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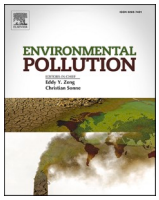

Hidden markers of health: how mercury affects gene expression in developing seabirds☆

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ABSTRACT

Mercury is a globally recognized environmental contaminant that bioaccumulates and biomagnifies in food webs, thereby causing adverse health effects in both humans and wildlife. While mercury exposure is known to impact several life-history traits in birds, the molecular mechanisms underlying these effects remain poorly investigated. In this study, we examined the association between blood mercury concentrations and the expression of 15 key genes involved in detoxification and oxidative stress regulation in magnificent frigatebird *Fregata magnificens* chicks from French Guiana. Specifically, we measured the expression of genes encoding glutathione-dependent enzymes (GSTA1, GSTA2, GSTK1, GSTM1, GSTT1_0, GSTT1_1, GPX1, GPX2, GPX3, GSS, GSR_0 and GSR_1), thioredoxin system (TXNRD1 and TXNRD3), and metallothionein (MT1). Our results revealed a significant decrease in GSTA1, GSTA2, and GSTT1_1 expression with increasing mercury concentrations. No significant associations were found for glutathione peroxidases, glutathione synthetase and reductases, thioredoxin reductases, or metallothionein expression. These findings might indicate a potential increase in toxicity and cellular damage due to the lower detoxification of glutathione S-transferases. We emphasize the need for further investigations into species-specific mechanisms of detoxification. Our study supports the utility of gene expression analysis in addition to traditional physiological measurements to assess contaminant induced disruptions.

1. Introduction

Mercury is a globally recognized environmental contaminant. To date, given its transport via oceanic and atmospheric currents, mercury is found everywhere on earth. Human activities have increased the concentrations of atmospheric mercury by 450 % compared to natural levels, and released thousands of tons of mercury into the environment (UNEP, 2019). Despite the ratification of the Minamata Convention on mercury in 2013, its emissions are still high in certain countries especially from Asia (which accounts for the 39 % of the global anthropogenic emissions, UNEP, 2019) and South America, particularly in the Amazon region, in relation to deforestation and biomass burning (Crespo-Lopez et al., 2021), industrial waste (Brocza et al., 2024), and artisanal and small scale gold mining ASGM (Brocza et al., 2024; Gerson et al., 2022; Legg et al., 2015). Mercury bioaccumulates in organisms as they age and it biomagnifies within food chains, leading to significant exposure for organisms feeding at the top of the food chain, particularly those in aquatic ecosystems, as seabirds (de Almeida Rodrigues et al., 2019).

Several studies have demonstrated that mercury exposure in birds is associated with a wide range of negative health effects including reproductive impairments, neurological dysfunction, and behavioral abnormalities (reviewed in Whitney and Cristol, 2017). But the molecular mechanisms driving such harmful effects remain poorly investigated. Therefore, transcriptomics - i.e. the analysis of the RNA transcripts produced by the genotype at a given time - represent a

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powerful tool for investigating the molecular pathways involved in the response to contaminant exposure (Bozinovic and Oleksiak, 2011; Kreitsberg et al., 2023; Pujolar et al., 2012). When compared to traditional physiological approaches, the use of transcriptomic analyses has the ability to provide a more comprehensive understanding of cause-effect relationships by measuring the activity of specific genes and by identifying genes that are upregulated or suppressed in response to contaminants, as it has been done previously in birds (Esperanza et al., 2024; Kreitsberg et al., 2023).

An area of increasing interest in mercury toxicity research involves its interaction with thiol-containing molecules (Ajsuvakova et al., 2020). Thiols represent chemical compounds similar to alcohols, but containing a sulfur atom in place of the oxygen atom (Ajsuvakova et al., 2020). They are abundant in glutathione, one of the major endogenous antioxidants, particularly involved in mitigating oxidative stress (Ulrich and Jakob, 2019). Despite the fact that organisms have evolved detoxification strategies, the high affinity of mercury for thiol-containing molecules can impair the action of such molecules, causing disruption of redox homeostasis and of detoxification processes(Ajsuvakova et al., 2020). In zebra finches *Taeniopygia guttata, in ovo* exposure of mercury was negatively associated with the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) which is indicative of oxidative stress (Henry et al., 2015). Similarly, in black-vented shearwaters *Puffinus opisthomelas*, birds showing higher blood mercury concentrations also showed a lower activity of the antioxidant enzyme glutathione peroxidase GPx (Soldatini et al., 2020), and mallards *Anas platyrhynchos* experimentally fed with dietary methylmercury (i.e. the organic and bioaccumulative form of mercury) showed decreased activities of GPx in both plasma and liver, and of glutathione S-transferase GST in the liver (Hoffman and Heinz, 1998). Furthermore, mercury binds strongly to the thioredoxin reductases, which are selenoenzymes whose function is highly inhibited by mercury and which are suspected to participate in methylmercury degradation (Branco and Carvalho, 2019). Although there is substantial evidence suggesting that mercury exposure has an impact on several physiological pathways, on thiol-containing molecules including glutathione, and on enzymes involved in detoxification processes, most previous studies have i) investigated the impact on a limited number of molecules without considering structurally or functionally related compounds (Oliveira et al., 2020); and ii) rarely examined the effects at the gene expression level (Oliveira et al., 2020). Therefore, we still lack a clear understanding of the relationship between mercury exposure and the molecules involved in detoxification and oxidative stress protection, especially in wild animals. Filling this research gap is crucial for our understanding of the impact of mercury exposure on wildlife health, to be able to predict population-level consequences and informing conservation strategies.

In French Guiana, magnificent frigatebirds *Fregata magnificens* are exposed to high concentrations of mercury (Sebastiano et al., 2016, 2017), thereby offering an opportunity to investigate the relationship between mercury exposure and transcriptomic consequences in free-living animals. In this article, we investigated the association between blood mercury concentrations and the expression of 15 key genes codifying for thiol-containing enzymes or proteins involved in detoxification processes from xenobiotic substances. Twelve of those genes codify for glutathione-dependent enzymes which are suspected to play a role in reducing the effect of mercury exposure (Balali-Mood et al., 2021; Gundacker et al., 2007; Martinez et al., 2017; Mlakar et al., 2021): glutathione S-transferases (GSTs) alpha family 1 and 2 (GSTA1 and GSTA2), glutathione S-transferase kappa family 1 (GSTK1), glutathione S-transferase mu family 1 (GSTM1), glutathione S-transferase theta family 1_0 and 1_1 (GSTT1_0 and GSTT1_1), glutathione peroxidases (GPXs) 1, 2, and 3 (GPX1, GPX2, and GPX3), glutathione synthetase (GSS), and of glutathione reductases (GSR_0 and GSR_1). We expected a decrease in glutathione transferases, peroxidases, synthetases, and reductases as the glutathione system is a target of mercury exposure (Franco et al., 2009; Martinez et al., 2017; Linšak et al., 2013). We also expected a disruption on the expression levels of thioredoxin reductases (TXNRD1 and TXNRD3), as mercury can induce inhibition of such enzymes (Branco and Carvalho, 2019). Finally, as the expression of metallothionein genes - important for inorganic mercury detoxification processes - is correlated to mercury concentrations (Schlenk et al., 1995), we further measured Metallothionein 1 (MT1) gene as we expected an increased expression of this gene.

2. Materials and methods

2.1. Data collection

We carried out this study in June 2022 on Grand Connétable island (4°49′30N; 51°56′00W), a natural reserve located 15 km off the coast of French Guiana, where about 1800 pairs of frigatebirds breed each year. We randomly captured and selected 18 chicks that were approximately of the same age at the nest. We then collected a blood sample of 1.5 mL with a 25G needle from the brachial vein within 3 min from capture, an aliquot of which was kept in RNAprotect Animal Blood collection tubes following the manufacturer instructions (Qiagen, Germany), while another aliquot was centrifuged to separate plasma and red blood cells (hereafter blood) to be used in contaminant analyses. While in the field, we stored the samples in dry ice, and we kept them at −80 °C until laboratory analyses. We measured body mass and skull length (two proxies of age in our species; Diamond, 1973) to control for any variation that might influence mercury accumulation patterns. This population is subjected to recurrent outbreaks of diseases likely linked to herpesvirus infections (Sebastiano et al., 2019, 2022) with chicks showing visible clinical signs (i.e. skin crusts). Although both chicks with and without visible clinical signs were sampled, the disease status was not taken into consideration in statistical analyses as any gene that showed a differential expression level between healthy and sick chicks (as highligthed in Sebastiano et al., 2024) was removed to avoid collinearity of results, and because birds with or without clinical signs of the disease show similar size and weight (Sebastiano et al., 2024). Therefore, in this work, we specifically focused on the association between mercury concentrations and gene expression of birds.

2.2. RNA isolation, quality control, and bioinformatics analyses

We used the RNeasy Protect Animal Blood Kit (Qiagen, Germany) for the purification of total RNA excluding miRNA (<200 nucleotides), accordingly to the detailed protocol on the manufacturer website. We stored the extracted RNA in collection tubes at −20 °C until they were shipped to Novogene Co. (UK) for sequencing and trancriptomic analyses. Here, the mapped reads of each sample were assembled by StringTie v1.3.3b (Pertea et al., 2015) in a reference-based approach. FeatureCounts v1.5.0-p3 (Liao et al., 2014) was used to count the reads numbers mapped to each gene. A detailed protocol can be found in Sebastiano et al. (2024). A detailed table on sample data quality and mapping results can be found in the Supplementary Table S1.

2.3. Contaminant analyses

Blood was freeze-dried and homogenised to powder, and mercury was quantified in subsamples of this powder (mean ± SD, 0.34 ± 0.07 mg dry weight (dw)) using an Advanced Mercury (Hg) Analyser (®Altec AMA 254 spectrophotometer) at the LIENSs laboratory. Mercury was quantified in duplicate such that the coefficient of variation between the two duplicates was below 10 % (mean 1.5 %). The retained concentration is the mean value of replicate measurements. A certified reference material (CRM) for trace elements was analysed under the same conditions of the samples: TORT-3 (lobster hepatopancreas, Hg-certified value: 0.29 ± 0.02 μg g^{-1} dry weight (dw) from NRCC, Willie et al., 2013). CRM recovery rate (±SD) was 103.1 ± 1.2 %. Blanks were measured before each run and the limit of detection of the AMA was 0.1

ng. Mercury concentrations are expressed in μg g^{-1} dw. One sample could not be analysed for mercury concentrations as we were not able to collect enough blood, and was thus excluded from the statistical analyses. The total number of samples for which both mercury concentrations and transcriptomic data were available is therefore 17 individuals.

2.4. Statistical analyses

Out of the mapped genes, we only selected 15 genes that are suspected to be influenced by mercury exposure according to the literature: glutathione S-transferase alpha 1 and 2 (GSTA1 and GSTA2); glutathione peroxidase 1, 2, and 3 (GPX1, GPX2, and GPX3); glutathione S-transferase theta 1_0 and 1_1 (GSTT1_0 and GSTT1_1); glutathione S-transferase kappa 1 (GSTK1); glutathione S-transferase mu 1 (GSTM1); glutathione synthetase (GSS); glutathione reductase 0 and 1 (GSR_0 and GSR_1); thioredoxin reductase 1 and 3 (TXNRD1 and TXNRD3); and metallothionein 1 (MT1). The association between mercury concentrations and gene expression was tested using DESeq2 package in R (Love et al., 2014) which fits gene-wise generalized linear models (GLMs) assuming a negative binomial distribution of read counts. As a general rule, filtering lowly expressed genes improves the false discovery rate and detection of differentially expressed genes, usually by filtering for FPKM >0.3 in more than 50 % of observations or a sum of at least 10 for count data for a specific gene (Deyneko et al., 2022; Love et al., 2014). However, the DESq2 package has a build-in function than enables to filter genes when those show almost no counts across all samples, which reduces the number of multiple comparison and improves the detection of true positives (Love et al., 2014). Furthermore, the package has a built-in outlier detection and control system based on Cook's distance (Cook, 1977), which automatically detects and handles outliers (Love et al., 2014). We centered and scaled the variables mercury and skull length (which was used to control for the size of each bird) in the models to increase model convergence and avoid collinearity. P-values were corrected to avoid multiple comparisons issues, and thus we considered a significant association when adjusted p-values were <0.05. We preferred to use the stricter Bonferroni correction instead of other less conservative methods (e.g. FDR) because this approach minimizes the risk of discovering false positives (type I error) in exploratory studies although there is also the risk of type II error (Nakagawa, 2004). This was done to ensure that only the most robust association between mercury and gene expression are highlighted. Any eventual removal of outliers is documented throughout the manuscript. For visual purposes, raw data of gene counts were used to plot the relationship between mercury levels and the expression of specific genes. All analyses were performed using R version 4.4.2 (R Core Team, 2021).

3. Results

Out of the 15 selected genes, GSTT1_0 and GPX2 showed very low expression levels (below 10 counts in >50 % of samples), but all genes were eventually retained during data analyses. Observed blood mercury concentrations ranged from 0.76 to 2.89 μg g^{-1} dw, with a mean concentration of 1.28 ± 0.51 μg g^{-1} dw. Increasing mercury concentrations were associated with decreasing levels of glutathione S-transferases alpha 1 (GSTA1; Wald statistics W = −6.98, p-adj = <0.001; Table 1, Fig. 1), alpha 2 (GSTA2; W = −6.72, p-adj <0.001; Table 1, Fig. 1), and theta 1_1 (GSTT1_1; W = −3.84, p-adj = 0.002; Table 1, Fig. 1).

There was no association between mercury concentrations and glutathione S-transferases of the kappa family, mu family, or theta 1_0 (GSTK1, GSTM1, and GSTT1_0, respectively; all W < 2.65, all p-adj >0.12; Table 1). Similarly, there was no association between mercury concentrations and i) glutathione peroxidases (GPX1, GPX2, and GPX3; all W < 0.94, all p-adj >0.99; Table 1); ii) glutathione synthetase (GSS; W = −2.07, p-adj = 0.57; Table 1); iii) glutathione reductases (GSR0 and GSR1; both W < 1.03, both p-adj >0.99; Table 1); iv) thioredoxine reductases (TXNRD1 and TXNRD3; both W < 0.54, both p-adj >0.99; Table 1); and v) metallothionein (MT1; W = −0.85, p-adj >0.99; Table 1). Effect sizes for non-significant genes were generally small, and none approached significance after correction for multiple testing.

4. Discussion

Our study provides the first correlative evidence for a potential interference of mercury with the expression levels of a specific set of genes linked to detoxification processes in wild birds. We were able to demonstrate an association between the levels of specific glutathione S-transferase genes and mercury concentrations in blood, underlying the importance of such genes in detoxification processes. Contrary to our expectations, we did not find an association with metallothionein levels nor with glutathione peroxidases, synthetases, reductases, and with thioredoxin reductases codifying genes.

Xenobiotic metabolism serves as the first line of defense against the harmful effects of chemical pollution exposure, facilitating the removal of toxicants from the organism through biotransformation. Glutathione S-transferases are therefore crucial in detoxifying environmental contaminants by catalyzing the conjugation of glutathione to xenobiotic-induced reactive compounds, for instance, by enhancing their solubility and by facilitating their excretion (Singh et al., 2024). Once inside the cell, the toxic molecules are recognized and targeted by different enzymes. Lipophilic molecules are metabolized by phase I enzymes, and then subsequently conjugated with GSH by phase II detoxification

Table 1
DESeq2 model outputs between gene expression and mercury concentrations in frigatebird chicks from French Guiana. P-values were adjusted with the Bonferroni correction method for multiple comparisons. BaseMean refers to the mean of normalized counts for each genes across all samples, where the higher the number, the higher the expression of a specific gene. An outlier was detected and removed from the model on GPX1, as it changed completely the relationship between mercury and GPX1 expression. All significant results are shown in bold. Genes are sorted based on increasing p-values.

	baseMean	Log2FoldChange	St. Err.	Wald statistics	p-value	adj p-value
GSTA1	519	−0.82	0.12	−6.98	<0.001	**<0.001**
GSTA2	438	−0.59	0.09	−6.72	<0.001	**<0.001**
GSTT1_1	41	−0.67	0.18	−3.84	<0.001	**0.002**
GSTK1	518	0.18	0.07	2.65	0.01	0.12
GSS	373	−0.17	0.08	−2.07	0.04	0.57
GSTT1_0	6	−0.59	0.36	−1.66	0.10	>0.99
GSR1	1049	0.08	0.08	1.03	0.30	>0.99
GPX3	51	0.14	0.15	0.94	0.35	>0.99
GPX2	11	0.20	0.23	0.88	0.38	>0.99
GSR0	1412	0.08	0.09	0.85	0.39	>0.99
MT1	12	−0.18	0.21	−0.85	0.40	>0.99
GPX1	26679	−0.07	0.08	−0.84	0.40	>0.99
TXNRD1	2181	−0.08	0.14	−0.54	0.59	>0.99
TXNRD3	627	−0.01	0.06	−0.21	0.83	>0.99
GSTM1	124	−0.02	0.10	−0.17	0.87	>0.99

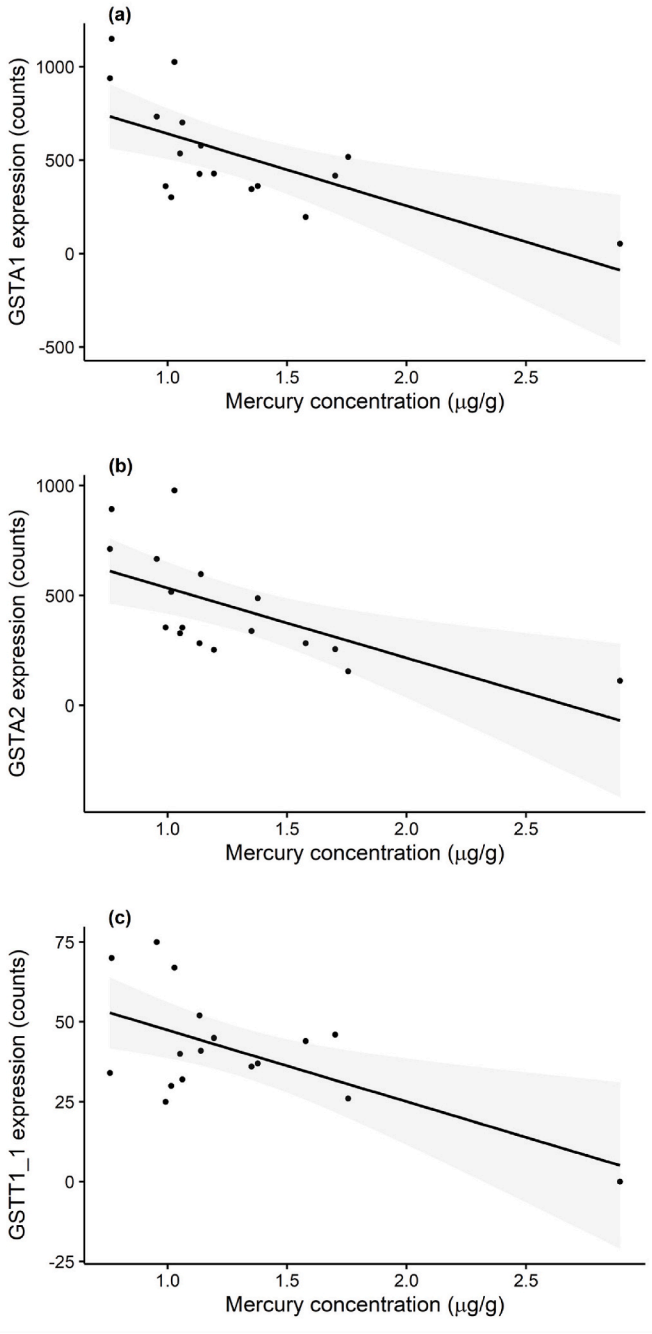

Fig. 1. Gene expression of GSTA1 (panel a), of GSTA2 (panel b), and of GSTT1_1 (panel c), in relation to mercury concentrations expressed as µg/g of dry weight. Data on gene expression is presented as raw counts. The regression line and 95 % confidence intervals are shown.

enzyme as glutathione S-transferases, and are finally exported out of the cell (Hayes et al., 2005). GSTs also seem to play a crucial role in neutralizing reactive oxygen species (ROS), thereby reducing oxidative stress generated by heavy metal exposure (Kumar and Trivedi, 2018).

Our results indicate a clear reduction in the expression of GSTA1, GSTA2, and GSTT1_1 in response to rising mercury concentrations. As GSTs are primarily involved in detoxifying organic xenobiotics and oxidative stress products (Landi, 2000), the suppression of both alpha and theta GSTs may result from the toxicity induced by circulating mercury, which would impair cellular defense mechanisms (Sheehan et al., 2001). Previous studies in birds have reported associations between GST gene expression and contaminant exposure (in cormorants *Phalacrocorax carbo*, between the levels of an organofluorine compound and GSTA3, Nakayama et al., 2008; in black-legged kittiwake *Rissa tridactyla* chicks, between polychlorinated biphenyls and GST, Helgason et al., 2010), suggesting contaminant disruption of GST genes. The observed decrease in GSTA1, GSTA2, and GSTT1_1 expression with increasing mercury concentrations also seem to support the hypothesis that mercury exposure impacts on the regulation of the redox status, but the complexity of such interactions clearly deserves further investigations. Indeed, since enzymatic activity was not measured in the current study, we cannot determine whether reduced mRNA expression corresponds to reduced GST activity, a limitation that should be addressed in future work combining transcriptomic and traditional physiological assays.

Interestingly, our results did not reveal any association between exposure to increasing concentrations of mercury and the expression of glutathione peroxidases. This result is somewhat unexpected and in contrast with several studies on birds and other animal models, where GPX1, GPX2, or GPX3 were significantly up- (Gibson et al., 2014) or down- (Franco et al., 2009) regulated in response to mercury exposure. Previous work *in vitro* demonstrated how GPX1 is an initial molecular target of low-dose methylmercury, whose decreased enzymatic activity is likely a consequence of mercury-selenium interactions (Farina et al., 2009). However, differences in mercury metabolism and detoxification strategies among species and/or the fact that GPX1 and GPX2 are primarily intracellular, while GPX3 is largely extracellular, may influence their responsiveness to mercury exposure (Brigelius-Flohé and Maiorino, 2013). Additionally, our results did not show any significant gene expression change in i) GSTK1 expression levels, a mitochondrial specific gene known to play a role in mitochondrial detoxification processes (Morel and Aninat, 2011; Raza, 2011); ii) glutathione synthetase and reductases, involved in the biosynthesis of glutathione (Dinescu et al., 2004) and in maintaining the supply of reduced glutathione (Couto et al., 2016), respectively; iii) thioredoxin reductases, which maintain an optimal cellular redox balance (Mustacich and Powis, 2000); and iv) metallothionein levels, crucial in maintaining metal homeostasis and in detoxification mechanisms (Schlenk et al., 1995). A possible explanation for our findings is that the detrimental effects of mercury may be context-dependent – e.g. it may be influenced by additional factors such exposure intensity and/or by selenium availability. For instance, it has been previously shown that the magnitude of mercury exposure determines gene expression changes, with mercury eliciting the activation of stress related genes after a threshold of exposure was reached (Sutton et al., 2002). Similarly, the induction of metallothionein is often more pronounced in organs like the liver and the kidneys compared to blood, as these tissues accumulate significant levels of mercury (Yasutake and Nakamura, 2011). It is important to note that transcriptomic in blood may not fully reflect gene expression in key detoxification organs, as the liver and kidneys, where metallothioneins and glutathione peroxidases are highly expressed. The absence of associations between mercury and those genes does not exclude potential responses in other tissues, as previously shown (Yasutake and Nakamura, 2011).

Finally, it is known that exposure to mercury can cause a "selenium-deficient-like" condition by binding to selenium and reducing its bioavailability (Ralston and Raymond, 2018). This may affect the synthesis and function of seleno-dependent proteins (Usuki et al., 2011) including the thioredoxin system (Branco and Carvalho, 2019), without necessarily triggering transcriptional changes. This would explain the lack of differential expression in these genes. However, selenium concentrations were not measured in this study, which reduces our ability to confirm the hypothesized mercury-selenium interactions and the consequent reduction of selenium availability. Future studies incorporating selenium analyses will prove useful to better interpret the regulation of thioredoxin reductases and glutathione peroxidases genes under mercury exposure.

Our results are important because identifying the patterns of expression associated with a specific stressor are necessary to elucidate

cause-effect relationships. For example, mercury-exposed individuals can suffer neurological damage (Farina et al., 2011), but using specific physiological measurements can hardly clarify whether the observed effects are due to oxidative stress, direct mitochondrial damage, or neuroinflammation. Gene expression analysis can help distinguish these mechanisms by identifying specific pathways activated in response to mercury. Our study results are limited to a single tissue (i.e. blood), thus we cannot assume that the observed molecular responses in blood would reflect responses in other mercury-accumulating tissues as the liver or the brain. While these tissues may likely provide more direct insights on the impact of mercury exposure at the organismal level, studies using such organs often require animal euthanasia, which is generally unsuitable for research involving wild animals, especially when the objective of the study is to identify sublethal effects.

5. Conclusions

Our study provides the first correlative evidence for a potential influence of mercury exposure on the expression of key genes directly involved in detoxification processes or thiol-containing proteins involved in the protection from the physiological effects of contaminant exposure. Although we did not find any significant associations between mercury and glutathione peroxidases, synthetases and reductases, methallotioneins, and thioredoxin reductases codifying genes, the expression levels of three out of six glutathione S-transferases codifying genes were negatively associated with increasing mercury concentrations.

Taken together, our results combined with literature findings suggest that the antioxidant and detoxification response to mercury exposure is complex, and may vary depending on species-specific metabolic pathways, exposure levels, and the type of tissue analysed.

As transcriptional changes often precede the manifestation of physiological symptoms associated to mercury toxicity, we suggest future studies to additionally include gene expression analyses when investigating the effects of contaminant exposure. Indeed, although the observed mercury concentration in frigatebird chicks fall within the category of background/low risk levels according to Ackerman et al. (2016), our gene expression approach was still able to detect changes associated with mercury exposure. A larger sample size would also increase statistical power to detect subtle gene expression changes. Gene expression might be a more sensitive approach for detecting early-stage toxic effects in comparison with physiological approaches, which often rely on detecting changes that may occur at later stages of exposure.

CRediT authorship contribution statement

Manrico Sebastiano: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Olivier Chastel:** Writing – review & editing, Validation, Resources. **Paco Bustamante:** Writing – review & editing, Validation, Methodology, Formal analysis. **Marcel Eens:** Writing – review & editing, Validation, Supervision, Conceptualization. **David Costantini:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the first author used ChatGPT only to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2025.127143.

Data availability

Data will be made available on request.

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