



Combined effects of PVC microplastics and thermal rise alter the oxidative stress response in Antarctic fish *Harpagifer antarcticus* and Sub-Antarctic *Harpagifer bispinis*

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ABSTRACT

This study evaluated the oxidative stress response in two cold-water fish species, the Antarctic *Harpagifer antarcticus* and the sub-Antarctic *H. bispinis*, following exposure to single and combined stressors: polyvinyl chloride (PVC) microplastics and thermal increase. Fish were exposed for 24 h to two temperature regimes (2 °C and 5 °C for *H. antarcticus*; 8 °C and 12 °C for *H. bispinis*) and were orally administered a PVC microplastic solution (200 mg/L). Oxidative stress was assessed through transcriptional and enzymatic activity analyses of key antioxidant markers: catalase, glutathione peroxidase, superoxide dismutase, and glutathione reductase. In *H. antarcticus*, gill antioxidant gene expression decreased in response to both stressors when applied individually, while *H. bispinis* exhibited transcriptional upregulation under the same conditions. In *H. antarcticus*, enzymatic activity in gill tissues increased for all four enzymes following exposure to both microplastics and elevated temperature. In contrast, *H. bispinis* showed a differential enzymatic response: thermal stress induced CAT activity, whereas exposure to microplastics specifically increased GR activity. At the hepatic level, *H. antarcticus* displayed increased transcription of antioxidant genes following exposure to both stressors. In *H. bispinis*, transcriptional upregulation was limited to GR and SOD in response to microplastics. However, under the combined exposure of multiple stressors, an inactivation of the antioxidant enzyme response was observed in the gills. This could indicate a functional collapse of the antioxidant system under conditions of exacerbated acute stress. The observed responses raise concerns about the potential vulnerability of polar and subpolar fishes, considering their ecological importance in trophic networks and the increasing pressure from multiple anthropogenic stressors in a rapidly changing climate.

1. Introduction

Over the past 60 years, the production of plastics and synthetic polymers increased significantly, from around 0.5 million tons in 1950 to 390 million tons in 2021. This implies a significant release of plastic waste into the environment, but in 2023, plastic production reached

413.8 metric tons. Plastic pollution has been detected in surface water (Zhou et al., 2021), seawater (Choy et al., 2020), and seafloor sediments (Walkinshaw et al., 2020). The presence of microplastics (MP) (synthetic polymer particles smaller than 5 mm in diameter), GESAMP (2015) and Arthur et al. (2009) in the marine environment and their potential toxic impact causes enormous concern due to their wide dispersion and long

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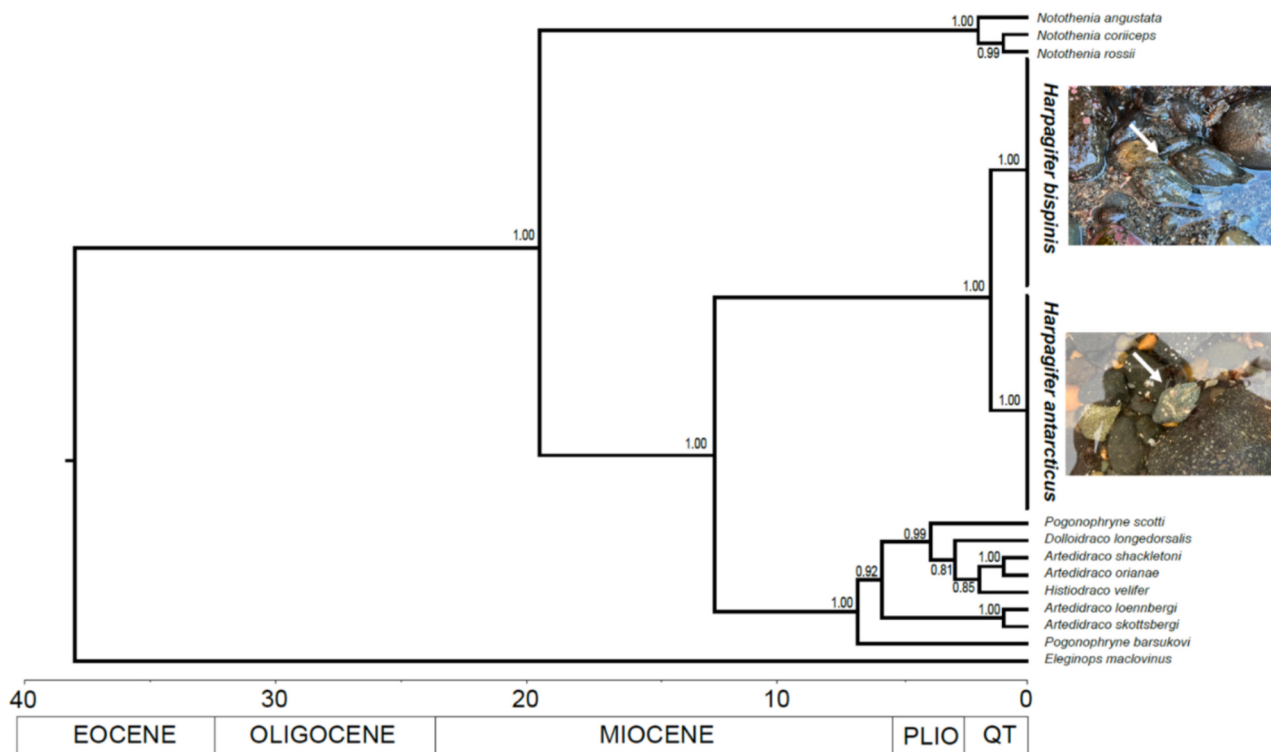


Fig. 1. Notothenioid Phylogeny shows the positions of the two species. Study species are shown in black text, and additional details are provided. The phylogeny shows the fourteen species from four families.

persistence (Ardusso et al., 2021). They are a concern even in remote polar regions, such as the Antarctic marine system, where cellulose fibers (Ergas et al., 2023), macro and microplastics have been detected in surface waters and marine-coastal areas (Lacerda et al., 2019; Suaria et al., 2020; Barboza et al., 2018; Shen et al., 2019; Li et al., 2021; Rebelein et al., 2021).

MPs are readily available to a wide range of organisms in the marine environment, and zooplankton, bivalves, shrimp, fish, cetaceans and whales, among other taxa, have been reported to ingest them (Farrell and Nelson, 2013; Ferreira et al., 2016; Solomando et al., 2022; Urbina et al., 2023). This poses a significant risk to organisms, as it has been observed that ingesting these microplastic particles can cross biological barriers and accumulate in tissues, cells, and organs (Strungaru et al., 2019; Meng et al., 2022). Some of the studied effects of acute and chronic MP exposure in fish include early mortality (Berg, 2017), inflammatory responses, growth inhibition, abnormal behaviors, stress, oxidative damage, reduced energy, and feeding activity (Huang et al., 2020; Rochman et al., 2013; Compa et al., 2024), immune dysfunction, and changes in lipid metabolism (Barboza et al., 2018; Chen et al., 2021; Lusher et al., 2013). Furthermore, the frequency and magnitude of temperature fluctuations are expected to increase globally over the next century (IPCC, 2023), partly due to anthropogenic climate change. Ocean temperature is changing due to global warming, and the projected increase in marine temperature ranges from 1 to 8 °C within the next 100 years (Pachauri and Meyer, 2014), and the occurrence of more extreme events may impose a challenge for many fish species (Ficke et al., 2007; IPCC, 2023). The Antarctic and subantarctic zone has been experiencing rapid regional warming since at least the 1950s; however, the impacts of this warming on the local scale are variable and nuanced (Goodell et al., 2024). Notothenioids are the predominant fish group in the Southern Ocean in terms of number of species and biomass (Gon and Heemstra, 1990). These fish possess a set of adaptations resulting from millions of years of evolution in the extremely cold and stable waters surrounding the Antarctic region (Eastman, 2005). Although notothenioids are highly endemic to Antarctica, several species also exist in

subantarctic waters (Gon and Heemstra, 1990; Angilletta et al., 2002). These sub-Antarctic notothenioids rarely experience temperatures below 5 °C, and having evolved under different environmental conditions, they may not share the same adaptations as their closely related Antarctic counterparts (Bilyk and DeVries, 2011; Hofmann et al., 2000). Closely related sister species pairs such as *Harpagifer antarcticus* (Antarctic) and *Harpagifer bispinis* (sub-Antarctic), which occur in distinct thermal regimes north and south of the Polar front, reveal insights into their evolutionary history as seen in Fig. 1, ecological adaptations, and responses to environmental changes (Hüne et al., 2015), being a good model for comparing responses to MP and temperature stress singly or simultaneously and considering their ecological importance in trophic networks. They are both stenothermic species that can be affected by but may not be able to adapt to these temperature changes. Physiological systems that may be influenced by temperature include outright survival, the stress response system, metabolism, food consumption, and the ability to maintain homeostasis in the face of changes in temperature, salinity, pollution, or solar radiation (Turner and Overland, 2009; Shivanna, 2022). Exposure to excessively high temperatures or other stressors like microplastics leads to loss of homeostasis, which can generate oxidative stress (Halliwell, 2006; Halliwell and Gutteridge, 2015; He et al., 2017; Samain, 2011; Troschinski et al., 2014). Oxidative stress is the imbalance between the production of reactive species and antioxidant defense. Saturation or compromise of these antioxidant processes results in ROS accumulation and damage to macromolecules (proteins, lipids, and nucleotides), failures in function, and cell death (Schieber and Chandel, 2014; Gulcin, 2020). Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) play critical roles in antioxidant defense mechanisms (Machado et al., 2014).

The increase in human activities in the ocean and nearshore environment, including shipping, fishing, and aquaculture, has increased the release of plastic waste into the marine environment (Jorquera et al., 2022; Urbina et al., 2021; Paredes-Osses et al., 2021). In addition, the expected increase in temperature due to climate change is a stressor for

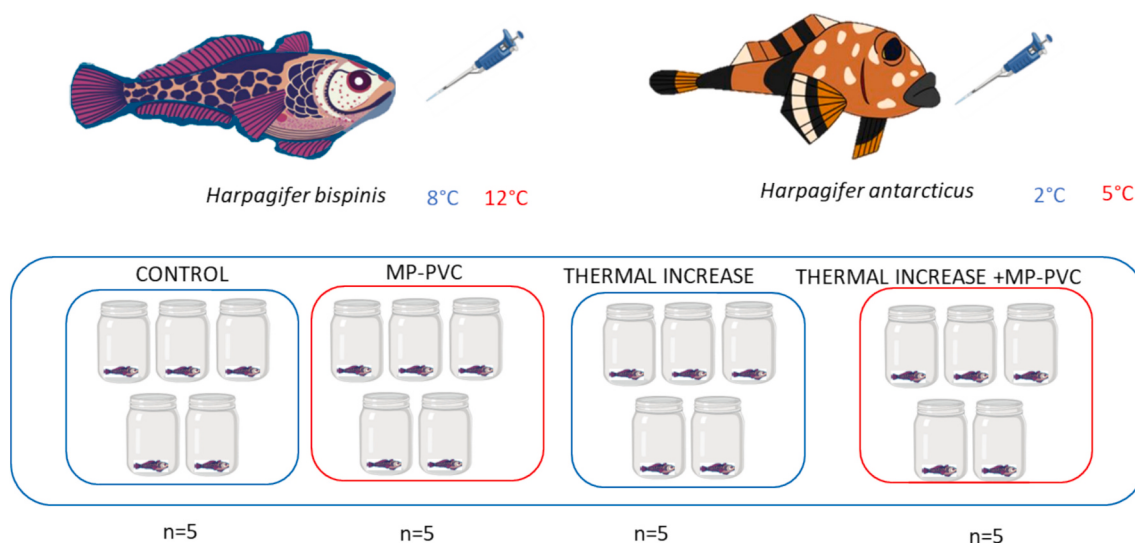


Fig. 2. Experimental design. Exposure experiment by cannula with PVC microplastic and thermal increase *Harpagifer antarcticus* (HAAN) and *Harpagifer bispinis* (HABI).

Table 1

Primer sequences for the immune system used in the experiments.

Primer	Nucleotide sequences (5'→3')	PCR product size	Efficiency Gills (%)	Efficiency Liver (%)	Genomic Locations
CAT Fw	CTGCCAGCAACCCAGATTAT	166 bp	102.91	102.92	CADEHL010000998.1 -
CAT Rv	ACCAAACCTTGGTGAGATCG				
GPx Fw	GAAGTGCAGCAATGGTGAGA	178 bp	101.6	101.7	CADEHL010001065.1
GPx Rv	CATGAGAGAGATGGGGTCGT				
GR Fw	CTGCACCAAGAAGCTGCAAA	162 bp	102.5	102.6	CADEHL010001271.1
GR Rv	CAGTCCACTTCCTGGATGGT				
SOD Fw	AGACCTGGGGAATGTGACTG	122 bp	101.9	101.10	CADEHL010001000.1
SOD Rv	TCATCATTCCTCTCTTTTCC				
b-actine Fw	AGGTCA TCA CCA TCG GAA ACG A	124 bp	102.7	102.8	Saravia et al., 2022
b-actine Rv	ACA GCA CGG TGT TGG CGT ACA				

all ectothermic species and particularly adverse for Antarctic notothenioids because they lack an inducible response to heat stress. We do not know if *H. bispinis*, due to its Antarctic ancestry and the young age of the lineage in the recolonization of non-freezing temperate southern waters. Fish can generally cope with single moderate stressors, which modify their homeostasis, however, multiple stressors may have additive or synergistic effects (Martínez et al., 2022), thus significantly increasing their negative impacts on fish. The physiological response of fish to these combined stressors and their consequences on the ability to survive and adapt to these conditions are currently unknown. This study used the independent and combined effects of PVC microplastic (MP) exposure and temperature rise to evaluate the impacts on the oxidative stress response in gill and liver tissues in *H. antarcticus* (HAAN) and *H. bispinis* (HABI). To monitor this response, we used enzyme activity and transcriptional gene expression of Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Reductase (GR), and Glutathione Peroxidase (GSH-Px), which are all involved in the oxidative stress response.

2. Methodology

2.1. Fish collection and acclimation

The experimental procedures and sample handling complied with ethical guidelines that regulate the use of fish in the laboratory, established by the National Commission for Scientific and Technological Research (CONICYT, Chile), FONDAP-IDEAL 15150003, Millennium Institute Biodiversity of Antarctic and Sub-Antarctic Ecosystems, BASE,

and the Universidad Austral de Chile Bioethical Committee (27/2020).

Adult specimens of *H. antarcticus* ($n = 20$, mean body mass = 14.5 g, mean body length = 9.8 cm) and *H. bispinis* ($n = 20$, mean body mass = 12.8 g, mean body length = 8.9 cm). Individuals were manually captured in situ by entering the water with insulated wading gear and turning over submerged rocks, a method that exploits the fish's natural tendency to hide among benthic substrates. Upon exposure, specimens were carefully caught by hand without the use of nets or traps from the intertidal Antarctic zone at Fildes Bay (King George Island) and Punta Arenas (ca. Port Bulnes), respectively. The collected fish were acclimatized in tanks for 72 h in seawater collected at the sampling site at 2 °C, 33 PSU, and 8 °C, 33 PSU, respectively, with natural summer photoperiod and constant aeration, and were fed ad libitum with amphipods collected at the site, which are part of their natural diet (Navarro et al., 2019). In two other tanks, the water temperature was raised to 8 °C and 12 °C, adapting the protocol used by Vargas-Chacoff et al. (2021). Once the desired temperature was achieved, the fish were randomly distributed into 2 L glass aquariums as seen in Fig. 2 (one fish per aquarium; $n = 5$ per condition). The temperatures chosen for the experiments are the conditions of thermal increase projected for the year 2050 by the IPCC, 2023 in the face of an unfavorable scenario of high greenhouse gas emissions due to climate change (RCP 4.5). We consider that our experimental thermal increase, 5 °C for Antarctic and 12 °C for Sub-Antarctic species, respectively, is relevant to projected warming and that they capture the wide thermal variation recorded in short time windows in some of the habitats in which *Harpagifer antarcticus* and *Harpagifer bispinis* are known to occur.

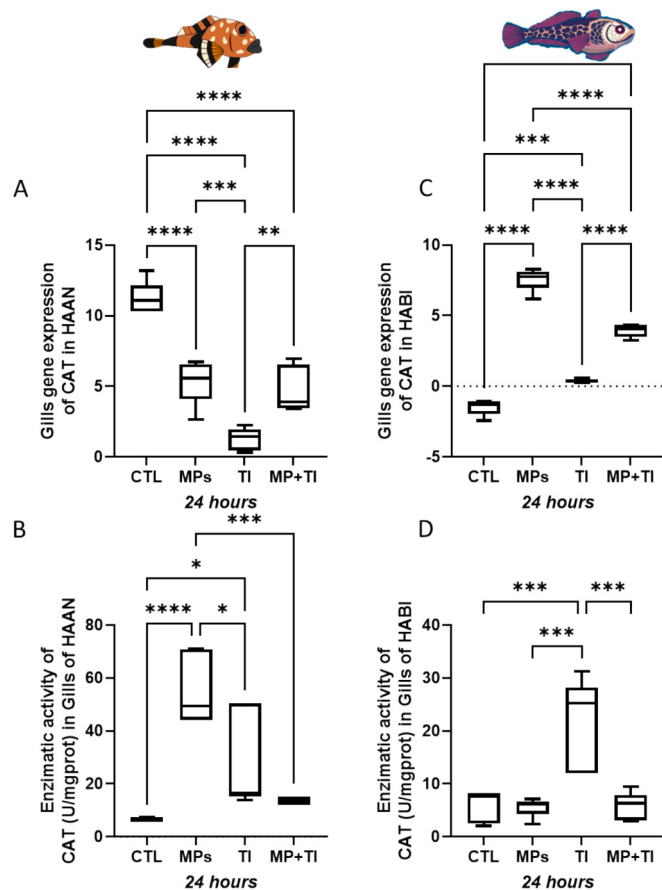


Fig. 3. Gill Catalase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Gill catalase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. One-way ANOVA followed by Tukey's test, (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$) $n = 5$ per sampling day/per treatment.

2.2. Cannulation of MP and experimental design

In the present study, spherical commercial PVC microplastics (3–10 μm in diameter; Sigma-Aldrich® (Product No. 189588, Polyvinyl chloride) were used for oral administration, following the protocol described by Gunaalan et al. (2023). A concentration of 200 mg/L of PVC microplastics was selected for this study, based on Brandts et al. (2018), who reported no lethality at this dose in fish. Although this concentration exceeds current environmental levels (estimated at ~ 0.01 g/L), it was intentionally chosen to elicit measurable physiological responses without causing mortality. This approach allows for a clearer assessment of sublethal cellular and molecular effects. Moreover, given the increasing global plastic input into marine environments, some projections suggest concentrations could reach or even surpass 0.1 g/L in hotspot areas, making the selected dose ecologically relevant for future scenarios of plastic pollution. All experimental materials and instruments were made exclusively of glass to minimize the risk of microplastic contamination. Fish were gavaged directly into the stomach using physiological saline (0.9 % NaCl), with volumes of 50 μL for HABI and 100 μL for HAAN. These volumes were adjusted according to species-specific body size, as *H. bispinis* is markedly smaller than *H. antarcticus*, requiring a lower gavage volume to avoid excessive gastric distension and ensure animal welfare. For the control group, only saline was administered, while the microplastic-exposed groups

received PVC particles resuspended in saline. For each species, individuals were randomly assigned to one of four experimental groups: i) Control (CTL): maintained at baseline temperatures (2 °C for *H. antarcticus*, 8 °C for *H. bispinis*), ii) Microplastics (MP): exposed to PVC microplastics at the same baseline temperatures, iii) Thermal Increase (TI): exposed to elevated temperatures (5 °C for *H. antarcticus* and 12 °C for *H. bispinis*), iv) Microplastics and Thermal Increase (MP + TI): exposed to both PVC microplastics and increased temperatures (5 °C and 12 °C, respectively). All groups were exposed to their respective treatments for 24 h to simulate an acute stress scenario and to capture the early molecular and enzymatic responses triggered by the organism's intrinsic defense antioxidant mechanisms. This short-term exposure period was selected specifically to evaluate the immediate oxidative stress response, rather than long-term adaptation. Moreover, in the absence of prior studies addressing the combined effects of microplastics and thermal stress in these species, a 24-hour exposure provides a controlled framework to identify early biomarkers of cellular stress. No mortality occurred in any of the experimental groups throughout the duration of the exposure.

2.3. Fish sampling

The specimens were anesthetized with a lethal dose of 2-phenoxyethanol (1 mL/L), weighed, and sampled. Liver and gills were extracted from each specimen, stored, and frozen after extraction in liquid nitrogen and stored at 80 °C until further analyses.

2.4. Gene expression analysis

2.4.1. Total RNA extraction

Total RNA was isolated from liver and gills using TRIzol reagent (Sigma) following the manufacturer's instructions and stored at -80 °C. Subsequently, RNA was quantified at 260 nm on a NanoDrop spectrophotometer (NanoDrop Technologies®), and RNA quality was evaluated using a 1 % agarose electrophoresis gel. Finally, total RNA (2 μg) was used as a reverse transcription template to synthesize cDNA, applying MMLV-RT reverse transcriptase (Promega) and the oligo-dT primer (Invitrogen) according to standard procedures.

2.4.2. qRT-PCR analysis of gene expression

Reactions were carried out on an AriaMx Real-time PCR System (Agilent). cDNA was diluted to 100 ng and used as a qRT-PCR template with reactive Brilliant SYBRGreen qPCR (Stratagene). Reactions were performed in triplicate, in a total volume of 14 μL , which contained 6 μL SYBRGreen, 2 μL cDNA (100 ng), 1.08 μL of primers mix, and 4.92 μL of PCR-grade water. The applied PCR program was as follows: 95 °C for 10 min, followed by 40 cycles at 90 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. Melting curve analysis of the amplified products was performed after each PCR to confirm that only one amplicon was amplified and detected. Expression levels were analyzed using the comparative Ct method ($2^{-\Delta\Delta\text{CT}}$) (Livak and Schmittgen, 2001). The data are presented as the fold change in gene expression normalized to an endogenous reference gene (β -actin) relative to the treatment control. The primers used for Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione Reductase (GR), and Superoxide Dismutase (SOD) are listed in Table 1. PCR efficiencies were determined by linear regression analysis of sample data using LinRegPCR (Ramakers et al., 2003) from the serial dilutions when Log dilution was plotted against DCT (threshold cycle number).

2.5. Enzymatic analysis

2.5.1. Homogenization

The liver and gill tissues were homogenized in 100 mM pH 7.4 phosphate buffer containing 0.15 M KCl, 1 mM EDTA, 0.1 mM PMSF, and 1 mM DDT in a 1:4 ratio (sample weight: buffer volume).

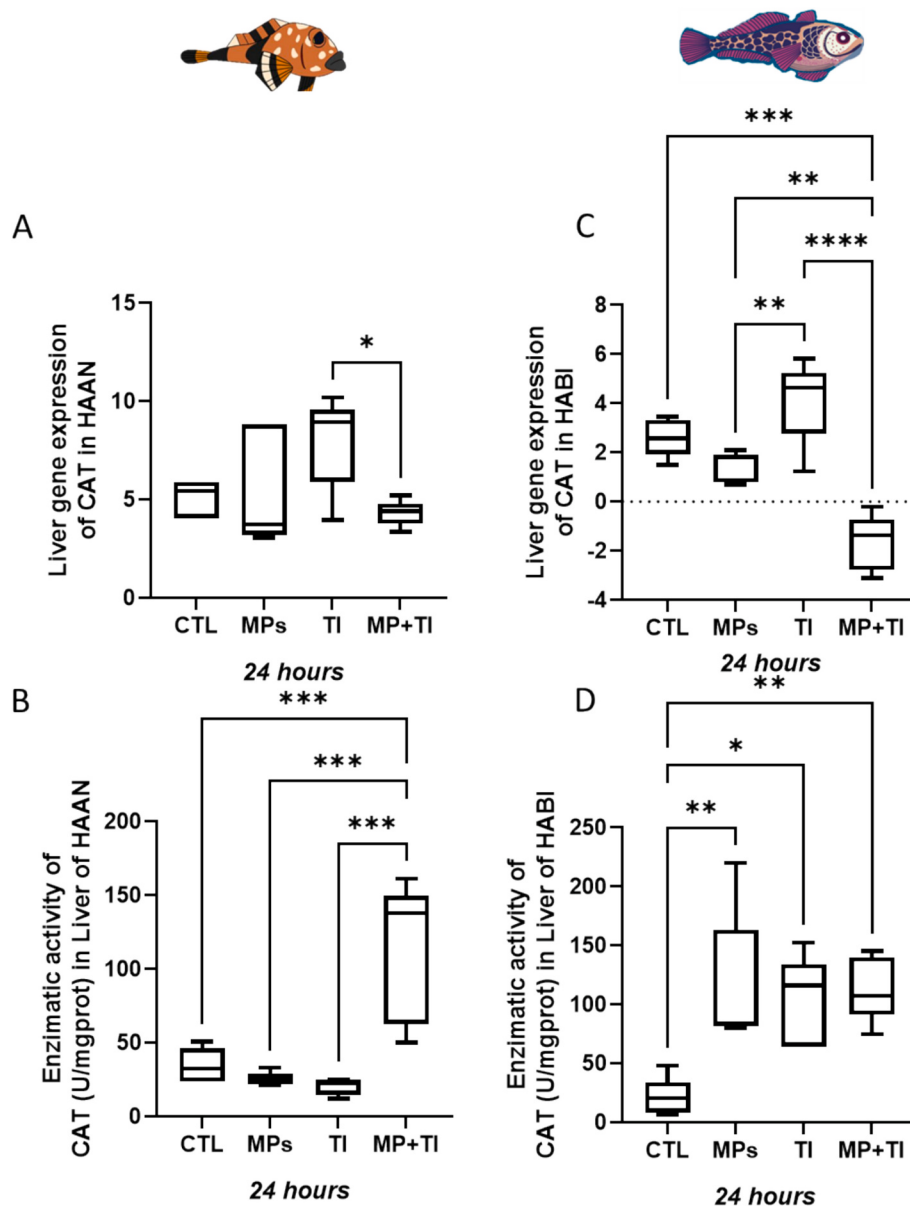


Fig. 4. Liver Catalase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Liver catalase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. One-way ANOVA followed by Tukey's test, (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001) n = 5 per sampling day/per treatment.

Subsequently, samples were centrifuged at 12,000 rpm at 4 °C for 30 min and supernatants were used to determine the enzymatic activities. The determination of enzymatic activities was performed in triplicate by spectrophotometric analysis at 20 °C using a microplate reader (Multiscan, Thermo Scientific), using Multiscan Scan 3.2 Software. Specific enzyme activities are expressed according to the concentration of proteins in each sample (U/mg prot).

2.5.2. Specific enzyme conditions

Catalase (CAT): Activity was determined using the Aebi protocol (Aebi, 1984) as modified by Nualart et al. (2025) and was quantified as a function of the degradation rate of the H₂O₂ substrate, monitored at 240 m for 5 min. The results were expressed considering that one unit of CAT activity equals the number of moles of H₂O₂ degraded per minute, per milligram of protein. **Glutathione Reductase (GR):** the rate of oxidation of NADPH by GSSG was used as a standard measure of enzymatic activity. Its activity was expressed as mmol/min/mg protein and was performed

at 340 m for 5 min, measuring the oxidation of NADPH according to the protocol of Carlberg and Mannervik (1975) and López-Galindo et al. (2010 a, b). **Glutathione Peroxidase (GPx):** The decrease in NADPH concentration is calculated using the appropriate extinction coefficient from the linear slopes of decreasing absorption. The glutathione peroxidase-dependent reaction rate is obtained when the nonenzymic and hydroperoxide-independent effects are subtracted from the overall reaction rate. The measurement consisted of the oxidation of NADPH at 340 m absorbance, as described by Flohé and Günzler (1984) and López-Galindo et al. (2010 a, b). **Superoxide Dismutase (SOD):** The reaction of xanthine with xanthine oxidase generates the superoxide anion (O₂⁻), which will be mutated by SOD into H₂O₂ and oxygen. According to the method described by Sun et al. (1988) and Livingstone (2001), SOD activity is based on its ability to inhibit the reduction of tetrazolium nitroblue (NBT) to formazan blue, measured at a wavelength of 560 m for 30 min. **Protein Quantification:** The total protein concentration of each sample was determined using a Pierce BCA protein kit (Bradford,

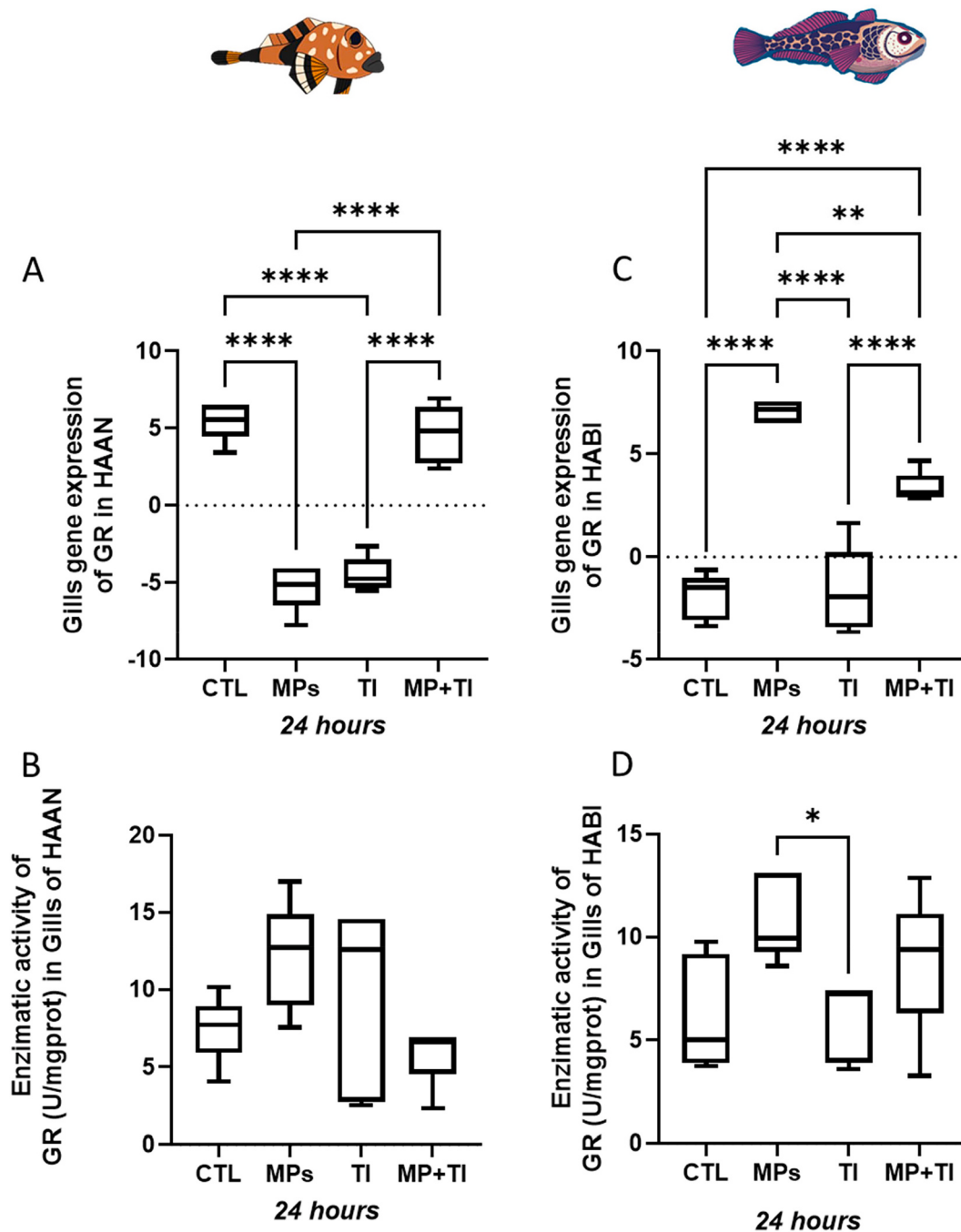


Fig. 5. Gill glutathione reductase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Gill glutathione reductase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. One-way ANOVA followed by Tukey's test, (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$) $n = 5$ per sampling day/per treatment.

1976) with bovine serum albumin (BSA) as standard, analyzed at 595 nm.

2.6. Statistical analysis

Linear categorical variable models were applied to evaluate the response of each treatment (condition and temperature variation) for all gills and liver parameters analyzed in both species. A Two-way ANOVA analysis of variance was used, followed by a Tukey's post-hoc test to identify different groups in qPCR analysis. The assumptions of normality, independence, and homogeneity of the residuals for the

variances between groups were also tested using a Shapiro-Wilk test and a Levene test. Symbols (*) over the bars indicate statistical differences between different treatments, and statistically significant differences were determined using * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Principal Component Analysis (PCA) was applied to aggregated gene expression and oxidative stress enzyme activity data from both gill and liver tissues to assess overall physiological responses to environmental stressors. Gene expression and enzyme activity values from the two tissues were combined for each individual to generate a single multivariate profile per specimen. This approach allowed us to integrate molecular and enzymatic responses across organs to analyze treatment

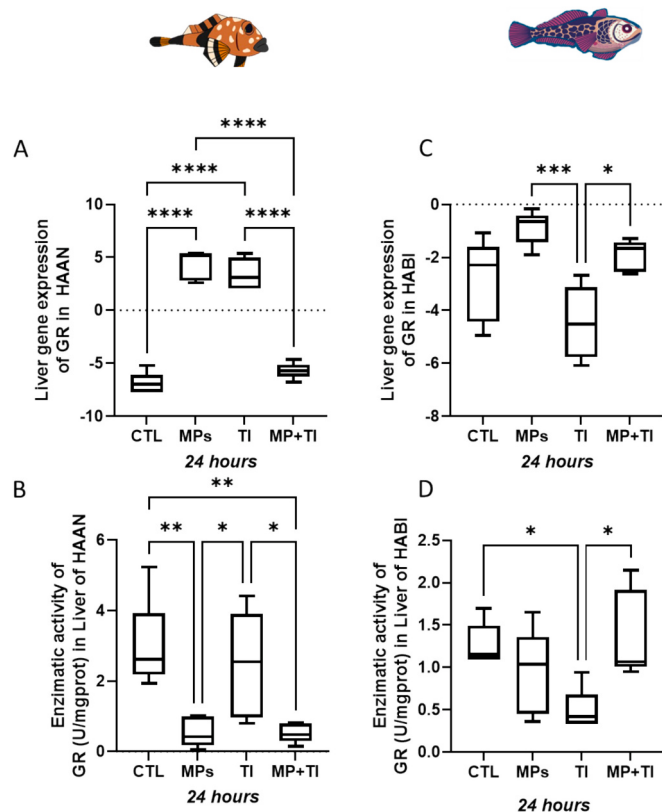


Fig. 6. Liver glutathione reductase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Liver glutathione reductase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. One-way ANOVA followed by Tukey's test, (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$) $n = 5$ per sampling day/per treatment.

effects comprehensively. A global PCA was first conducted to evaluate overall patterns of variation across all individuals and treatments, including both *Harpagifer antarcticus* and *Harpagifer bispinis*. To further investigate the species-specific responses to each treatment, additional PCAs were performed separately for each species.

Data were standardized (z-score normalization) to account for differences in measurement scales. PCA was performed on the correlation matrix to ensure equal weighting of all variables. Component selection was set at those explaining up to 75 % of the total variance. The proportion of variance explained by each principal component was examined, PCA biplots were generated to visualize clustering of individuals according to treatment conditions and species, and to evaluate whether distinct physiological response profiles emerged under different stress scenarios (control, thermal increase, microplastic exposure, and combined thermal increase + microplastics). All statistical analyses and graphs presented as boxplots or PCA score distribution biplots were performed using GraphPad Prism 9 (GraphPad Software, LLC, USA).

3. Results

3.1. Gene expression and enzyme activity

3.1.1. Catalase in gills

CAT expression in gills decreased significantly in *H. antarcticus* (HAAN) when exposed to microplastics (MPs), subjected to a thermal increase from 2 to 5 °C, as well when fish were exposed to both stressors

(MP + TI) (Fig. 3A). However, despite the reduction in gene expression in experimental conditions, the catalase enzymatic activity in HAAN's gills was significantly greater (6-fold) when exposed to MPs. Although still significant, the increase in activity was less pronounced in response to the thermal increase and did not differ statistically from control values in the MP + TI group (Fig. 3B).

Contrary to HAAN, gene expression for catalase in *H. bispinis* (HABI) was significantly increased when the fish were exposed to MP, exposed to thermal increase (TI), and when both stressors were combined (MP + TI; Fig. 3C). In HABI, significant changes in catalase activity were observed only in response to temperature alone, whereas fish exposed to either MPs alone or in combination with a temperature rise had CAT activity rates similar to those of control fish (Fig. 3D).

3.1.2. Catalase in liver

Catalase mRNA levels in the liver had a narrower range of values than in the gill. In HAAN catalase, gene expression increased slightly but not significantly in fish exposed to MPs or TI, and both groups showed a large dispersion of individual values. These effects were not seen when these two stressors were combined (Fig. 4A). The enzymatic activity showed an opposite pattern, with slightly lower values in MPs and TI, and a statistically significant increase was observed only in fish exposed to both stressors (MP + TI) during the 24-hour period, despite high variability compared to the other three groups (Fig. 4B).

In HABI liver, catalase mRNA levels did not change significantly when exposed to either MPs or TI alone but were significantly reduced in MP + TI fish (Fig. 4C). In contrast to gene expression, catalase enzymatic activity increased in all treatment groups compared to the control group. In HAAN, similar to what was observed for the gills, liver CAT activity treatment groups (MPs, TI, and MPs + TI) showed significant increases and had large increases in the dispersion of activity among fish (Fig. 4D).

3.1.3. Glutathione reductase in gills

The GR mRNA expression in HAAN gills decreased significantly when fish were exposed to MPs or TI. Still, no significant differences were observed in fish exposed to a combination of both stressors (Fig. 5A). No significant changes were observed in the overall average values of GR activity in relation to the control fish, regardless of the treatment group; however, notable changes in individual variability were observed in the groups exposed to a single factor (Fig. 5B).

In HABI, a significant increase in glutathione reductase was observed in *H. bispinis* when exposed to MPs and MP + TI combined, but not to TI temperature alone (Fig. 5C). In this species, Gill GR activity followed a profile identical to that observed in gene expression, although the slight increases in activity observed in both groups exposed to MPs were not significantly different from the control group or the group exposed to a thermal challenge alone (Fig. 5D), likely on account of the significant individually variability in all groups, including the control.

3.1.4. Glutathione reductase in liver

The expression of mRNA for glutathione reductase in the liver increased significantly when HAAN was subjected to a temperature increase and exposed to PVC microplastics (Fig. 6A), but not when both conditions were combined. In turn, GR activity in the liver of HAAN did not change in response to a temperature challenge alone, and activity decreased significantly in fish cannulated with PVC microplastics, as well as in those subjected to both thermal increase at 5 °C and PVC microplastics simultaneously (Fig. 6B).

In HABI, there were no significant changes in GR gene expression between the control and all three experimental groups. However, those groups exposed to MPs showed less individual variability and slightly higher average expression than the control and the increased temperature group (Fig. 6C). Regarding GR activity, only the increased temperature group showed significantly altered enzymatic rates, which were significantly lower than those of the control and the combined exposure group. Contrary to the gene expression, both groups containing

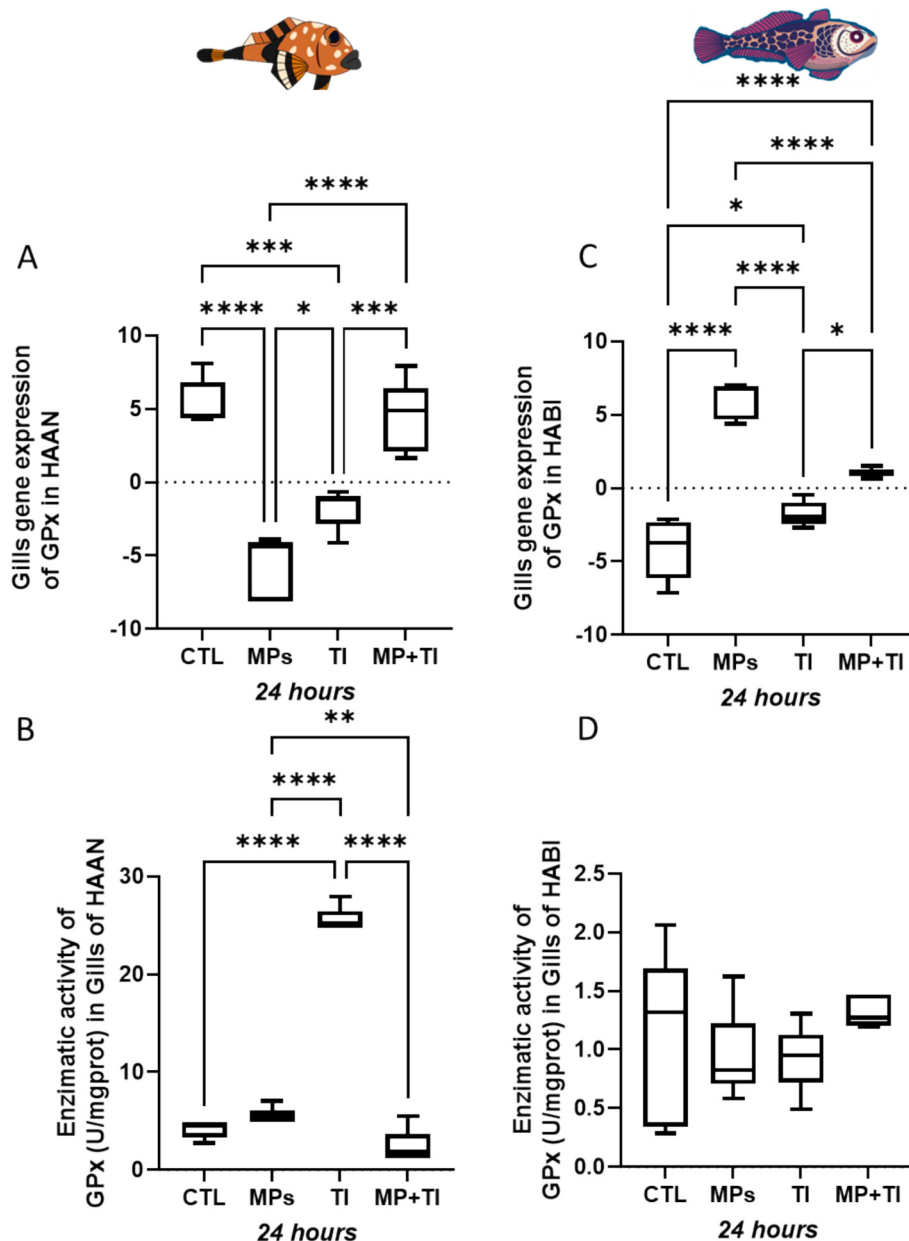


Fig. 7. Gill glutathione peroxidase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Gill glutathione peroxidase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. Two-way ANOVA followed by Tukey's test, (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001) n = 5 per sampling day/per treatment.

MPs showed the highest individual variability in GR activity in the liver (Fig. 6D).

3.1.5. Glutathione peroxidase in gills

Significant downregulation of the glutathione peroxidase transcript was observed in gills when HAAN was subjected to MPs and TI alone, but not with both stressors combined (Fig. 7A). In contrast, only the increased temperature evoked a significant change in actual enzymatic activity, resulting in a 5-fold increase, while activity due to temperature in combination with MPs or to MPs alone did not differ from the control (Fig. 7B). Despite these relevant changes in transcript expression, no activity changes were observed in this species' gills. The measured values were significantly lower than those observed in HAAN, with the control group in HABI exhibiting average values that were less than half of those seen in HAAN, to which they were subjected (Fig. 7B). HABI

mRNA levels changed significantly in the three experimental conditions, with higher rises observed in the two groups gavaged with MPs and less exposed solely to thermal challenge (Fig. 7C). Similarly, in *H. antarcticus*, it was observed that activity in the gills increases significantly only with a thermal increase to 5 °C (Fig. 7C). In the case of *H. bispinis*, no changes in the enzymatic activity were observed when cannulated with the microplastic PVC, nor with the thermal increase (Fig. 7D).

3.1.6. Glutathione peroxidase in liver

When exposed to MPs and TI, HAAN showed significant upregulation of glutathione peroxidase transcription in the liver (Fig. 8A). Effects were more pronounced in fish exposed to increased temperature than in those gavaged with microplastics, either alone or in warm conditions. Such changes in mRNA expression were not accompanied by

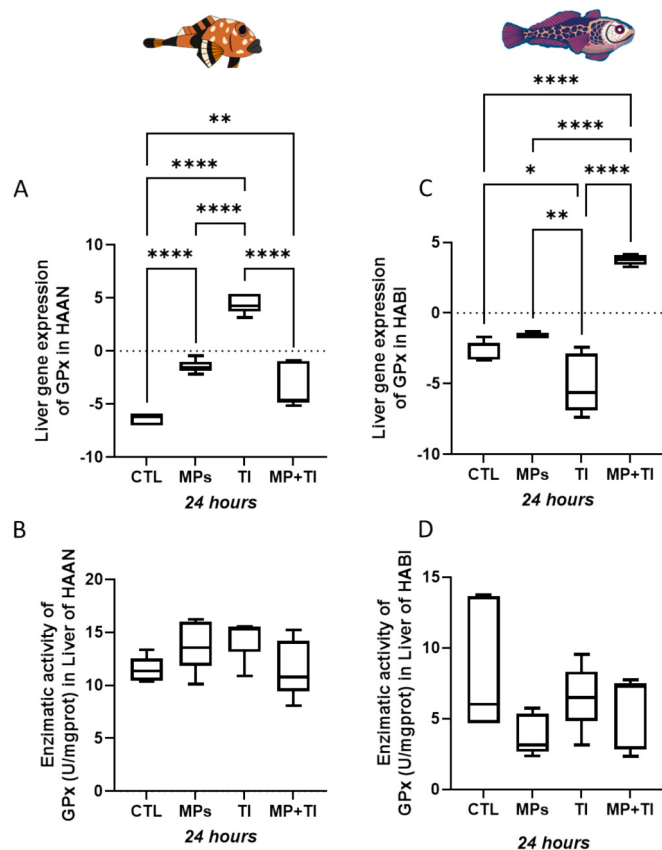


Fig. 8. Liver glutathione peroxidase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Liver glutathione peroxidase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. Two-way ANOVA followed by Tukey's test, (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$) $n = 5$ per sampling day/per treatment.

modifications in enzyme activity, and no changes were observed in GPx rates in either treatment group compared to the control fish (Fig. 8B). In HABI, only the group exposed to the combined effect of MPs and increased temperature showed a significant increase in GPx gene expression, whereas those exposed to increased temperature alone showed a slight decrease in average expression, but with higher individual variability. No significant changes occurred for the MPs group (Fig. 8C). As for HAAN, no significant changes were noted in the activity of GPx in HABI liver, regardless of the treatment. However, it is worth noting that there was considerable individual variability in the readings of the control group (Fig. 8D).

3.1.7. Superoxide dismutase in gills

The average expression for the gene coding for SOD in HAAN gills did not change in any of the experimental groups, although the variability increased significantly when compared with the control group (Fig. 9A). Similarly, no significant effect was observed in the enzyme activity in relation to either temperature, MPs, or both in this species (Fig. 9B). In HABI, significant changes in expression were observed in association with MPs, either alone or combined with increased temperature, but not when fish were exposed to the temperature increase alone (Fig. 9C), and these changes in expression were not reflected in enzyme activity (Fig. 9D). Interestingly the combined effect of MPs and temperature seems to decrease the variability of the individual SOD activity in both species but without modifying its meaning in relation to

control or other groups.

3.1.8. Superoxide dismutase in liver

Expression of SOD mRNA in the liver was increased in both species by each stressor but less so when both were applied together. In HAAN there was a highly significant upregulation in transcription of the SOD gene by MPs and when fish were exposed to 5 °C. The combination of these two factors still evoked a stimulation of SOD transcription but a lower significance level (Fig. 10A). However, measurements of actual protein enzymatic activity showed a high individual variability in the control group, and a slight reduction in activity in all other groups compared to the control. This reduction was only statistically significant in the MPs gavaged group (Fig. 10B). In HABI, the pattern for SOD mRNA was similar to that observed in HAAN, although transcript levels were higher and also was the individual variability in all but especially in experimental groups (Fig. 10C). Thus, the significance level of the increase was lower, and the change in the MPs and temperature group was not statistically different from the control. In this case, no differences were observed in the liver SOD enzymatic activity between any of the groups tested (Fig. 10D).

3.1.9. Principal component analysis (PCA)

The PCA score plot using the combined responses of both species to control, to MP exposure, to high temperature, or to both stressors exhibited that the first two dimensions present only 54 % of the total variances (PC1, 34.1 %, and PC2, 19.9 %, Fig. 11A). Based on the evaluated parameters, the analysis showed separated clusters among the treatment groups and species. Such separations were also observed for each species individually when the 16 parameters were plotted into the reduced dimensional space of the PCA. Much like for the combined species analysis, total variance explained by PC1 and PC2 was low, amounting to 62.2 % (PC1, 48.8 %, PC2, 13.4 %) and 59.2 % (PC1, 41.3 %, PC2, 17.8 %) for *H. antarcticus* and *H. bispinis* respectively (Fig. 11B and C). While gill-related indicators showed more extreme PC1 scores and liver-related indicators presented higher PC2 scores in *H. bispinis* (data not shown), there was no consistency between loadings of specific parameters or tissues in determining PC scores in *H. antarcticus*. Overall, the outcomes of this analysis indicated that, regardless of a considerable variation in the direction and magnitude of indicators among tissues and species, a combined response appears to aggregate individuals in relation to treatment groups, suggesting specific responses to each exposure condition that could not be easily perceived by evaluation of individual indicators alone.

We included a resume table “Table 2” to simplify the results.

4. Discussion

The present comparative study revealed differences in the activities of antioxidant enzymes and the expression of mRNA in tissues of two nototheniids fish, *H. bispinis* and *H. antarcticus*. Overall, our results showed that either single stress or multifactorial stresses, which combined MPs and thermal challenges, elicited complex responses between the transcriptional and the functional levels which warrant further disentanglement. However, results also show that despite the large dispersal in direction and scale of the responses among the parameters tested, fish exposed to the same treatments cluster together, although the trends taken appear to be species specific.

4.1. Impact of microplastics on oxidative stress

Microplastics are a severe problem due to the high impact of their presence and effects at the trophic level, and their presence has recently been confirmed in several Antarctic fish species (Zhang et al., 2022; Ergas et al., 2023; Mancuso et al., 2023). However, the potential physiological effects of their accumulation are yet unknown, namely at the level of the cellular protection mechanisms. Microplastics are known to

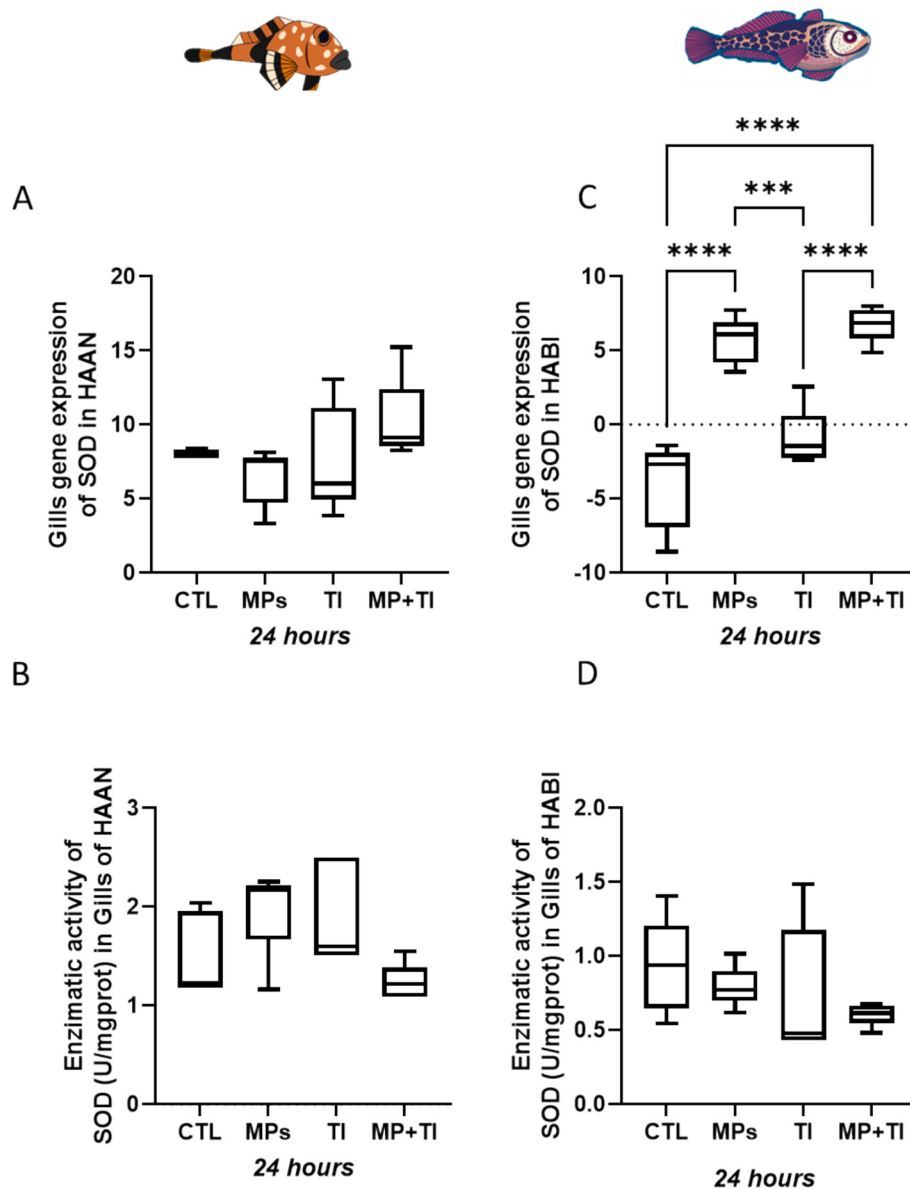


Fig. 9. Gill superoxide dismutase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Gill superoxide dismutase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. Two-way ANOVA followed by Tukey's test, (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001) n = 5 per sampling day/per treatment.

prompt oxidative stress by raising ROS directly and altering the action of antioxidant enzymes, reducing the efficiency of ROS removal (Kadaczapska et al., 2024). We observed a similar trend in the gill of *H. bispinis*, where gene expression for the four oxidative stress markers appeared to be up-regulated upon treatment with MP, but the contrary occurred in the gill of *H. antarcticus*, in which the transcripts for all enzymes were reduced in MP-treated fish in relation to the control. Despite the significant downregulation in gene expression the activity for CAT showed a marked increase, and therefore, it can also be inferred that exposure to MPs produces more ROS, such as hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-), related to the activities of the transcripts and antioxidant enzymes evaluated here. The cause of oxidative stress, as was observed in the liver of both species exposed to MP, may be related to a systemic response to a potential inflammatory situation in the intestine, as the physical presence of microplastics may cause tissue damage as was previously described in *Oryzias melastigma* and *Sparus aurata* (Bhuyan, 2022). However, it cannot be ruled out that MP may

directly affect the hepatic tissue. We have not assessed the presence of MPs in tissues and cannot indicate whether they may have been absorbed at the intestinal level or breached the intestinal barrier. The response of gill tissue in *H. antarcticus* was increased by exposure to PVC-type MPs, whereas, on the contrary, in *H. bispinis*, no significant changes were observed at the level of enzymatic activity. Indeed, the two tissues separate quite well in their overall responses, in which liver and gill were well separated by PC2 in the PCA and the treatment groups by PC1, especially in the case of *H. bispinis*. In this species, the response to MPs differed from the control parameters, a difference driven mainly by the genes expressing enzymes in gills, while in the liver, no clear separation was observed between these treatments.

4.2. Impact of temperature on oxidative stress

Exposure to warmer temperatures, compared to those at which an organism lives optimally, whether brief or prolonged, can alter its

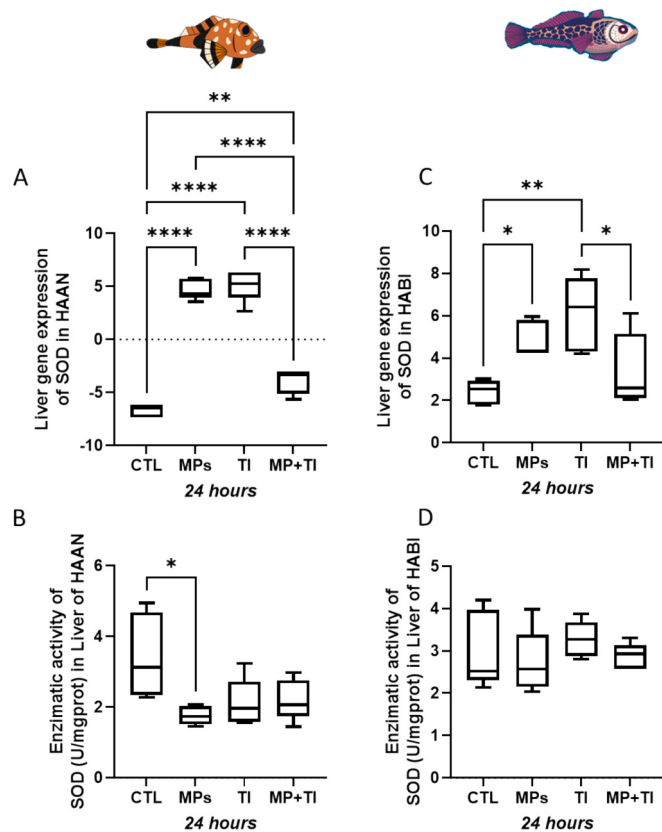


Fig. 10. Liver superoxide dismutase gene expression A) *Harpagifer antarcticus* (HAAN) and C) *Harpagifer bispinis* (HABI). Liver superoxide dismutase enzyme activity B) *Harpagifer antarcticus* (HAAN) and D) *Harpagifer bispinis* (HABI). The boxplots represent different treatments. CTL; treatment control, MP; treatment microplastics, TI; treatment thermal increase, MP + TI; treatment thermal increase and microplastics. Symbols (*) over the bars indicate statistical differences between different treatments. Two-way ANOVA followed by Tukey's test, (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$) $n = 5$ per sampling day/per treatment.

metabolic functioning at the genetic and enzymatic levels and affect how it responds to new stressors, potentially impacting its ability to cope with them in the long term (Alfonso et al., 2021). Impacts of increased temperature on oxidative stress challenges are well documented in ectotherms, including studies Antarctic fish. The activity and expression of antioxidant enzymes and other markers of oxidative stress have been used for the evaluation of Antarctic fish *Chaenocephalus aceratus* (Lönnerberg 1906), *C. rastrospinosus* (DeWitt and Hureau 1979), *G. gibberifrons* (Lönnerberg 1905) and *N. coriiceps* (Mueller et al., 2012). In *H. antarcticus*, experimentally induced rises of 3 to 6 °C significantly increased oxygen consumption, lowered feeding efficiency, and reduced osmoregulatory capacity despite increased enzymatic activity (Navarro et al., 2019) and in warm acclimated Antarctic *Notothenia rossii* and the sub-Antarctic *Lepidonotothen squamifrons*, differences were observed in mitochondrial efficiency (Strobel et al., 2013). Increases in respiration rates associated with these losses in cellular and functional efficiency have the potential to negatively impact the organism through elevated ROS production (Lesser, 2006) and increases in temperature in *H. antarcticus* and *H. bispinis* produced significant increases in markers of ubiquitination and apoptosis in gill and liver (Saravia et al., 2024), which can also be indicative of unresolved oxidative stress (Todgham et al., 2017; Song et al., 2023). In the case of *H. antarcticus* we recorded an increase in antioxidant enzymes at the gill level when these fish were exposed to increased temperature. Catalase activity increased in the gills of both species and the liver of *H. bispinis*, GR decreased in the liver of *H. bispinis*, and GPx only rose in the gills of the Antarctic species, and

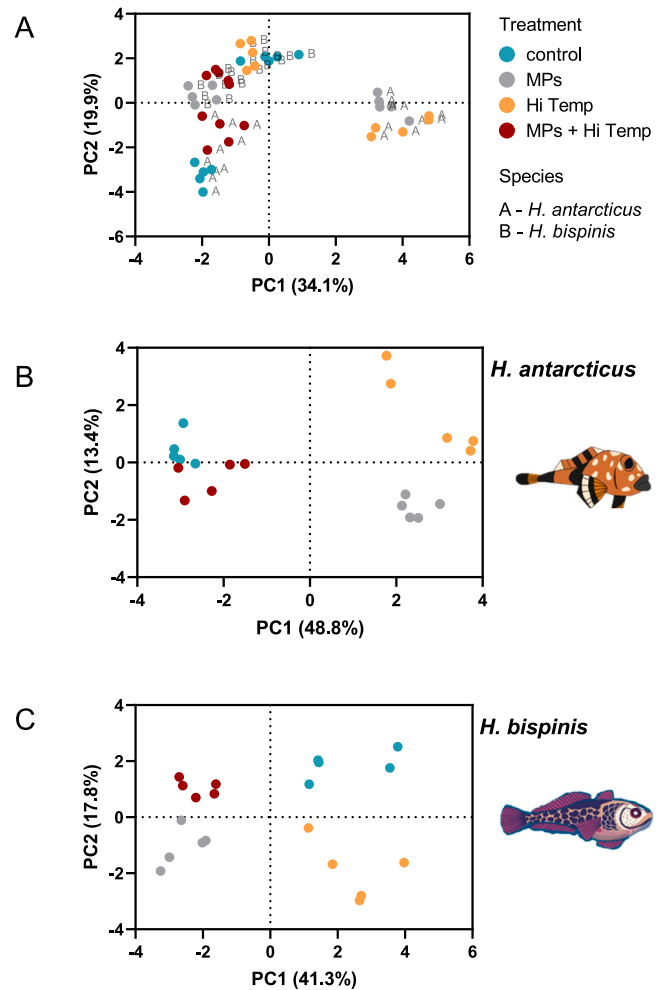


Fig. 11. Principal Component Analysis based on measured parameters in the gill and liver of *Harpagifer antarcticus* and *Harpagifer bispinis*. Combined distribution for the four treatment groups in both species A), and for *H. antarcticus* B) or *H. bispinis* C) alone. Factor scores of the observations plotted on the first two components. $n = 5$ per treatment per species and 16 variables analyzed per individual.

none of the other tested enzymes showed differences in the tissues of heated fish relative to the control groups. This aligns with minimal changes in enzymatic activity observed by Klein et al. (2017) when testing the effect of rapid increasing temperature on the antioxidant defense system and oxidative stress parameters in the Antarctic fish *Notothenia coriiceps* and *Notothenia rossii* at the gill, brain, liver, and white muscle levels. In these fish significant differences mainly were observed in the heart and muscle, where the regulation of SOD, CAT and GPx varied depending on the temperature (4–8 °C) and the exposure time (12–144 h). In the present work, most genes showed down-regulation in the gill and up-regulation in the liver of *H. antarcticus* and an inverse trend in tissues of *H. bispinis*. Nonetheless, when the parameters tested were analyzed in a PCA, the control group and the temperature increased group formed separate clusters, especially for *H. antarcticus*, for which the rise from 2 to 5 °C may be more extreme than the change from 8 to 12 °C in *H. bispinis*.

Thus, the antagonistic effects on transcription and activity require clarification, and since the fish have been exposed to the increased temperatures for only 24 h, our sampling should not represent a time window of debilitation and turnover of the oxidative stress responses. Rapid enzymatic changes have previously been observed in several fish species with increases and declines in activity occurring within periods of <24 depending on the heat stress inflicted (Mueller et al., 2011;

Table 2
Resume table.

Species	MPs				Temperature				MPs + Temperature			
	<i>H. antarcticus</i>		<i>H. bispinis</i>		<i>H. antarcticus</i>		<i>H. bispinis</i>		<i>H. antarcticus</i>		<i>H. bispinis</i>	
	Gill	Liver	Gill	Liver	Gill	Liver	Gill	Liver	Gill	Liver	Gill	Liver
Expression												
<i>cat</i>	↓	=	↑	=	↓	=	↑	=	↓	=	↑	↓
<i>gr</i>	↓	↑	↑	↑	↓	↑	=	↓	=	=	↑	=
<i>gpx</i>	↓	=	↑	=	↓	↑	↑	↓	=	↑	↑	↑
<i>sod</i>	=	↑	↑	↑	=	↑	↑	↑	=	↑	↑	=
<i>Overall</i>	↓	↑	↑	↑	↓	↑	↑	↓	=	↑	↑	=
Activity												
CAT	↑	=	=	↑	↑	=	↑	↑	=	↑	=	↑
GR	=	↓	=	=	=	=	=	↓	=	↓	=	=
GPX	↑	=	=	=	↑	=	=	=	=	=	=	=
SOD	=	↓	=	=	=	=	=	=	=	=	=	=
<i>Overall</i>	↑	↓	=	=	↑	=	=	=	=	=	=	=

Madeira et al., 2013; Machado et al., 2014; Klein et al., 2017; Zutshi et al., 2020) which suggest that oxidative stress biomarkers should be analyzed with caution and can be highly dependent on tissue, species, challenge and exposure time.

4.3. Impact of multiple stressors on oxidative stress

Multiple stressors can interact to produce complex responses that can be additive, synergistic, and/or antagonistic, and are difficult to predict (Jackson et al., 2016). For example, combined exposure to microplastics and higher temperatures increased microplastic concentrations in fish bodies, but had antagonistic interactive effects, as temperatures reduced their impacts on post-exposure predatory performance (Wen et al., 2018). When exposed to multiple stressors, organisms are likely already performing outside of optimal conditions (Van Straalen, 2003) and therefore more sensitive to pollutants (Noyes et al., 2009), including microplastics and their associated toxic substances (Fonte et al., 2016). Increases in temperature may generate a higher frequency of intake of MPs, either through ventilation or through the active search for food to meet metabolic needs (Hasan et al., 2023), and in the present case, could accelerate the passage of the MPs through the gastrointestinal tract and/or stimulate increased absorption. However, we observed that when the fish were confronted with both stressors simultaneously, the overall response in gills and liver was not generally significant, and the levels of enzyme activity were similar to those measured in the control condition and in many cases lower than those in the MP or temperature treatments alone. Only catalase activity was increased in the liver of both species, which may indicate that an antioxidant response was required in this organ to restore the levels of reactive species generated due to exposure to both stressors simultaneously. The CAT levels in the liver of *H. antarcticus* in response to the combined stressors were about three-fold higher than the control and any of the other individual stressors. In *H. bispinis*, hepatic CAT was similar to the other treatment groups, all significantly different from the control. However, no other enzymes were stimulated, which suggests the two factors counteract each other, leading to a weaker overall effect than expected, or that this challenge exhausted the initial antioxidant responses in these organs, at least within the time frame of our sampling. Studies on Antarctic fish are contradictory on the onset and duration of the oxidative stress response, which may be species and condition-related. Enzor and Place (2014) focused on three Antarctic species: *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi* and found that short-term exposure to higher temperatures and elevated pCO₂ led to increased oxidative damage, but after long-term acclimation, the fish exhibited a significant reduction in oxidative damage, suggesting an adaptive physiological response to prolonged environmental stress. On the other hand, Klein et al. (2017) observed increased antioxidant responses when

fish were exposed to increased temperatures over 6 days, compared to a 24 h exposure. These studies provide evidence that Antarctic fish can respond to rising temperatures and microplastics; however, there is little information on the energy cost of this physiological process at the molecular and cellular level and is unclear whether this ability entails physiological trade-offs. This is the first time that these species, and to our knowledge, any notothenioid species, are subjected simultaneously to these multiple stressors (MP and TI).

In general, relevant differences were observed in several antioxidant components, including expression levels and enzymatic activity, between *H. bispinis* and *H. antarcticus*. It was observed that both species responded differently when faced with a stressor separately; however, when they were faced with both variables in combination (MP + TI), the levels were similar to the control condition. The sub-Antarctic fish enzymatic activity does not vary significantly in the different treatments, whether combined or applied separately, while the transcripts in the gill respond to all conditions and in the liver only to MPs. Nonetheless, using dimensional reduction analysis, using the activity and gene expression of four enzymes in two organs as continuous variables, the fish exposed to each treatment cluster well together, indicating differential responses to each condition. The Antarctic fish show larger responses, with gill antioxidant enzymes upregulated in response to MPs and temperature, and similar to control in the combination group, and a reduction in liver activity in response to MPs. This is reflected well in the groups clustered by the PCA, distancing MPs and temperature alone from control and combination. Species-specific tissue responses are also observed but less evident, with differences more accentuated in gills than in the liver.

5. Conclusion

Our findings in the present study show that the liver appears to have a more active role than the gills, inducing the oxidative stress response in the notothenioid fish examined. This was primarily due to increased catalase activity. The activity of several antioxidant enzymes was higher in both species when exposed to temperature and microplastics separately. This is the first study to demonstrate that Antarctic and Subantarctic fish can induce an oxidative stress response in their tissues when subjected to these stressors. Although the findings corroborate the vital role of this antioxidant system in maintaining reactive oxygen species at levels necessary for proper physiological functioning, the magnitudes of the overall responses are not significant. Conversely, a substantially different response is observed when the fish are exposed to both stressors in combination; the levels (enzyme activity) are similar to the controls, indicating a highly complex response to combined stressors in these species. This complexity is added by mismatches between transcriptional levels and functional activities for the different enzymes and

tissues. These results warrant further studies, which should also consider different exposure periods, potential inflammatory effects in the intestinal tract and impacts over food digestibility and absorption, to better understand the long-term consequences of microplastic exposure on fish health in natural environments and the potential effects of combinations of stressors on the physiology of Antarctic and sub-Antarctic fishes in climate change scenarios.

CRedit authorship contribution statement

Daniela P. Nualart: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kurt Paschke:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Pedro M. Guerreiro:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Stephen D. McCormick:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Claudio González-Wevar:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Chi-Hing Christina Cheng:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Luis Vargas Chacoff:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, 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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