Biochemical and molecular biomarkers and their association with anthropogenic chemicals in wintering Manx shearwaters (*Puffinus puffinus*)

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Short Title: Biomarkers of plastic ingestion and ocean pollution in Manx shearwater

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ABSTRACT

Anthropogenic pollution poses a threat to marine conservation by causing chronic toxic effects. Seabirds have contact throughout their lives with pollutants like plastic, metals, polychlorinated biphenyls (PCBs), and organochlorine pesticides such as hexachlorocyclohexanes (HCHs). We assessed 155 Manx shearwaters (*Puffinus puffinus*) stranded along the Brazilian coast, analyzing associations between organic pollutants, plastic ingestion, biomarkers (transcript levels of aryl hydrocarbon receptor, cytochrome P450-1A-5 [CYP1A5], UDP-glucuronosyl-transferase (UGT1), estrogen receptor alpha-1 (ESR1), and heat shock protein-70 genes) and enzymes activity (ethoxy-resorufin O-deethylase and glutathione *S*-transferase (GST)]. Plastic debris was found in 29% of the birds. The transcription of *UGT1* and *CYP1A5* was significantly associated with hexachlorobenzene (HCB) and PCBs levels. *ESR1* was associated with HCB and Mirex, and GST was associated with Drins and Mirex. While organic pollutants affected shearwaters more than plastic ingestion, reducing plastic availability remains relevant as xenobiotics are also potentially adsorbed onto plastics.

Keywords: Biotransformation, Gene transcripts, Ocean pollution, Procellariiformes, Xenobiotics

1. Introduction

Marine top predators play a crucial role in marine ecosystems and in understanding the long-term impact of ocean pollution (Hazen et al., 2019). Most seabird species are long-lived, and their life histories are generally characterized by low fecundity and high survival rates (Dias et al., 2019), which limits their adaptability to rapid environmental change and renders their health status a valuable indicator of the conservation threats experienced by their populations (Phillips et al., 2023). Seabirds are exposed to contaminants from marine, terrestrial and atmospheric sources, which can accumulate and magnify along marine food webs (e.g. Lima et al., 2023). These characteristics make them powerful sentinels of marine ecosystem changes, and their declining populations in recent years highlight the urgency of understanding the underlying mechanisms, including the sublethal and populational effects of pollutants (Burger and Gochfeld, 2004; Rivers-Auty et al., 2023). Shearwaters, for example, have the potential to be exposed and transport contaminants over a vast area within their migratory range (Robuck et al., 2022).

The Manx shearwater (*Puffinus puffinus*) is a medium-sized (body mass 350–575 g, wingspan 76–89 cm) burrow-nesting Procellariidae species, a family that includes petrels, shearwaters and prions. This species breeds in the North Atlantic Ocean (mainly Great Britain and Ireland) and performs a long-distance trans-equatorial migration to spend the austral spring and summer (October–April) in Southwest Atlantic Ocean, on the continental shelf waters of Brazil, Uruguay and Argentina (Guilford et al., 2009; Prado et al., 2022). The diet of the Manx shearwaters is predominantly composed by shoaling fish, which are captured through pursuit or plunge diving, although they may also consume cephalopods, crustaceans and insects (Petry et al., 2008; Brooke, 2013). Manx shearwaters have long been known to ingest plastics (e.g. Moser and Lee, 1992; Colabuono et al., 2009), and this species may be at increased risk for plastic ingestion through intergenerational transfer (Alley et al., 2022).

Strandings of juvenile Manx shearwaters are frequent in their migratory wintering grounds, with one study revealing that plastics are present in the digestive tract of 60% of individuals stranded in southern Brazil, representing 83% of all items ingested (prey and plastic) (Colabuono et al., 2009). The risks associated with plastic waste from various sources on a global scale are heightened due to changes in their physical and chemical properties caused by weathering, as well as the presence of hazardous pollutants introduced as chemical additives and through adsorption

(Rai et al., 2022). Hence, enhancing comprehension on the impacts of pollution on seabirds is imperative, with biomarkers of exposure or impact serving as potent tools for elucidating sublethal effects.

Seabirds are subject to daily exposure to a diverse spectrum of chemicals found in their surrounding environment and diet (Provencher et al., 2020). As with all vertebrates, they have developed a repertoire of enzymes and transporters to facilitate the biotransformation and elimination of these compounds (Walker et al., 2012). Amongst the biochemical biomarkers used to study the biological response to xenobiotics, the cytochrome P450 (CYP) superfamily of enzymes catalyzes the oxidation of a diversity of pollutants, playing a key role in the detoxification and elimination of lipophilic compounds from cells (Goldstone et al., 2007). Members of the CYP1A subfamily are known to be induced by polycyclic aromatic hydrocarbons (PAHs) and other organic pollutants, that may be adsorbed by plastics (Bucheli and Fent, 1995; Taniguchi et al., 2016; Rai et al., 2022). Thus, CYP1A are recognized as useful biomarkers of exposure, which can be assessed by the content of protein levels and their associated 7-ethoxyresorufin *O*-deethylase (EROD) enzyme activity (Bachman et al., 2015).

In addition to the aforementioned CYP complex of phase I biotransformation system, uridine diphosphate (UDP) UDP-glucuronosyl-transferase (UGT) is a xenobiotic metabolizing enzyme that plays an important role in the phase II metabolism of birds (Kawai et al., 2019). Glutathione *S*-transferases (GSTs) enzymes also play a crucial role in the detoxification of numerous xenobiotics. GSTs participate in phase II biotransformation reactions by conjugating the thiol group of endogenous reduced glutathione with the electrophilic centers produced in phase I reactions and/or contaminants, rendering them more hydrophilic (Walker et al., 2012). This process enhances their excretion, safeguarding the cell against their toxic effects, which can include mutagenicity and carcinogenicity (Fitzpatrick et al., 1997; Konishi et al., 2005).

Moreover, a significant portion of the enzymatic and transporter systems that facilitate the biotransformation and elimination of pollutants can be regulated through the activation of xenobiotic receptors, acting as transcription factors that govern the expression of their target genes, notably those encoding xenobiotic-metabolizing enzymes (Nakayama et al., 2006). Notably, the aryl hydrocarbon receptor occupies a central position in orchestrating xenobiotic metabolism in birds (Doering et al., 2018; Larigot et al., 2018). Meanwhile, estrogen receptor 1 regulates the endocrine system, and alterations might indicate disruption caused by pollutants in birds (Felton et al., 2020). In turn, heat shock proteins (HSP) are involved in responding to various types of metabolic stress, preventing cellular damage (Woodruff et al., 2022).

In recent decades, gene transcription profiles (*i.e.* transcriptomics) have emerged as a promising approach allowing researchers to analyze thousands of genes and pathways to identify potential health effects of individual toxicants and pollutant combinations (Schirmer et al., 2010), which has also proven valuable for ecotoxicological studies in seabirds (Kreitsberg et al., 2023). Specifically, the transcription of genes encoding proteins belonging to the phase I and II biotransformation system, such as *cytochrome P450 1A5* (*CYP1A5*) and *UDP-glucuronosyl-transferase* 1 (*UGT1*), along with genes of the *aryl hydrocarbon receptor* (*AhR*), *estrogen receptor alpha 1* (*ESR1*), and the gene encoding *heat shock protein 70* (*HSP70*) comprise useful biomarkers to investigate the molecular responses to xenobiotics in a wide variety of organisms (*i.e.* Kreitsberg et al., 2023).

In this context, we hypothesize that biochemical and molecular biomarkers in hepatic samples of the pelagic migratory Manx shearwater are influenced by organic pollutants and plastic ingestion. Therefore, this study aimed to explore how the biomarker responses can be used to detect sublethal effects of ocean pollution and guide future management decisions for conservation of seabirds.

2. Material and methods

2.1. Sample collection

Manx shearwaters were recovered from the coastline of southeast and south Brazil through the Santos Basin Beach Monitoring Project (*Projeto de Monitoramento de Praias da Bacia de Santos* – PMP-BS), a requirement by federal environmental authorities for licensing of offshore oil and natural gas exploration and production activities (PETROBRAS, 2021). Between 2016 and 2020, liver tissue samples were obtained from 155 Manx shearwaters found ashore from Saquarema (22°56'13"S; 42°29'27"W) to Laguna (28°29'43"S; 48°45'38"W), spanning *c.* 1140 km of coastline (Fig. 1). Only fresh carcasses (presumably deceased within 24 h; n = 123) and birds that were found alive but died during transport to the rehabilitation facility (n = 32) were evaluated; oil-fouled individuals were not included.

Carcasses were stored in ice and were necropsied immediately upon arrival to the rehabilitation facility (within 12 h from carcass collection). Body mass was measured with a scale (precision ± 1 g), and the age class (juvenile or adult) was inferred from plumage. Approximately 5 – 10 g of liver were collected (using heat-treated scalpel blades and tweezers, while wearing clean nitrile gloves), and placed in heat-treated aluminum foil. Additionally, approximately 0.5 g of liver tissue was placed in sterile RNAse-free cryotubes. Liver samples were then frozen in liquid nitrogen, and later stored at –80°C until processing. Sex was determined through the dissection of gonads. During necropsy, the gastrointestinal tract of the animal was dissected, and its contents were processed following the established PMP-BS protocol (Gallo et al., 2021; Baes et al., 2024). Visual sorting was conducted to identify marine debris, and plastic materials were recorded following the methodology outlined by Baes et al. (2024). The analysis primarily focused on determining the prevalence of debris ingestion among the studied animals. Thus, comprehensive data regarding quantity, mass, size, and color were lacking. The digestive tract was visually inspected also for the presence of gastrointestinal parasites visible to the naked eye. The presence of kidney trematode parasites (*Renicola* sp.) was also recorded (Matos et al., 2021).

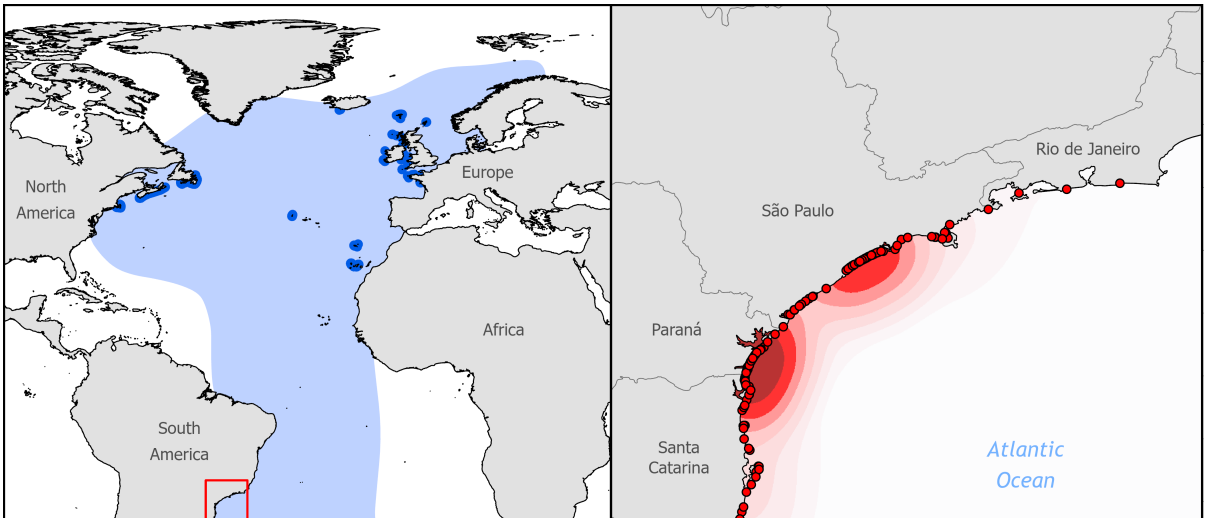

Fig. 1. Geographic distribution of the sampling effort relative to the natural distribution of Manx shearwater (*Puffinus puffinus*). Light blue areas represent the species' at-sea distribution, and dark blue areas represent the species' breeding distribution data (Ridgely et al., 2003). Red circles represent the stranding location of sampled individuals along the southern and southeastern coast of Brazil (red shaded areas are used to illustrate the density of sampled individuals).

2.2. Contaminant analysis

Detection and quantification of organic pollutants was performed at the Laboratory of Marine Organic Chemistry of the Oceanographic Institute of the University of São Paulo. The analytical procedure was conducted following the method outlined by MacLeod et al. (1985) with minor adjustments. In brief, liver samples underwent drying with anhydrous Na_2SO_4 and extraction via a Soxhlet apparatus. Surrogates, namely 2,2',4,5',6-pentachlorobiphenyl (PCB 103) and 2,2',3,3',4,5,5',6-octachlorobiphenyl (PCB 198), were utilized. Extract purification involved gravity flow through a glass column packed with silica and alumina, succeeded by size exclusion liquid chromatography. The eluate was concentrated, and 2,4,5,6-tetrachlorometaxylene (TCMX) served as the internal standard. Extracts were analyzed by using gas chromatography coupled to a mass spectrometer (PETROBRAS, 2021). The following PAHs were analyzed: 2-methylnaphthalene, 1-methylnaphthalene, C2-naphthalene, C3-naphthalene, C4-naphthalene, acenaphthylene, acenaphthene, fluorene, C1-fluorene, C2-fluorene, C3-fluorene, dibenzothiophene, C1-dibenzothiophene, C2-dibenzothiophene, C3-dibenzothiophene, phenanthrene, C1-phenanthrene-anthracene, C2-phenanthrene-anthracene, C3-phenanthrene-anthracene, C4-phenanthrene-anthracene, anthracene, fluoranthene, pyrene, C1-fluoranthene-pyrene, C2-fluoranthene-pyrene, benz[a]anthracene, chrysene, C1-chrysene, C2-chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, benzo[g,h,i]perylene. The following organochlorine pesticides (OCPs) were analyzed: hexachlorobenzene (HCB), hexachlorocyclohexane (HCH - α, β-, δ- and γ-isomer), chlordanes (heptachlor, heptachlor epoxide A and B, and α and γ chlordane), dichlorodiphenyltrichloroethane (DDTs, o,p'-DDT, p,p'-DDT, o,p'-DDD, p,p'-DDD, o,p'-DDE, and

p,p'-DDE), Drins (aldrin, isodrin, dieldrin, and endrin), endosulfan I and II, methoxychlor, and Mirex. The following polychlorinated biphenyls (PCBs) were analyzed: PCB49, PCB52, PCB66, PCB77, PCB81, PCB95, PCB101, PCB110, PCB114, PCB118, PCB123, PCB138, PCB141, PCB149, PCB151, PCB153, PCB156, PCB157, PCB169, PCB174, PCB177, PCB180, PCB189, PCB194, PCB195, and PCB206. The following polybrominated diphenyl ethers (PBDEs), also known as flame retardants, were analyzed: PBDE28, PBDE47, PBDE99, PBDE100, PBDE153, PBDE154, and PBDE183.

Limits of detection were as follows: 0.3 ng g^{-1} lipid weight for PAHs, 0.07 ng g^{-1} lw for PCBs, 0.05 ng g^{-1} lw for Drins and PBDEs, 0.04 ng g^{-1} lw for DDTs and HCB, and 0.02 ng g^{-1} lw for Mirex. For subsequent quantitative analyses, samples below the detection level were assigned a concentration equal to the limit of detection divided by the square root of 2 (Tekindal et al., 2017).

2.3. Biochemical biomarkers

Hepatic enzymatic activity of EROD and GST, as well as the levels of CYP1A protein were analyzed. Liver samples (50 mg) were homogenized in Tris buffer (Tris-HCl 50 mM, pH 7.4, 150 mM KCl, 1 mM DTT, and 0.5 mM PMSF) and subjected to centrifugation at 9,000 g (30 min, 4°C), and the supernatant from this first step was subjected to a second centrifugation at 100,000 g (1 h, 4°C). The resulting pellet, corresponding to the microsomal fraction, was used for quantifying EROD activity and immunochemical detection of CYP1A, while the supernatant was used for GST activity analysis (Focardi et al., 1992).

EROD activity quantification was based on the conversion of the substrate 7-ethoxyresorufin (7-ER) to the fluorescent compound resorufin in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), whose fluorescence can be quantified at 530/585 nm (ex/em) by fluorimetry (Burke and Mayer, 1974). EROD activity assays employed a temperature of 37°C, Tris/NaCl buffer (50 mM/0.1 M, pH 7.4), 1.25 µM 7-ER and 1 mM NADPH concentrations, and a quantification range of 219.96 to 9,020.61 $mRFU.min^{-1}$. GST enzyme activity was quantified by a photometric kinetic assay (Keen et al., 1976), through the evaluation of the formation of a GS-DNB conjugate from reduced glutathione (GSH) and 2,4-dinitrochlorobenzene (CDNB) substrates. GST activity assays employed a temperature of 37°C, potassium phosphate buffer (0.1 M, pH 7.0), 2.5 mM GSH and 2.5 mM CDNB concentrations, and a quantification range of 252.17 to 761.44 $mAbs.min^{-1}$.

The immunochemical detection of CYP1A protein was performed using the Western blotting technique with chemiluminescence detection (V3 Western Workflow system, Bio-Rad, Hercules, California, USA), following manufacturer instructions. CYP1A immunochemical detection employed 20 µg liver protein mass, 1:5,000 primary antibody anti-fish CYP1A produced in rabbit (Biosense CP-226, Cayman Chemical, Ann Arbor, Michigan, USA), 1:25,000 secondary antibody anti-rabbit IgG conjugated with peroxidase (NIF824, Cytiva Life Sciences, Amersham, UK), and the Clarity Max (Bio-Rad) detection method. A 1 µg sample of liver microsomes from mullet (*Mugil liza*) exposed to oil for 48 h was used as a positive control.

Total protein determination was performed using the Bradford method (Bradford, 1976). Enzyme analyses and total protein determination were performed in triplicates on the 96-well plate reader Spectramax M5 (Molecular Devices, Sunnyvale, California, USA).

2.4 Molecular biomarkers

Hepatic total RNA was extracted using Qiazol (Qiagen, Hilden, Germany) following manufacturer instructions, and stored at –80°C. The quantity and purity of the obtained RNA were assessed by spectrophotometry using NanoDrop 1000 (Thermo Fisher Scientific, Waltham,

Massachusetts, USA). The absorbance at 260 nm was used to estimate RNA concentration, and purity was assessed using the 260/280 nm ratio for proteins (acceptable values between 1.8 and 2.0), and the 260/230 nm ratio for other interfering substances (acceptable values above 1.7) (Wieczorek et al., 2012). Fluorimetry was also used to verify the RNA quality of each sample (Qubit 4.0 and Qubit RNA IQ assay kit, Thermo Fisher Scientific).

The transcript levels of the following genes were analyzed: *aryl hydrocarbon receptor* (*AhR*), *cytochrome P450 1A5* (*CYP1A5*), *UDP-glucuronosyl-transferase* (*UGT1*), *estrogen receptor alpha 1* (*ESR1*), and *heat shock protein 70* (*HSP70*). Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, Iowa, USA), and primer pairs were evaluated for their potential to form dimers and hairpin-like structures using OligoAnalyzer (Integrated DNA Technologies) and FastPCR 6.5 (PrimerDigital, Helsinki, Finland). Primers used for qPCR in this study are provided in Supplementary Table S1. Complementary DNA (cDNA) was synthesized from the total RNA extracted using the QuantiTect® Reverse Transcription kit (Qiagen). Real-time PCR (qPCR) assays were performed on a Rotor-Gene Q thermocycler (Qiagen) using the QuantiNova SYBR Green kit (Qiagen). Blanks and a standard curve with known copy numbers of the target genes for Manx shearwater were included in all assays. For each sample, the number of copies was normalized by the cDNA concentration in each reaction, and quantified fluorometrically using the Quant-iT OliGreen ssDNA assay kit (Thermo Fisher Scientific).

2.5. Statistical analysis

We ran analyses using R 4.2.2 (R Core Team, 2023), multiple tests and different scenarios were implemented to detect the most important predictor variables for determining the observed pollution biomarkers responses in birds. A hierarchical clustering of specimens using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used to explore patterns and relationships within individuals, building a hierarchy of interactions by iteratively merging the most similar clusters until all data points were grouped. Data normality was assessed by D'Agostino and Pearson test, and the distribution was not considered normal for $p > 0.05$. The homogeneity of variances (homoscedasticity) was tested using Levene's test. The data obtained were considered homoscedastic when $p > 0.05$. Spearman's rank correlation analysis among variables was used, particularly in the context of dimensionality reduction and to avoid collinearity within statistical models. Kruskal-Wallis tests were used to evaluate association between quantitative and categorical variables. Linear regression was used to evaluate relationship between two quantitative variables and was considered strong when $p < 0.05$ and $R^2 > 0.4$. Chi-square tests and Cramer's V statistic were used to evaluate association between two categorical variables.

Packages used for these analyses included DHARMa 0.4.6, dplyr 1.1.2, gam 1.22-3, GGally 2.2.0, ggeffects 1.3.4, ggplot2 3.4.4, mgcv 1.8-42, and MuMIn 1.47.5 (Wood, 2017; Hartig, 2022; Bartón, 2023; Hastie, 2023; Wickham et al., 2023; Lüdecke et al., 2024; Schloerke et al., 2024). Variables were considered predictors or modulators of biomarkers responses through a Generalized Additive Model (GAM), an extension of Generalized Linear Models (GLMs), capturing complex nonlinear relationships between independent and dependent variables (Tredennick et al., 2021). GAMs were chosen over GLMs after an initial exploratory modeling assessment because GAMs exhibited a better fit and the opportunity to better explore data variability as a function of explanatory variables. The GAM approach was selected as it allowed modeling the relationship between the explanatory variables and the response variable as parametric and/or additive nonparametric (smooth) terms (Rigby and Stasinopoulos, 2005; Stasinopoulos et al., 2017; Prado et al., 2022). The double penalty approach was used for variable selection, an alternative to avoid the problems normally associated with stepwise variable selection procedures (Marra and Wood, 2011).

The models produced allowed inference of patterns explaining the qualitative-quantitative relationships between different degrees of exposure to environmental contamination, parasites, and other impacts as predictors or modulators of responses of biotransformation enzymes activity levels and differentially transcribed genes.

Quantitative variables considered as predictors in our models and their respective measurement units were year (integer value), day of the semester (Julian days counting from 1 July of each year), body mass (Kg), PAHs (ng g^{-1} lw), PCBs (ng g^{-1} lw), HCB (ng g^{-1} lw), Drins (ng g^{-1} lw). DDTs were not included as predictors because they were strongly correlated to PCBs (R = 0.833, p < 0.001, Fig. S1). Chlordanes, endosulfans, methoxychlor and PDBEs were not included in the analyses because all samples were below the limit of detection. Categorical variables included region (SC = Santa Catarina state, PR = Paraná state, SP/RJ = São Paulo and Rio de Janeiro states), sex (male, female), age class (juvenile, adult), gastrointestinal parasites (true, false), renal parasites (true, false), plastics (true, false). Response variables included biomarkers as the enzyme activity for EROD and GST (pmol.min^{-1}.$mgprt^{-1}$) and immunodetection of CYP1A protein (relative fluorescence units); and number of transcripts for *AhR, ESR1, HSP70, CYP1A5* and *UGT1* (trancripts.ng^{-1} cDNA). Significance level (alpha) was 0.05.

3. Results

Necropsies, molecular and biochemical analysis were performed for 155 Manx shearwaters stranded along the southern and southeastern coast of Brazil (Fig. 1) in the states of Santa Catarina (43 individuals; 27.7%), Paraná (39 individuals; 25.2%), São Paulo (69; 44.5%) and Rio de Janeiro (4; 2.6%). These individuals were sampled in 2016 (2 individuals, 1.3%), 2017 (20; 12.9%), 2018 (33; 21.3%), 2019 (50; 32.3%) and 2020 (50; 32.3%). On average, individuals were found 116.0 ± 25.6 days (mean ± SD; range = 8 to 179) into the semester (days counting from July 1st of each year); in other words, the studied individuals stranded on average on October 25th ± 25.6 days. Overall, 76 were females (49.0%) and 79 were males (51.0%); 115 were juveniles (74.2%) and 40 were adults (25.8%). Average body mass (mean ± SD) was 0.253 ± 0.041 kg (range = 0.15 to 0.40 kg). Post-mortem examination revealed that 45 individuals (29.0%) had ingested plastics, 49 individuals (31.6%) had gastrointestinal parasites, and 93 individuals (60.0%) had kidney parasites.

Regarding associations among variables (Table S2), we found that the body mass was significantly associated to the age class (H = 4.094, p = 0.043), with juveniles being generally lighter (0.250 ± 0.040 kg) than adults (0.264 ± 0.042 kg). Body mass was also associated with the presence of kidney parasites (H = 3.967, p = 0.046), with parasitized individuals being generally lighter (0.248 ± 0.043 kg) than individuals without such parasites (0.261 ± 0.038 kg). Plastic ingestion was associated with the age class (V = 0.264, p = 0.001,) and the presence of gastrointestinal parasites (V = 0.222, p = 0.006,), with plastics more frequent in juveniles (36.5%) and individuals with gastrointestinal parasites (44.9%) than in adults (7.5%) and individuals without such parasites (21.7%). Plastic ingestion was also associated with the region (V = 0.212, p = 0.031), being more frequent in individuals collected at Paraná (38.5%) and São Paulo/Rio de Janeiro states (32.9%) than in those collected in Santa Catarina (14.0%). Linear regression found a significant but weak negative association between the day of the semester and body mass (r = -0.263, p < 0.001).

Table 1 summarizes the results found for the levels of bioaccumulated pollutants, genes transcription levels, enzymes activity and protein expression in the liver samples from the studied shearwaters. The limit of detection of pollutants was exceeded by 46 individuals (29.7%) for PAHs (most naphthalene), 135 individuals (90.6%) for PCBs, 144 individuals (96.6%) for total DDTs (most

p,p'-DDE), 38 individuals (25.5%) for HCB, 70 individuals (46.9%) for Drins, and 15 individuals (10.1%) for Mirex. Although Drins analyses encompassed several compounds such as aldrin, dieldrin, isodrin, and endrin ketone, in the Manx shearwaters studied only dieldrin was detected (n = 70). The predominance of naphthalene was also recorded within total PAHs (n = 40).

All samples were below the limit of detection for chlordanes, endosulfans, methoxychlor and PDBEs. Total concentrations of DDTs were strongly correlated to those of PCBs (r = 0.833, p < 0.001); DDTs concentrations were also weakly but significantly correlated to the concentrations of HCB, Drins and Mirex (Fig. S1). For these reasons, DDTs were not included in subsequent statistical analyses. A correlation was also found between Drins and Mirex (r = 0.642, p < 0.001). There were some significant correlations among the biochemical and molecular biomarkers measured in this study (Fig. S2). Although these correlations were not considered strong (r > 0.4), all statistical models were conducted separately for different biomarkers, in order to understand the best predictors for each one.

Table 1. Concentration (ng g^{-1} lw) of total polycyclic aromatic hydrocarbons (PAHs), total polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs), total hexachlorobenzene (HCB), total Drins and Mirex, transcription levels (transcripts ng^{-1} cDNA) of the genes *aryl hydrocarbon receptor* (*AhR*), *UDP-glucuronosyl-transferase 1* (*UGT1*), *cytochrome P450 1A5* (*CYP1A5*), *estrogen receptor alpha 1* (*ESR1*), *heat shock protein 70* (*HSP70*), the activity (pmol.min^{-1}.$mgprt^{-1}$) of the 7-ethoxyresorufin *O*-deethylase (EROD) and glutathione *S*-transferase (GST), and the expression levels (relative fluorescence units) of CYP1A protein in liver samples of Manx shearwaters sampled in Brazil from 2016 to 2020.

Contaminant	**N**	**Mean ± SD**	**Median (range)**
Total PAHs	155	569.92 ± 1344.77	0.30 (0.30 – 9725.00)
Total PCBs	149	22,636.52 ± 3419.85	10,908.00 (0.07 – 247,970.00)
Total DDTs	149	5827.88 ± 9225.85	2909.08 (0.04 – 70,687.71)
HCB	149	283.05 ± 809.62	0.04 (0.04 – 7660.93)
Total Drins	149	603.49 ± 1289.55	0.05 (0.05 – 11,178.20)
Mirex	149	333.16 ± 1729.60	0.02 (0.02 – 15,073.41)
Biomarker	**N**	**Mean ± SD**	**Median (range)**
AhR	155	323.88 ± 310.92	238.14 (7.61 – 1834.38)
UGT1	155	1104.68 ± 871.70	882.72 (25.72 – 4723.21)
CYP1A5	155	533.57 ± 511.85	389.32 (9.56 – 2890.87)
ESR1	155	21.48 ± 18.14	16.80 (0.54 – 99.73)
HSP70	155	41.36 ± 146.25	16.64 (0.41 – 1762.23)
EROD	155	16.79 ± 14.88	13.75 (0.02 – 100.03)
GST	155	0.38 ± 0.12	0.36 (0.13 – 0.71)
CYP1A	155	0.04 ± 0.04	0.02 (0 – 0.27)

Figure 2 summarizes the effects of variables identified through GAM as significant predictors of biochemical biomarkers (further details provided in Tables S3 and S4). Total concentrations of Mirex (p = 0.028; Fig. 2A) and Drins (p = 0.037; Fig. 2B) and the year of sampling (p = 0.046; Fig. 2C) were significant predictors of GST enzymatic activity. The year of sampling (p < 0.001; Fig. 2D) and the day of the semester (p = 0.001; Fig. 2E) were significant predictors of EROD activity. For the immunodetection of the CYP1A protein, the day of the semester (p = 0.011; Fig. 2F), the region (p = 0.026 for Paraná, p = 0.051 for São Paulo/Rio de Janeiro; Fig. 2G), and Mirex concentration (p = 0.038; Fig. 2H) were identified as significant predictors. The only pollutants with significant associations with enzymatic activity included Mirex and Drins. Plastic ingestion and parasites prevalence were not good predictors of biochemical biomarkers (all p > 0.05).

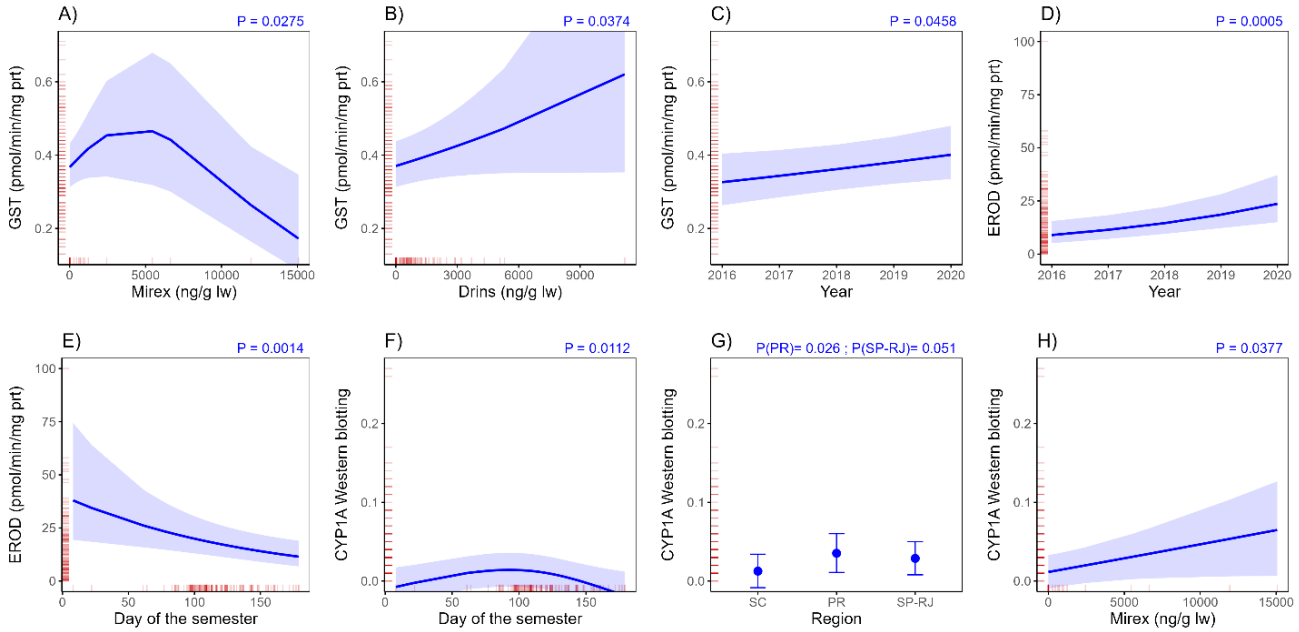

Fig. 2. Effect plots for significant variables ($p < 0.05$) in General Additive Models (GAM) for the liver enzymatic activity of glutathione *S*-transferase (GST) and 7-ethoxyresorufin *O*-deethylase (EROD) and the immunodetection level of the cytochrome P450 1A proteins (CYP1A) in Manx shearwaters (*Puffinus puffinus*) stranded along the southern and southeastern coast of Brazil. Blue lines or circles represent the model predictions, with their 95% confidence intervals represented by shaded blue areas or blue error bars. Red rugs along the margins of the plots represent the data distribution used to generate the model.

Figure 3 summarizes the effects of variables identified through GAM as significant predictors of molecular biomarkers (further details provided in Tables S4 and S5). *AhR* transcription level was predicted by the region ($p = 0.035$ for Paraná, $p = 0.681$ for São Paulo/Rio de Janeiro; Fig. 3A). *CYP1A5* transcription level was predicted by the day of the semester ($p = 0.001$; Fig. 3B), HCB concentration ($p = 0.001$; Fig. 3C), region ($p = 0.021$ for Paraná, $p = 0.054$ for São Paulo/Rio de Janeiro; Fig. 3D), and PCBs concentration ($p = 0.034$; Fig. 3E). *ESR1* transcription level was predicted by the presence of kidney parasites ($p = 0.031$; Fig. 3F), Mirex concentration ($p = 0.038$; Fig. 3G), presence of gastrointestinal parasites ($p = 0.041$; Fig. 3H), and HCB concentration ($p = 0.048$; Fig. 3I). *HSP70* transcription level was predicted by the body mass ($p = 0.002$; Fig. 3J) and year of sampling ($p = 0.004$; Fig. 3K). *UGT1* transcription level was predicted by the region ($p < 0.001$ for Paraná, $p = 0.001$ for São Paulo/Rio de Janeiro; Fig. 3L), HCB concentration ($p < 0.001$; Fig. 3M), PCBs concentration ($p = 0.003$; Fig. 3N), age class ($p = 0.013$; Fig. 3O), presence of kidney parasites ($p = 0.015$; Fig. 3P), and year of sampling ($p = 0.022$; Fig. 3Q). The only pollutants with significant associations with molecular biomarkers were polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), Mirex and Drins (primarily dieldrin).

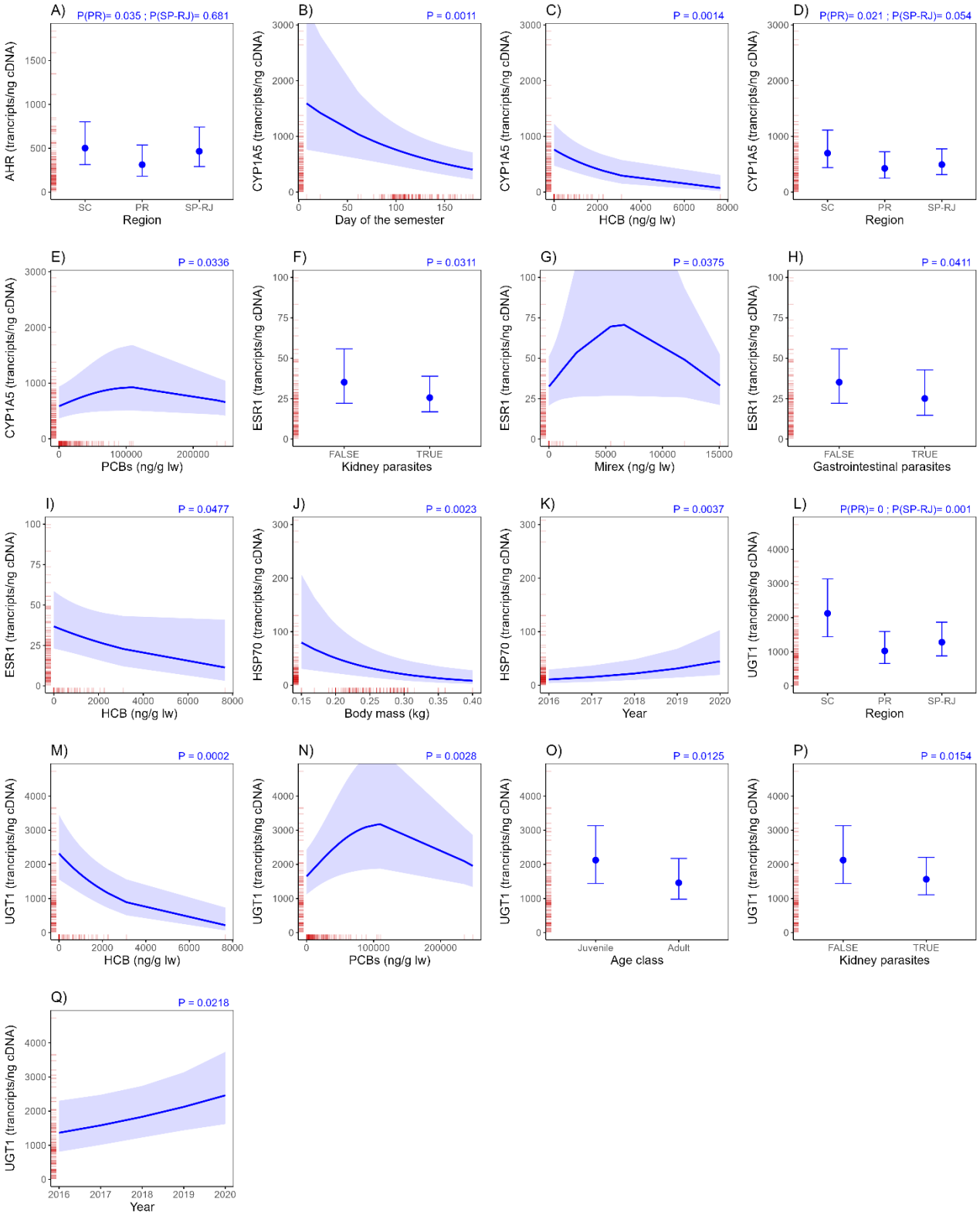

Fig. 3. Effects plots for significant variables ($p < 0.05$) in General Additive Models (GAM) for the liver transcription levels of *aryl hydrocarbon receptor* (*AhR*), *cytochrome P450 1A5* (*CYP1A5*), *estrogen receptor alpha 1* (*ESR1*), *heat shock protein 70* (*HSP70*) and *UDP-glucuronosyl-transferase* (*UGT1*) genes in Manx shearwaters (*Puffinus puffinus*) stranded along the southern and southeastern coast of Brazil. Blue lines or circles represent the model predictions, with their 95% confidence intervals represented by shaded blue areas or blue error bars. Red rugs along the margins of the plots represent the data distribution used to produce the model.

4. Discussion

In the present study, we demonstrated that the Manx shearwaters stranded on Brazilian coast have a high prevalence of bioaccumulated pollutants, such as PCBs, that affected their hepatic biotransformation system, namely the transcript levels of *CYP1A5* and *UGT1* genes. Additionally, Drins (i.e. dieldrin) were detected in 46.9% of the sampled birds and affected the GST enzyme activity. The response of enzymes and genes observed in our study represented a biotransformation defense mechanism developed by organisms exposed to contaminants. They are considered good indicators of sublethal effects because they had the potential to reflect subtle variations on contaminant levels, corroborating its use as biomarkers for assessing both exposure to and the effects of environmental pollutants (Homolya et al., 2003; Walker et al., 2012). Additionally, both male and female birds across all age classes exhibited low body mass and a high prevalence of parasitosis, suggesting they are already facing environmental challenges. This situation could be further compounded by contaminants exposure, posing an additional threat.

On the other hand, the prevalence of plastic ingestion in our study (29%) was relatively low when compared to the findings of Alley et al. (2022), who observed that 71% of Manx shearwaters sampled in the Northern Hemisphere had plastic in their digestive tracts, with 24 out of 34 individuals affected. We found no consistent correlation or significance of association between plastic ingestion with observable effects in all chosen biomarkers. This implies that the main source of contaminants in this case may not be linked directly to plastics. This is the first study to explore effects using these methods in Manx shearwater, but further investigation into the potential for nano- and microplastics to serve as sources of adsorbed contaminants remains necessary.

Sources of organic pollutants in the ocean can be numerous. In our study, we detected a strong association of OCPs (i.e. HCB, Drins and Mirex) and PCBs with the seabird's response in xenobiotic biotransformation classical biomarkers, specifically *CYP1A5* and *UGT1* gene transcription. It is well known that PCBs and OCPs exhibit an affinity for polymeric particles, such as plastic, and tend to undergo adsorption onto their surfaces (Mato et al., 2001; Endo et al., 2005; Rios et al., 2007; Taniguchi et al., 2016; Provencher et al., 2018). Both contaminants have been detected in plastic fragments ingested by seabirds in the Southwest Atlantic Ocean (Colabuono et al., 2010). Nevertheless, we did not detect a direct significant relationship between plastic ingestion and *CYP1A5* and *UGT1* individual response.

CYP genes play a pivotal role in the interplay between environmental lipophilic pollutants and animal health and have been demonstrated to be impacted by contaminant exposure in seabirds (Nelson et al., 2013; Kreitsberg et al., 2023). Our results corroborate this, as *CYP1A5* transcription was related to PCBs and HCB levels. Findings from previous avian and mammalian studies demonstrate some effect of pollutants on biotransformation pathways, particularly those mediated by the modulation of *CYP* genes, such as *CYP1A4* and *CYP1A5* (but also *CYP3A* and *CYP2*) (e.g. Head and Kennedy, 2019). In particular, a study conducted on cormorants revealed that concentrations of perfluorooctane sulfonate and perfluorononanoic acid were negatively correlated with levels of *CYP2C45* and *CYP2J25* gene transcripts, suggesting downregulation of expression by these environmental pollutants (Kubota et al., 2011). Furthermore, European herring gull (*Larus argentatus*) embryo hepatocytes exhibited upregulation of *CYP1A4* and *CYP1A5* genes following exposure to dioxins (Hervé et al., 2010). Our results for the transcription of *CYP1A5* corroborate that *CYP* genes have a pivotal role as both biomarkers of exposure and xenobiotic biotransformation.

Particularly, *CYP1A* is widely employed as a biomarker for wildlife exposure to substances binding to the *AhR*, initiating the transcription of several enzymes involved in the chemical biotransformation (Xia et al., 2020). Transcriptional induction of *UGT1*, a gene encoding phase II

xenobiotic metabolizing enzyme, via the *AhR* pathway, was also described to be induced after exposure to classical environmental pollutants that play a role as inducers of *CYP1A* activity (Bugiak and Weber, 2010). In the present study, elevated levels of bioaccumulated HCB were significantly related to decreased *CYP1A5* and *UGT1* transcription, showing a downregulation pattern for both genes. The downregulation effects are potentially related to chronic HCB exposure and/or bioaccumulation. Interestingly, the response of both classical biomarkers to PCB levels was similar, showing an initial upregulation of gene transcription followed by a downregulation associated with higher pollutant levels. Levels of PCBs found for some birds in our study are high when compared to mean hepatic concentrations of total PCBs in other seabirds and higher than those estimated to elicit immunosuppressive effects and possibly increase susceptibility to parasitosis (Malcolm et al., 2003; Naso et al., 2003; Sakellarides et al., 2006). Thus, our findings indicate that very high levels of PCBs seem to no longer stimulate biotransformation gene transcription. It is also worth considering that, in contrast to PAHs, synthetic organic chemicals like PCBs are only partially metabolized and can induce different effects according to the byproducts and rates of metabolization (Grimm et al., 2015).

In our study, PAHs levels were not significantly related to any biomarker of exposure or effect, and this pollutant was only marginally associated with the *AhR* gene transcription, which was also slightly influenced by plastic. PAHs sources comprise natural (e.g. creosote, forest fires, volcanoes) and anthropogenic (fossil fuel combustion, accidental and intentional oil spills, waste incineration, asphalt production) origins, with human activities as the predominant sources. PAHs are ubiquitously found in the global environment and source contributions to total PAHs in the marine environment are mainly petrogenic (related to petroleum or hydrocarbon-based substances), with subsequent contributions from combustion of coal/wood, fossil fuels, and engine emissions (González-Gaya et al., 2016). PAHs have a wide array of adverse effects on organisms; however, their toxicity thresholds are not available. We consider that in our study the weak *AhR* response to PAHs is more likely from a diffuse source than an acute petrogenic exposure because the concentrations of PAHs in hepatic tissues from the Manx shearwaters were relatively low, when compared to the concentrations found in seabirds from other regions with higher industrial pollution (Provencher et al., 2020; Waszak et al., 2021) or other seabird species in the region (e.g. Quinete et al., 2020). It has also been shown that sites near coastal regions exhibited elevated contributions from petrogenic sources (Zhang et al., 2021). A significant difference of *AhR* transcription levels by region might explain spatial influences of exposure and response to contaminants, being the Paraná state the most influential region for this biomarker.

We also found that the *ESR1* gene transcription was mainly influenced by HCB, with a significant response being demonstrated also for Mirex. *ESR1* transcription levels have previously been found to correlate with total PCBs in ringed seals (Brown et al., 2014) and harbor seals *(*Noël et al., 2017), but we did not find association reported between *ESR1* with HCB in literature. As our statistical models could not prove a strong association of females presenting the highest transcription levels, our findings corroborate the need of future use of other genes, as those encoding vitellogenin, to delve deeper into the endocrine disruption effect of pollutants (Felton et al., 2020). That is important because hormonal distinctions play a role, with endogenous estrogen potentially being more efficacious in females as a defense against pollutants effects (Nakayama et al., 2008). Sex-specific distinctions, like the maternal transfer of contaminants to eggs, have been well-documented and even quantified (Ackerman et al., 2016); however, we were not able to detect a clear sex-related pattern for both bioaccumulation and gene transcription. Additionally, considering that most shearwaters we have analyzed were juveniles (74.2%), that might be the reason for unrevealing sex-specific variations in pollutant detoxification, which could be better

explained by metabolic differences between adult males and females during reproduction (Gibson et al., 2014).

In the present study, the *HSP70* transcription levels were associated with body mass and the stranding year. Our data showed that for Manx shearwaters, none of the contaminants significantly modulated the *HSP70* gene transcription. Our predictive models showed an upregulation of *HSP70* transcripts over the years, potentially related to an increase in environmental stressors at the study area over time. The downregulated levels of *HSP70* by body mass may reflect a reduced capacity for response to stressors in emaciated birds, which potentially experienced severe food deprivation after migration. Heat shock proteins, particularly those from the HSP70 family, have long been associated with the response to general environmental stressors (Mahmood et al., 2014). Most members of this family of proteins are typically present in the normal cellular state, where they serve a fundamental role in maintaining native polypeptide folding and facilitating their transfer to various cellular compartments (Feder and Hofmann, 1999). Nevertheless, when cells encounter conditions that induce cellular stress, there is a notable increase in protein misfolding. In such instances, HSPs are frequently upregulated to aid in either the refolding of proteins or their targeted removal from the cell. The latter is crucial because the accumulation of denatured proteins within the cell can be cytotoxic (Fink, 1999).

Overall, other studies carried out with vertebrates also showed that exposure to xenobiotics leads to gene expression downregulation rather than upregulation (i.e. Hook et al., 2006). For instance, extended exposure to PCBs revealed detrimental effects on the CYP1 system, evidenced by the downregulation of the *CYP1A1* gene, as demonstrated by Celander et al. (1996) in rainbow trout (*Oncorhynchus mykiss*). Granby et al. (2018) also observed a significant downregulation of fish biotransformation-related genes following a 40-day exposure to PCBs. The downregulation of *CYP1A1* and *GST* genes during the initial phase of bioaccumulation suggests an impairment of the detoxification process, as noted by Maradonna et al. (2014). Furthermore, the downregulation of biotransformation genes serves as an indicator of oxidative stress, a phenomenon supported by various studies demonstrating the involvement of reactive oxygen species in the downregulation of certain CYP isoforms (Reynaud et al., 2008). Thus, the putative downregulation of biotransformation-related genes of organisms chronically exposed may increase their susceptibility to pollutants. On the other hand, heightened levels of enzymatic activity in the liver could potentially trigger a negative feedback loop. It could also be hypothesized that the downregulation of biotransformation-related genes following exposure to cumulative concentrations of contaminants such as total PCBs and OCPs may be characteristic of a chronic response causing bioaccumulation adaptive changes in gene transcription (Kreitsberg et al., 2023). However, whether the Manx shearwaters are chronically exposed to organic pollutants in their habitats needs further investigation.

Considering the biochemical responses, we found a positive and significant relationship between the levels of Drins, Mirex and the GST activity. The occurrence of Drins has been already reported for albatrosses and petrels in the South Atlantic Ocean (Colabuono et al., 2012; Quadri Adrogué et al., 2019). Drin pesticides were synthesized from pentadiens obtained as secondary products of petrochemistry through the Diels-Alder reaction; they were historically used as insecticide, as well as a rodenticide and piscicide (Thiombane et al., 2018). Significant correlations between activities of antioxidant enzymes (i.e. GST isoforms) and concentrations of various OCPs in liver of waterbirds was described by Kocagöz et al. (2014), providing additional support for oxidative toxicity of pollutants. The increase in GST activity found in the present study is probably related to the stimulation of the detoxification mechanism imposed by the organochlorine compounds, as proposed by Thiombane et al. (2018).

Among markers of health, the results obtained for prevalence of parasites showed significant associations with *ESR1* and *UGT1* transcription levels. Limited understanding exists regarding the factors influencing parasite prevalence and load in wild birds. Nevertheless, some studies showed that environmental contaminants and parasites serve as widespread stressors, potentially impacting animal physiology synergistically (i.e. Carravieri et al., 2020a). Immunocompetence and energetic constraints likely play crucial roles in mediating individual responses to contaminants and parasites, warranting further investigation (Bustnes et al., 2006). Our results highlight a pressing need for studies elucidating the impact of gastrointestinal parasites on contaminant kinetics and dynamics in avian hosts, particularly concerning organochlorine pollutants, given their potential interaction with parasites and adverse effects on fitness.

The present study showed an ontogenetic association of the *UGT1* gene regulation, with higher gene transcription levels in older Manx shearwaters stranded. However, the predominance of juveniles in our sample may have influenced this outcome. The age of the bird was proposed by Kreitsberg et al. (2023) as one of the most pivotal factors influencing the pollution burden, along with the activation of genomic pathways in seabirds. Therefore, we suggest that further studies might be necessary to understand patterns of contaminant bioaccumulation and related response in different age classes (Lima et al., 2023).

Finally, it is important to mention that, transfer through the food chain may be the primary source of pollutants to seabirds (Carravieri et al., 2020). Nevertheless, the ingestion of microplastics and nanoplastics, and even their bioaccumulation in top predator birds cannot be discarded as adsorption can be an additional source for the contaminants observed in the Manx shearwaters (Rai et al., 2022). Specially because Procellariiformes are particularly prone to be exposed, as they have been reported as one of the seabird groups most affected by plastic pollution (Colabuono et al., 2010; Dias et al., 2019; Daudt et al., 2023).

5. Conclusions

Our findings endorse shearwaters as suitable model species for assessing differences in gene transcription and pinpointing early indicators of adverse health effects caused by pollutants, but not for plastic ingestion. Their extended lifespan, vast geographical range, and top-predator status make them susceptible to the processes of contaminant bioaccumulation and biomagnification within the food chain (Burger and Gochfeld, 2004). They offer opportunities to explore a broader spectrum of health outcomes and delve into a wider array of the physiological trade-offs that result from the response to ocean pollution, such as diminished investments in reproduction or parasite protection.

Studies involving biomarkers in long-lived animals exposed to pollution have the potential to yield more efficient early detection methods for sublethal effects, before population impacts are seen. In fact, early indicators of impact make it possible to inform management decisions (e.g. Zahaby et al., 2021). Our findings regarding associations between gene transcription and GST activity to PCBs, HCB, Drins and Mirex serve not only as baseline data for Manx shearwaters, but also as valuable tools for decision-makers and conservationists who aim to take proactive measures and anticipate actions on mitigation and regulation of pollutant sources.

Author contributions

Patricia Pereira Serafini: formal analysis (lead), investigation (lead), data curation (equal), methodology (equal), validation (equal), writing–original draft (lead), writing–review and editing (lead). **Bárbara P.H. Righetti**: data curation; formal analysis; investigation; methodology; writing–review and editing. **Ralph E.T. Vanstreels**: formal analysis; investigation; methodology; writing–review and editing. **Leandro Bugoni**: formal analysis; investigation; methodology; writing–review and editing. **Daína Lima**: data curation; methodology; writing–review and editing. **Jacó J. Mattos**: data curation; methodology; writing–review and editing. **Clei E. Piazza**: data curation; methodology; writing–review and editing. **Cristiane K.M. Kolesnikovas**: data curation; writing–review and editing. **Alice Pereira**: writing–review and editing. **Marcelo Maraschin**: writing–review and editing. **Isadora Piccinin**: writing–review and editing. **Tim Guilford:** writing–review and editing. **Luciana Gallo:** methodology, writing–review and editing. **Marcela Uhart:** formal analysis; methodology; writing–review and editing. **Afonso C.D. Bainy**: conceptualization; supervision; writing–review and editing. **Karim H. Lüchmann**: conceptualization; supervision; writing–review and editing.

Conflict of interest

The authors have no conflicts of interest to declare.

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Ethics and permissions

This project was undertaken with all the necessary permits issued by SISBIO/ICMBio and IBAMA, environmental agencies of Brazil.

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Appendix A. Supplementary data

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