LPO. GST correlated positively with SOD and micronuclei. SOD correlated positively with GSH and LPO. CAT correlated negatively with SOD and LPO, and positively with GSH. GSH correlated positively with AChE (Table 2).

Blebbed and notched nuclear abnormalities correlated negatively with PHE, while micronucleus correlated positively with NAP, PHE, and CHR.

3.5. Fish densities based on visual census

Stegastes fuscus was recorded in the sampling areas ($N = 930$), with mean densities per transect (mean ± standard error) varying among the reefs of Janga (3.92 ± 0.41), Cupe (5.17 ± 0.99), Paiva (5.5 ± 0.48), Serrambi (14.25 ± 0.33) and Mamucabas (30.67 ± 0.65).

3.6. Integrated Biomarker Response Index

The Integrated Biomarker Response Index (IBRv2i) calculated for T1 was significantly higher in samples from JA-T1 (median = 7.1), PV-T1 (median = 6.3), CA-T1 (median = 7.9), and MA-T1 (median = 6.1), when compared to samples from the CU-T1, reference area (median = 4.8) (Fig. 7A). The IBRv2i applied to the T2 samples was significantly higher for all reef areas when compared to the samples from the Cupe reference area (Fig. 7B). The medians found were 6.5, 5.6, 7.9, 8.0, 4.5, 5.9, 6.4, and 5.6 for JA-T2, PV-T2, SU-T2, MU-T2, CU-T2, SE-T2, CA-T2, and MA-T2, respectively. The IBRv2i applied to T3 samples was significantly higher for all reef areas when compared to samples from the Cupe reference area (Fig. 7C). The medians found were 7.0, 6.3, 5.5, 5.6, 3.5, 6.3, 6.4, and 6.1 for JA-T3, PV-T3, SU-T3, MU-T3, CU-T3, SE-T3, CA-T3, and MA-T3, respectively.

4. Discussion

Monitoring of eight reef areas in the state of Pernambuco at 17, 24 and 34 months after the oil spill indicated elevated values of biliary PAHs and biochemical alterations in *S. fuscus* fish from the Janga (JA), Paiva (PV), Suape (SU), Muro Alto (MU), Serrambi (SE), Carneiros (CA) and Mamucabas (MA) reef areas, compared to samples from the Cupe (CU) reference area. Genotoxic effects were also observed in samples from the Paiva, Suape, Serrambi and Mamucabas reef areas.

The verified gradient of *S. fuscus* population density among the five reef areas analyzed is probably due to the influence of anthropogenic disturbances beyond chemical exposure to contaminants, including habitat degradation and structural complexity at each location. More structurally complex habitats can positively influence reef fish density (Graham and Nash, 2013; Komyakova et al., 2018). However, the lowest *S. fuscus* population density was found in Janga reef area, directly influenced by urbanization and consequently by losses of reef habitat, but also an area where increases in bile PAHs and biochemical alterations were evident, suggesting that chemical exposures including PAHs is also a relevant factor.

4.1. Biliary PAHs

In an earlier study focused on the accumulation of biliary PAHs and biochemical changes in *S. fuscus* before and shortly after the oil spill on the Brazilian coast in 2019, Serrambi reef area was used as a regional reference area (Melo Alves et al., 2024). The medians found for NAP_{bile}, PHE_{bile}, and CHR_{bile} were 39.0, 43.0, and 11.3 (Melo Alves et al., 2024). However, in this study, it was observed that fish samples collected in Serrambi (SE-T3) presented fluorescence values of NAP_{bile}, PHE_{bile}, and CHR_{bile} of 19.2, 10.7, and 2.8, respectively, and in Cupe (CU-T3) 10.4, 8.2, and 2.1, for NAP_{bile}, PHE_{bile}, and CHR_{bile}, respectively. The values found in SE-T3 were higher than in samples from CU-T3. The samples were collected at similar depths, from 1.0 to 1.5 m. It should be noted that the oil hit a deeper area surrounding shallower reefs at Cupe where *Stegastes fuscus* reside and were sampled, and significant amounts of oil were removed by cleanup operations. However, in this study it was observed that samples collected in Serrambi reef area (SE-T3) presented higher bile PAH fluorescence values than in samples from Cupe reef area (CU-T3) included in this study, which was hit by oil in 2019. As discussed by Melo Alves et al. (2024), significant spatial variability was verified in affected areas at a relatively small spatial scale in Japaratinga Beach reefs, a feature that probably also occurred at Cupe reef area (CU-T1, CU-T2, and CU-T3). For this reason, in this study Cupe reef area was chosen as the basal regional reference.

The analysis of PAHs in bile is effective because it provides information on the acute exposure of fish to oil spill events, as well as on the subsequent recovery of these environments through monitoring of the affected areas, showing good positive correlation with environmental PAH exposure levels (de Souza et al., 2024; Dearnley et al., 2022; Silva et al., 2021). Melo Alves et al. (2024) quantified biliary PAHs in *S. fuscus* from Paiva and Suape reef areas which were also sampled during this study shortly after the oil spill. It is possible to observe a reduction in PAH levels in samples from Paiva reef (PV-T1), 17 months after the oil spill, with decreases of 57.0 %, 88.0 %, 92.2 %, 82.2 % and 44.1 % in NAP_{bile}, PHE_{bile}, CHR_{bile}, PYR_{bile}, and BAP_{bile}, respectively (Table S1). In samples from Suape (SU-T1), 17 months after the oil spill, the reduction reached 74.8 %, 49.2 %, 30.6 %, and 77.7 % for NAP_{bile}, PHE_{bile}, CHR_{bile}, and $PYR_{bile,}$ respectively (Table S1). Therefore, biliary PAHs in *S. fuscus* provided important information on the decrease in environmental exposure to PAHs 17 months after the oil spill.

Concentration of 16 priority PAHs in water samples from the Paiva and Suape reef areas decreased from 266.6 ng L^{-1} and 548.2 ng L^{-1} shortly after the oil spill in 2019 (Melo Alves et al., 2024) to 0.6 and 4.1 ng L^{-1}, respectively, 17 months after the oil spill (Choueri et al., 2024), corroborating the decay pattern found in biliary PAHs. However, this decay observed in water concentrations of 99.8 % and 99.3 % at Paiva and Suape, respectively, were higher than the decay in biliary PAHs (Table S1).

The concentration of the 16 priority PAHs in water which were measured at the same locations and dates on which *S. fuscus* was sampled (T1 and T2), ranged from not detected to 5.9 ng L^{-1} (Choueri et al., 2024), and did not differentiate the locations in terms of PAH contamination. Fish significantly bioconcentrate PAHs in bile reaching bile concentrations much higher than environmental water concentrations according to their octanol water partition coefficient (Torreiro-Melo et al., 2015). Bile fluorescence in fish from areas with different types of anthropic influence was different and distinguished sites where PAH bile bioconcentration is higher, such as Janga (urban influence), Paiva (influence from contaminated Jaboatão river), Suape (industrial and port), and Carneiros (touristic recreational boating) (Fig. 2). Similarly, NAP and PHE in the bile of the reef fish *Ocyurus chrysurus* were higher in samples collected near the city and a port region, influenced by untreated sewage discharges and ship waste discharges (Gold-Bouchot et al., 2017).

Higher values of biliary PAHs were found in T1 samples during the rainy season compared to T2 during the dry season in samples from Janga, Paiva and Suape. These values may have been elevated by a greater runoff of water from continental areas with urban influence during the rainy season influenced by greater hydrodynamics, causing the resuspension of sediments and mobilization of PAHs, returning to lower contamination levels in October, as hypothesized by Choueri et al.

Table 2
Spearman correlation coefficients between biliary PAHs and biochemical biomarkers evaluated in *Stegastes fuscus* individuals.

	CHR	PYR	BaP	EROD	GST	CAT	SOD	GSH	AChE	LPO	BLEBBED	NOTCHED	MICRONUCLEI	TGD
NAP	0.512	0.621	0.393	0.0872	−0.0496	−0.02	0.00535	−0.103	0.0314	0.0266	−0.092	−0.123	0.218	0.0672
	<0.001	**<0.001**	**<0.001**	0.138	0.312	0.702	0.913	**0.0376**	0.609	0.587	0.169	0.0627	**<0.001**	0.311
PHE	0.646	0.515	0.493	0.123	−0.0085	0.0149	−0.0163	−0.142	0.0499	−0.01	−0.135	−0.171	0.278	0.0787
	<0.001	**<0.001**	**<0.001**	**0.036**	0.862	0.777	0.74	**0.004**	0.416	0.839	**0.0438**	**0.010**	**<0.001**	0.235
CHR		0.599	0.566	0.156	−0.0619	−0.0666	0.0335	−0.0914	0.147	−0.136	−0.0793	−0.0517	0.178	0.0819
		<0.001	**<0.001**	**0.00795**	0.207	0.203	0.495	0.0649	**0.016**	**0.006**	0.236	0.437	**0.007**	0.217
PYR			0.692	0.161	−0.253	−0.098	0.0528	0.0232	0.158	−0.037	−0.0866	−0.044	0.111	0.0208
			<0.001	**0.006**	**<0.001**	0.0607	0.283	0.639	**0.010**	0.451	0.195	0.509	0.0927	0.754
BaP				0.224	−0.263	0.0989	−0.124	0.0759	0.168	−0.182	−0.025	−0.0472	0.125	0.0908
				<0.001	**<0.001**	0.058	**0.012**	0.126	**0.006**	**<0.001**	0.709	0.478	0.0596	0.171
EROD					0.0184	0.281	−0.251	−0.117	0.038	−0.12	0.091	0.132	0.0772	0.16
					0.752	**<0.001**	**<0.001**	**0.045**	0.537	**0.039**	0.241	0.0868	0.315	0.0362
GST						0.0408	0.217	0.0732	−0.0331	−0.0294	−0.113	−0.147	0.247	0.0759
						0.428	**<0.001**	0.134	0.585	0.544	0.0887	**0.026**	**<0.001**	0.252
CAT							−0.24	0.256	−0.0004	−0.113	0.0751	0.091	−0.125	0.0248
							<0.001	**<0.001**	0.995	**0.027**	0.293	0.2	0.0766	0.727
SOD								0.181	−0.002	0.155	−0.0358	−0.0318	0.115	0.0116
								<0.001	0.974	**0.001**	0.593	0.632	0.0813	0.861
GSH									0.197	−0.0669	0.105	0.111	−0.00158	0.0579
									0.001	0.169	0.118	0.0939	0.981	0.383
AChE										−0.0238	0.0144	0.0865	−0.169	−0.0724
										0.695	0.856	0.273	**0.031**	0.358
LPO											0.0106	−0.0284	−0.0198	−0.0651
											0.874	0.668	0.765	0.324
BLEBBED												0.72	−0.191	0.589
												0.001	**0.004**	**<0.001**
NOTCHED													−0.171	0.617
													<0.001	**<0.001**

Spearman correlation coefficients indicate *p*-values for each combination of endpoints.
p-values in bold are statistically significant ($p < 0.05$).
TGD = Total Genomic Damage.

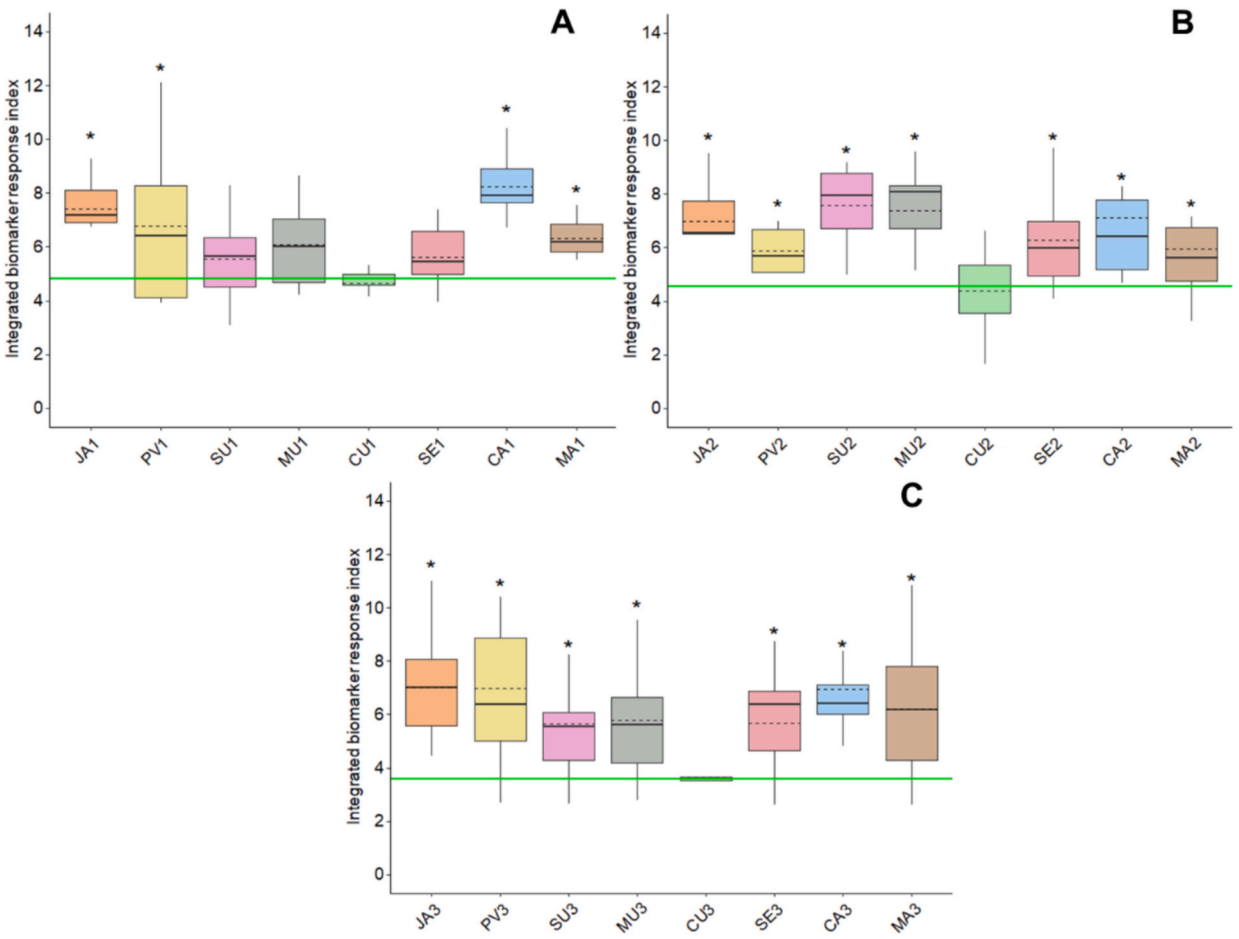

Fig. 7. Integrated biomarker response index for *Stegastes fuscus* sampled in the reef areas of Janga (JA), Paiva (PV), Suape (SU), Muro Alto (MU), Cupe (CU), Serrambi (SE), Carneiros (CA), and Mamucabas (MA). Numbers 1, 2 and 3 after the reef area code indicate samples from March 2021, October 2021 and August 2022, respectively. The green line indicates the median for the data from the Cupe reference reef area. *: significant difference in relation to the Cupe reference area (Permanova, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(2024) and Frapiccini et al. (2020). Sediment PAH concentrations measured at the same locations and dates on which *S. fuscus* was sampled (T1 and T2), ranged from 0.2 to 143.3 ng g^{-1}, with the highest concentrations observed at the Janga, Paiva, and Suape reef areas (Choueri et al., 2024). Our results also indicate higher PAH concentrations in the bile of fish from Janga, Paiva, and Suape (Fig. 3), concurring with the above results in sediments.

4.2. Biochemical biomarkers

Biotic factors related to sex and maturation stage/reproductive period of individuals can influence the biochemical responses of organisms, and this factor may confound interpretation of results comparing samples from different sites (Sadauskas-Henrique et al., 2024; van der Oost et al., 2003). Significant differences between sexes (males and immature individuals, and reproductively active females) were found in the present study, where male and immature individuals presented higher enzymatic activity for EROD, GST, and CAT and lower values for SOD and GSH. Significantly higher EROD activity in male fish compared to females is well documented (Arukwe and Goksøyr, 1997; Bucheli and Fent, 1995; Chiang et al., 2012; Gagnon and Rawson, 2017; Nicolas, 1999; Whyte et al., 2000). The hormone 17β-estradiol, responsible for stimulating the synthesis of vitellogenin in females, causes a decline in EROD from the beginning of ovulation until spawning (Nicolas, 1999; Whyte et al., 2000).

EROD activity in fishes tends to be high and more similar among reproductively active and inactive males, and in reproductively inactive females, but reproductively active females have lower EROD activity (Whyte et al., 2000). In this study, we separated the EROD analysis by females, males, and individuals without developed gonads (immature), to reduce the confounding factors caused by the influence of sex and seasonal factors. The period between September and January was identified as the reproductive period of *S. fuscus*, while females with developing and resting gonads predominated in the rainy season, between March and August (Canan et al., 2011). However, we found females with developed gonads in all sampling periods in this study, including the rainy season.

Ratios of EROD between males and females have been reported for fish species *Limanda limanda* (ratios from 1.32 to 5.0) (Kirby et al., 1999; Stagg et al., 1995), *Percilia gillissim* (ratio = 1.34) (Chiang et al., 2012), *Pleuronectes platessa* (ratio = 1.58) (Kirby et al., 1999), *Acanthopagrus butcheri* (ratios from 1.13 to 4.64) (Webb et al., 2005), *Gasterosteus aculeatus* (ratio = 2.03) (Sanchez et al., 2008) and *Plagioscion squamosissimus* (ratio = 3.74) (Wunderlich et al., 2015). Our study verified ratios ranging from 2.01 to 6.67 for *S. fuscus*, similar to the ranges reported. Our results also indicate that the higher EROD activities in males and immature individuals were more efficient in detecting significant differences among samples from different reef areas compared to the regional reference Cupe. However, the fold induction relative to the reference area is greater in females (mean = 4.20) than in males (mean = 1.83). This result is similar to that found for the species *Limanda limanda*, in which females showed a 20-fold induction while males

showed a 4-fold induction (Stagg et al., 1995). Although females present higher induction values, our results suggest a better suitability of using males and immatures of this species in environmental monitoring studies to avoid sexual influences in EROD.

High concentrations of PAHs with 4 or more aromatic rings, such as fluoranthene, PYR, BAP, benzo(*a*)anthracene, CHR, benzo(*k*)fluoranthene, and indeno(1, 2,3-*cd*)pyrene were observed in Janga sediments, suggesting a pyrolytic origin of these PAHs, whereas sediments from Carneiros were dominated by lighter contaminants, indicating a petrogenic origin (Choueri et al., 2024). PAHs with higher molecular weight are considered aryl-hydrocarbon-receptor (AhR) agonists in fish and are potent inducers of EROD (Barron et al., 2004). In the present study, the highest EROD induction was verified in Janga and Carneiros (Fig. 3), and EROD correlated positively with the biliary PAHs PHE, CHR, PYR and BAP (Table 2). Sediment PAH contamination in Janga and Carneiros suggest pyrolytic and petrogenic origin, respectively (Choueri et al., 2024), and our results from Carneiros and Janga indicate that both pyrolytic and petrogenic sources of PAHs can lead to EROD induction. This finding could be explained by the fact that bile increases in high molecular weight PAHs CHR, PYR, and BAP were verified in both locations, and by evidence that exposure to PHE mixed with BAP potentiates CYP1A1 synthesis induced by BAP (Bramatti et al., 2023), a situation that possibly occurred in fish from Janga and Carneiros reef areas.

GST activity was inhibited in the liver of male individuals collected in Janga, Paiva, Suape, Muro Alto, Serrambi, Carneiros and Mamucabas compared to samples from Cupe reef area, and this inhibition occurred in most samples collected at T3. GST activity may be inhibited at higher concentrations of PAHs, and is related to phase II biotransformation, acting in cellular detoxification and excretion, in addition to assisting as a cofactor in the antioxidant defense system (Bramatti et al., 2023; Briaudeau et al., 2021; Lushchak, 2011). Our results agree with previous studies that reported the inhibition of GST activity in fish from sites contaminated by PAHs, such as in *Diplodus annularis* (Bagnasco et al., 1991) and *Liza aurata* (Oliveira et al., 2010). In addition, laboratory exposure to PAHs also inhibited GST in *Clarias gariepinus* (Otitoloju and Olagoke, 2011), *Hippocampus reidi* (Delunardo et al., 2015) and *Oryzias melastigma* (Zeb et al., 2024). When GST inhibition is accompanied by simultaneous induction of EROD activity, the probability of DNA damage may increase due to the accumulation of reactive PAH metabolites (Bramatti et al., 2023).

SOD inductions were observed in samples collected 17 months after oil (T1), in females from the Janga and Serrambi reef areas, and in males from Suape, while CAT was inhibited in these same samples. SOD inhibitions were observed in samples collected 34 months after oil (T3), in females from the Paiva, Muro Alto, and Carneiros reef areas, and in males from the Paiva reef area, while CAT was induced in these same samples. Pan et al. (2022) reported that there may be divergences between the activities of different antioxidant enzymes after contamination by PAHs, with opposite patterns of induction and inhibition occurring in individuals of *Boleophthalmus pectinirostris* exposed to petroleum. The increase in CAT or SOD activity in samples from these areas can be explained by the increase in reactive oxygen species, which need to be converted by these antioxidant enzymes to maintain cellular redox balance (Soltani et al., 2019). The decrease in SOD activity observed in fish from impacted places in this study may be related to excessive pollutant presence, which leads to increased reactive oxygen species (ROS) overloading and inhibiting enzymatic function (Li et al., 2025). On the other hand, the reduction of CAT activity may occur by the initial inhibition caused by $O_2 \bullet^-$; And as SOD operates and increases the formation of H_2O_2, CAT activity tends to intensify (Pan et al., 2022).

GSH is considered an efficient non-enzymatic antioxidant, used to control the levels of ROS, through direct interaction between them, or as a substrate for GST and antioxidant enzymes (Lushchak, 2016; Santos et al., 2020). The generation of PAH metabolites by phase I and the probable increase in ROS may have caused an increase in GSH concentration in fish due to its action as an antioxidant.

Lipid peroxidation was observed in samples from areas that also presented higher biliary PAHs, such as Janga, Suape, and Carneiros compared to reference, suggesting an increase in this biomarker with increased oil contamination (Pan et al., 2022). The presence of lipoperoxides is related to serious tissue damage, causing rupture of biological membranes and irreversible damage to tissues. In addition, lipid peroxidation occurs when ROS levels increase to the point of overcoming defense mechanisms and the lipids that form cell membranes are oxidized, potentially causing their rupture and leading to cell death (Marinsek et al., 2024). LPO showed a negative correlation with CAT, where samples that presented greater lipid damage had lower CAT activity. According to Macedo et al. (2024), a decrease in CAT activity, which is the first line of defense to prevent oxidative stress, can lead to increased lipid damage.

AChE activity has been used to assess the impacts of oil spills, and it is suggested that high molecular weight PAHs are inhibitors of AChE activity (Jung et al., 2011). However, a pattern of AChE induction was observed in the brain of individuals collected in T1 in the Paiva reefs and inhibitions in T2 in Serrambi and Carneiros. He et al. (2012) observed induction of AChE with a reduction in the levels of acetylcholine (ACh) and choline acetyltransferase (ChAT) in embryos of the fish *Sebastiscus marmoratus*, exposed to concentrations of 0.5 and 5 nmol L^{-1} of BAP. The complex response patterns of AChE activity in this study concur with the suggestion by Olivares-Rubio and Espinosa-Aguirre (2021) that further studies are needed on the mechanisms involved between exposure to PAHs and the diversified responses in AChE activity.

4.3. Genomic damage

The presence of micronuclei and nuclear abnormalities was frequent mainly in fish samples from the reef areas of Suape (SU-T1, SU-T2, and SU-T3) and Serrambi (SE-T3). Higher levels of genotoxic and cytotoxic effects have already been found in fish collected in areas close to oil platforms (Baršienė et al., 2013; Pietrapiana et al., 2002; Rybakovas et al., 2009). The Suape region is predominantly a port area, and this may have influenced the greater occurrence of damages to fish from this reef area. The changes in genotoxicity biomarkers found in samples from the Serrambi reef area can possibly be attributed to tourism activities, including PAH exposure, as both bile PAHs and total genomic damage increased in SE-T3. Furthermore, this is the only area in this study in which the possible adverse effects of the 2019 oil spill are not considered, as no crude oil was observed in that region.

Samples from the Janga reef area, a location with the greatest influence of urbanization, did not show significant increases in micronuclei and nuclear abnormalities compared to samples from the reference area. However, frequency of micronuclei in fish from Cupe reference area (median of 34 micronuclei per 1000 erythrocyte cells) was high compared to the basal value for *Oncorhynchus mykiss* of 0.5 micronuclei per 1000 erythrocyte cells (Williams and Metcalfe, 1992). This may indicate that basal frequencies of micronuclei in *Stegastes fuscus* are relatively high, or that fish from the reference area are also under genotoxic stress. In addition, the chronic exposure of fish from Janga to PAHs and other contaminants present in the reef environment may have influenced the lower responses observed for micronuclei. The decrease in the frequency of micronuclei and nuclear abnormalities in fish chronically exposed to xenobiotics may be explained by the organism's cell renewal processes, in which damaged erythrocytes are replaced by new cells (Cariello Delunardo et al., 2019). Micronuclei decrease trends were observed in *Hippocampus reidi* and *Prochilodus lineatus* after chronic exposures to water-accommodated fraction (WAF) of diesel fuel (Cariello Delunardo et al., 2019; Vanzella et al., 2007). Furthermore, micronuclei analysis correlated positively with the PAHs NAP and PHE, typical of petrogenic contamination, while the Janga area is mainly influenced by pyrogenic contamination.

4.4. Integrated index of biomarker responses

The highest medians for IBRv2i were observed in individuals from Janga, with medians of 7.1, 6.5, 7.02 for JA-T1, JA-T2, and JA-T3, respectively, and in individuals from Carneiros, with medians of 7.9, 6.4, and 6.4 for CA-T1, CA-T2, and CA-T3, respectively. These areas, with a high degree of anthropic influence, presented greater biological changes compared to individuals from the reference Cupe, with medians of 4.8, 4.5, and 3.5 for CU-T1, CU-T2, and CU-T3, respectively. The index provides an additional interpretation of the health status of organisms, where higher IBR values indicate a greater biomarker response in fish across locations, regardless of the occurrence of induction or inhibition of biochemical biomarkers (Hou et al., 2016; Lyu et al., 2024).

Although the IBRv2i index is based only on our results on biochemical biomarkers, it indicated a clearer pattern of differences at sampled sites compared to our regional reference, compared to the analysis of each of the biochemical parameters, which indicated more variation and complexity. Cupe presented the lowest median values in the analysis, indicating suitability for use as a reference area. The reef areas with the lowest medians, excluding the reference area, varied over time: SE-T1 (median = 5.4), MA-T2 (median = 5.6), and SU-T3 (median = 5.5, indicating a spatiotemporal variation in the contamination of these environments.

The use of IBRV2 index also proved efficient in identifying larger effects in the fishes *Gobioides broussonnetii* and *Atherinella brasiliensis*, resident in areas with strong anthropic influence (Salgado et al., 2021; Salgado et al., 2019). Integrated biomarker indices are considered useful tools to examine pollution levels and to simplify the interpretation of data, being useful for environmental agencies (Broeg and Lehtonen, 2006; Mattos et al., 2024).

5. Conclusion

The results indicate that the monitored reef areas are not being significantly influenced by the oil that arrived in 2019. However, contamination by PAHs occurs chronically in the reef areas of Janga, Paiva, Suape, and Carneiros, especially in the rainy season. The use of males or immature individuals is recommended for the evaluation of biochemical parameters in this species. The biliary biomarkers, biochemical EROD, GST, CAT, SOD, GSH, and LPO and genotoxic biomarkers proved to be sensitive and suitable for environmental monitoring of the eight reef areas. IBRv2i was effective in identifying contaminated areas with greater changes in biochemical biomarkers. Continued monitoring with the species *Stegastes fuscus* is necessary to obtain information on the contamination status of these environments over the years.

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CRediT authorship contribution statement

Maria Karolaine de Melo Alves: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Célio Freire Mariz:** Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shaieny Marcela Ventura dos Santos:** Investigation. **João Victor Gomes do Nascimento:** Investigation. **Thalita Joana Bezerra de Melo:** Investigation. **Natallia Vivian da Silva Maia:** Investigation. **Romulo Nepomuceno Alves:** Investigation. **Eliete Zanardi-Lamardo:** Writing – review & editing, Supervision, Investigation. **João Lucas Leão Feitosa:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Mônica Lúcia Adam:** Methodology, Conceptualization. **Paulo S.M. Carvalho:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, 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.

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Data availability

Data will be made available on request.

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