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Article

Metallothionein expression as physiological response against metal toxicity in the striped rockcod *Trematomus hansoni*.

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Abstract: Metal bioaccumulation and metallothionein (MT) expression were investigated in gills and liver of the red-blooded Antarctic teleost *Trematomus hansoni* with the aim to evaluate the possibility for this species to face, with adequate physiological responses, an increase of copper or cadmium concentrations in the environment. Specimens of this Antarctic fish were collected from Terra Nova Bay (Ross Sea) and used for a metal exposure experiment in controlled laboratory conditions. The two treatments lead to a significant accumulation of both metals and an increase of gene transcription only for the MT-1. The biosynthesis of MTs was verified especially in specimens exposed to Cd, but the majority of these proteins were soon oxidized, probably because they were involved in cell protection against the risk of oxidative stress, by reactive oxygen species scavenging. The obtained data highlighted the phenotypic plasticity of *T. hansoni*, a species evolved in an environment characterized by natural high concentrations of Cu and Cd, and maybe the possibility for the Antarctic fish to face the challenges of a world that is becoming every day more toxic.

Keywords: Antarctica; antioxidants; cadmium; copper; fish; metallothioneins

1. Introduction

Antarctic environment has unique characteristics related to its distance and isolation from other continents of our planet. Anthropogenic contamination is considered negligible, even if contaminants can reach Antarctica by long-range atmospheric transport [1].

Antarctic marine organisms evolved in this environment, isolated for 10–12 million years and exposed to peculiar physical and chemical conditions, such as a very low and constant temperature as well as a high oxygen concentration. Such conditions probably affected the metabolic adaptive strategies during the evolution of these organisms [2] and in particular the physiological defense systems against the risk of oxidative stress [3,4].

One of the main characteristic of Antarctic seawater is also a natural occurrence of high cadmium (Cd) concentrations, about 70 ng L^{-1} in the soluble fraction and 0.05-0.49 μg/g dry mass in surface sediments along the coast of Terra Nova Bay, Ross Sea [5,6]. Cd is a typical recycled metal, uptaken by biological systems and subsequently discarded as detritus and fecal pellets [7]. It is not an essential metal to biota, but biological processes also influence its distribution in seawater column.

Antarctic seawaters are also characterized by a natural occurrence of high copper (Cu) concentrations, about 150 ng L^{-1} [5]. Cd, unlike Cd, is an essential metal for biota but can lead to toxicity if present at high concentrations.

As a consequence of these elevated environmental metal levels, organisms may accumulate metals, which penetrate their tissues by various mechanisms, according to the chemical speciation of the metal. The main pathway for metal uptake in fish seems to be through gills and intestine, but the relative extent of these routes varies, partly depending on the chemical and physical characteristics of water and sediments [8]. Antarctic vertebrates can accumulate metals also feeding mollusks and epibenthic crustaceans, because these preys frequently have high tissue metal concentrations [6,9].

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It is well known that metal ions widely interact with biological molecules. Cd has a high affinity for the sulfhydryl groups of cysteine, and competes against zinc (Zn) and Cu for the structural and active sites of various enzymes, thus impairing their catalytic activities. Cd also exerts several toxic effects on at both cellular and systemic levels [10]. On the other hand, essential trace metals, such as Cu and Zn, are required in various physiological and metabolic processes, but they become toxic at excessive concentrations, damaging plasma membrane and other cell components. Cu is also a redox-active metal and can act as a catalyst in the Fenton reaction, facilitating the conversion of superoxide anion and hydrogen peroxide to hydroxyl radical, the most dangerous among reactive oxygen species (ROS) and mainly responsible of oxidative stress [11].

To limit the problems related to metal exposure, organisms evolved metallothioneins (MTs), a group of ubiquitous low molecular weight metal-binding proteins (6–1.9 kDa), the first line of defense against metal toxicity [12,13]. These proteins are characterized by an unusually high cysteine content (30%) and generally by the lack of both aromatic amino acids and histidine [14]. Their biosynthesis may be induced in tissue by various stimuli, especially metal ions or several stress conditions, having many functions, such as the regulation of essential metal content, the detoxification of essential and non-essential metals and the non-enzymatic scavenging of ROS [15]. Tissue expression MTs in fish primarily occur in liver, kidney and gills [16].

One of the main scientific question, related to the physiological adaptation of Antarctic organisms, is whether they have evolved specific acclimatization capacities towards a variation of the environmental concentrations of metals, given that they evolved under an important selective pressure represented by the elevated concentrations of these chemical elements. In particular, in this paper we aimed to verify whether and how an increase in the environmental concentrations of Cu or Cd can be reflected in an implementation of the gene expression of MTs. *Trematomus hansoni*, an Antarctic teleost widespread in the coastal marine areas of the ice continent, was chosen as the experimental organism, and used in exposure experiments under controlled laboratory conditions. The data of metal accumulation in gills and liver have been correlated with the MT gene expression, evaluated at both transcriptional and post-transcriptional levels.

2. Results and Discussion

Cu treatment led to a statistically significant accumulation of this metal ($p < 0.001$) in both gills and liver, with an increase of 53% and 61%, respectively (Figure 1a). The accumulation of Cd was much more consistent in percentage (Figure 1b). In fact, after treatment with this metal the concentrations measured in the liver almost quadrupled, while in the gills they became about 100 times higher than controls ($p < 0.001$).

These results can be correlated to the different degree of metal absorption in different tissues [17]. It is also possible that the higher percentage accumulation of Cd is related to the fact that it is a non-essential metal. In fact, for non-essential metal cells favour a system of detoxification based on chelation (and therefore storage in molecular structures that reduce its solubility and bioavailability) rather than on excretion, having no membrane transport systems that are certainly present for Cu which is instead an essential metal [18,19].

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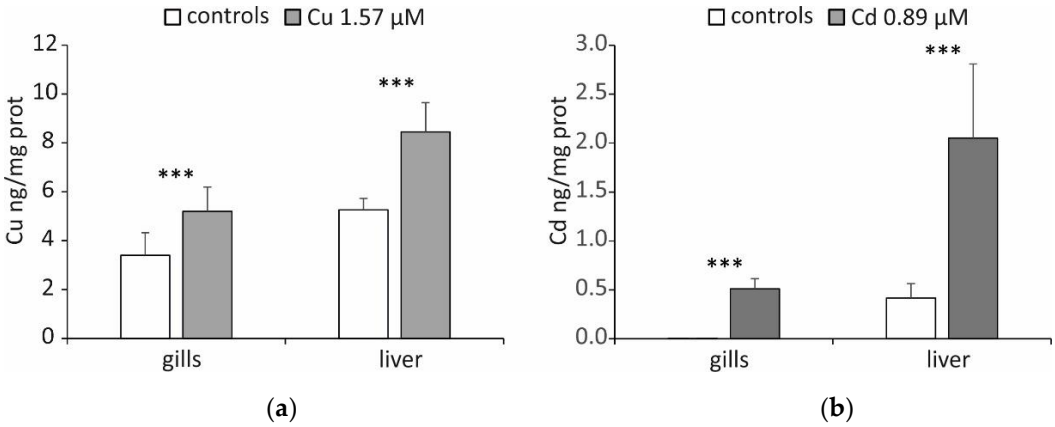

Figure 1. (a) Cu and (b) Cd concentrations (ng/mg of total proteins) determined in gills and liver of *T. hansoni*. Data are reported as mean of 5 specimen s± SD. Asterisks indicate differences between controls and treated specimens ($p < 0.001$).

Another possibility is that the greater percentage increase in the gills is the result of acute exposure to high concentrations of this metal. This effect should be emphasized in gills because they are directly exposed to the external environment, being also lined with a simple epithelium, which favours diffusional processes towards body fluids. In any case, the obtained results confirm that liver plays an important role in the detoxification of xenobiotics, accumulating them in greater quantities than other organs and tissues even in Antarctic fish. For example, Dalla Riva et al. [7] determined higher Cd concentrations in the liver than in white muscles and spleen of *Trematomus bernacchii*. Santovito et al. [20] found a greater accumulation of Cu and Cd in the liver compared to other tissues (gills, heart, white muscle) in both *T. bernacchii* and *Trematomus newnesi*. Furthermore, *T. bernacchii* experimentally treated with different metals showed a significant increase in the hepatic concentration of these elements [21].

As a consequence of metal accumulation, there is an increase in the transcription of genes encoding MTs, but only in the liver and exclusively for the MT-1 isoform (Figure 2a), with mRNA concentrations increasing relatively little, by 15% after exposure to Cu, and by 10% after treatment with Cd ($p < 0.05$).

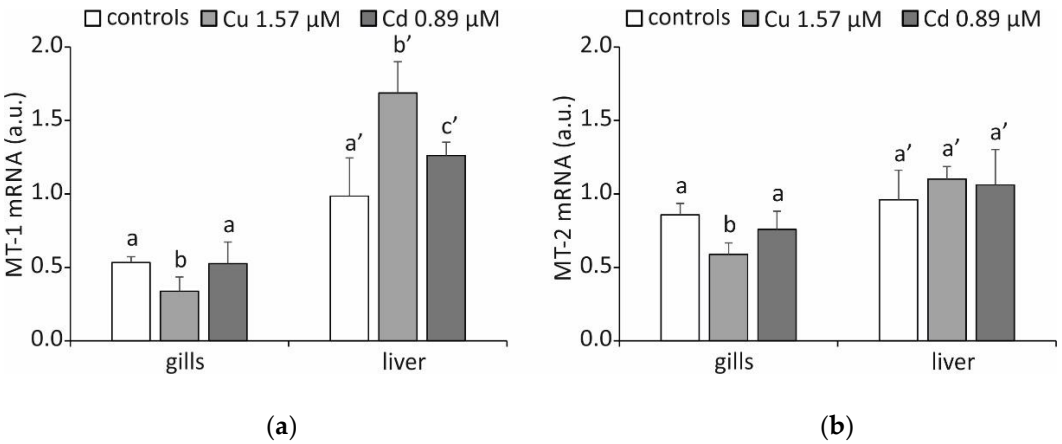

Figure 2. MT mRNA accumulation (arbitrary units) in gills and liver of *T. hansoni*. The results are expressed as mean of 5 specimens ± SD. Different letters with the same index correspond to significant statistical differences among the different experimental conditions for $p < 0.05$. (**a**) MT-1; (**b**) MT-2.

This result is partially confirmed by literature data, as Cd exposure studies using the icefish *Chionodraco hamatus* showed that MT-1 transcript was preferentially accumulated

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in liver [22]. However, it is generally assumed that isoform 1 plays a significant role in detoxification from Cd [17].

It is however probable that the preferentiality in expression of the different MT isoforms is not exclusively tissue-specific, but may vary according to the inducing metal and the species considered, as shown by recent studies carried out in *Trematomus eulepidotus* [23]. More likely, structural and functional differences in the promoter regions of the genes encoding these proteins are responsible for the differential expression of the two MT isoforms [24].

A rather singular result was obtained by measuring the tissue levels of MT with the silver saturation method [25]. As can be seen in Figure 3a, the specimens exposed to both Cu and Cd do not show an increased concentration of MT either in the gills or in the liver, and even the levels are statistically lower in the treated specimens compared to the controls ($p < 0.05$). Given the role played by MTs as metal-binding proteins, it was unlikely that an increase in the accumulation of Cu and Cd would lead to a physiological response characterized by a reduction in the presence of these proteins at cellular level. Therefore, we wanted to verify the hypothesis that the biosynthesis of MTs had actually occurred, but the presence of these proteins could not be highlighted with the silver saturation method as they were partially oxidized. In fact, by applying Santovito et al.'s method [26], which is able to measure also oxidized MTs, an increase in MT expression is evident ($p < 0.05$), in particular in response to Cd (Figure 3b). This is in line with what was previously highlighted by the analysis of metal accumulation. The only exception is represented by the gills of the specimens exposed to Cu, in which the TM levels are comparable to those measured in the controls. It is possible that in this particular circumstance other chelating molecules play a more important role, such as glutathione (GSH).

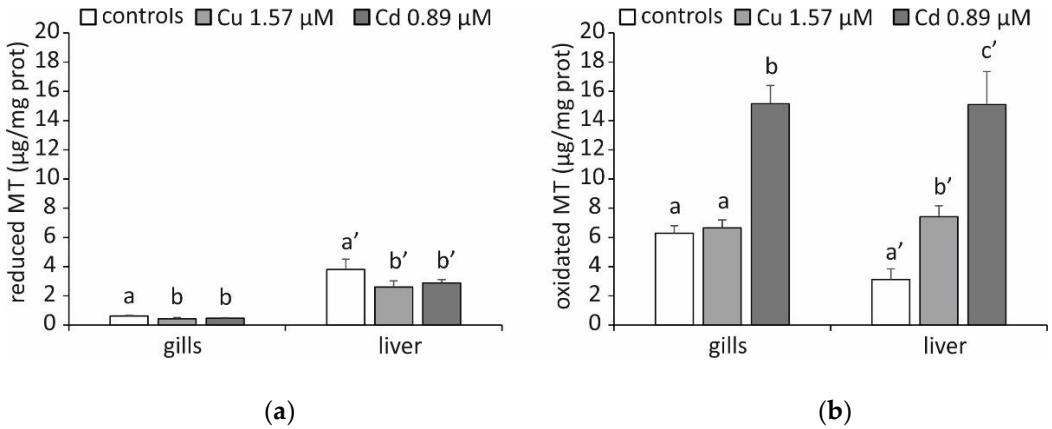

Figure 3. MT content (µg/mg total proteins) in gills and liver of *T. hansoni*. The results are expressed as mean of 5 specimens ± SD. Different letters with the same index correspond to significant statistical differences among the different experimental conditions for $p < 0.05$. (**a**) Reduced MTs; (**b**) oxidized MTs.

It is well known that this tripeptide plays an important protective role against the toxic effects of metals, acting as a primary chelating molecule in an early phase of the intracellular accumulation of these elements, as indeed occurs during acute exposure [27,28]. Furthermore, it may not be surprising that this occurs precisely in the gills of specimens exposed to Cu. In fact, Cu is a metal with redox properties and may be involved in Haber-Weiss and Fenton reactions, producing ROS [29,30]. GSH is known to form stable GSH-Cu (I) complexes, preventing further redox cycling and the generation of free radicals, and this may explain the complete protection afforded by GSH against the effects of Cu [27]. Moreover, this preventive action in the establishment of a condition of oxidative stress is very important in an organ such as gill, in which high partial pressures of oxygen are physiologically present, a condition that in itself favours the formation of ROS.

The increase in MT biosynthesis was confirmed by a Western blot assay only in the liver, as a sufficient amount of cell-free extract was available only for this organ. As can

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be seen in Figure 4, there is a higher concentration of MT in the liver of treated specimens compared to the controls, and in particular in the fish exposed to Cd.

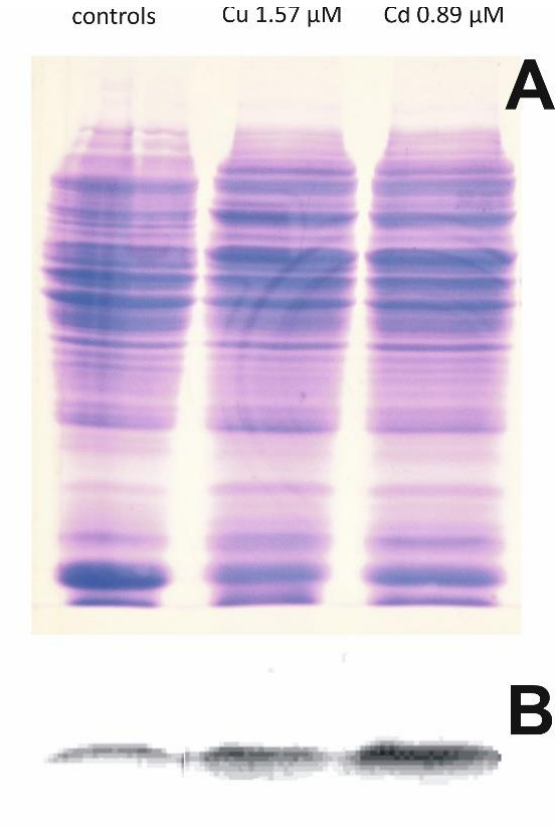

Figure 4. (**A**) Analysis of the total protein content, separated in polyacrylamide gels and stained with Coomassie brilliant blue, and (**B**) of MT by Western blotting, in liver cell-free extracts from *T. hansoni* specimens treated with Cu, with Cd and control.

Among the protein alterations affecting MTs, whose characteristics are known, is the oxidation of these molecules with the formation of disulfide bridges between the thiol groups of the cysteines. This protein modification is linked to the antioxidant defense function characteristic of MTs [31,32]. Under our experimental conditions, this is probably determined by an increase in the rate of ROS formation, the production of which is notoriously enhanced by the presence of an excess of metals in the cell [11].

Another peculiar result is that the concentrations of MTs in the liver are not correlated with their mRNA levels. In particular, the rate of protein biosynthesis is higher than the rate of messenger transcription, suggesting that in controls there is a relatively high mRNA concentration, part of which is not translated to protein. This type of result is widely documented in literature, and many authors attribute it to a post-transcriptional control on MT synthesis, first identified in the rat liver [33]. This control seems to be based on the formation of stress granules (SGs), cytoplasmic foci in which the messengers can be stored, undergoing future translation [34]. This condition is a common feature in organisms that live in stressogenic conditions, but without acute stress [35-39], allowing an extremely rapid response by the tissues toward the sudden onset of acute stress. This can easily occur in liver of *T. hansoni* experimentally exposed to metals.

Nucleation proteins are involved in SG formation, such as the T-cell-restricted intracellular antigen (TIA) proteins, TIA-1 and TIA-1-related protein (TIAR), which both self-associate to promote the growth of SGs, directly binding target RNAs. Recently, we characterized these proteins and their expression in relation to the expression of anti-stress proteins in *C. hamatus* and *T. bernacchii*. Preliminary data indicated that, in both species, high levels of expression of the messenger of TIA-1 correspond to low levels of biosynthesis of antioxidant enzymes, such as peroxiredoxins, supporting the hypothesis of a post-

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transcriptional control operated by stress granules [40]. Similar results we obtained studying SG proteins in the solitary ascidians *Ciona robusta* experimentally exposed to metals [41].

4. Materials and Methods

4.1. Ethical procedures

The sample collection and animal research conducted in this study comply with Italian Ministry of Education, University and Research regulations concerning activities and environmental protection in Antarctica and with the Protocol on Environmental Protection to the Antarctic Treaty, Annex II, Art. 3. All experiments have been performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU and Italian DL 2014/26 for animal experiments.

4.2. Experimental animals

Adult specimens of *T. hansoni* were collected in the proximity of Mario Zucchelli Station in Terra Nova Bay, Antarctica (74° 42' S, 167°7' E) and kept in aquaria supplied with aerated seawater at approximately 0°C. After a distress period of seven days, 10 specimens were randomly distributed in two tanks (five for each tank), where they were exposed to Cu (1.57 M) or Cd (0.89 μM), sub-lethal doses previously used in similar experimentations on fish of the same genus [42]. Five untreated specimens were used as a control group. After 5 days all the fish were euthanized (tricainemethanesulfonate, MS-222; 0.2 g L^{-1}) and samples of gills and liver tissues were excised, quickly frozen in liquid nitrogen and stored at −80°C.

4.3. Primers Design, Total RNA extraction, mt-1, and mt-2 cDNA synthesis.

Primers were designed in the coding regions of the *mt-1* and *mt-2* cDNA sequences previously characterized in of *T. hanasoni* and published in the NCBI database (GenBank accession numbers FJ870679.1 and FJ870680.1, respectively). Primer sequences were analyzed with the IDT Oligo Analyzer tool (https://eu.idtdna.com/pages/tools/oligoanalyzer). Primer sets are shown in Table S1.

Total RNA was purified from tissues using PRImeZOL™ reagent (Canvax, Córdoba, Spain) according to the manufacturer's protocol. Further purification was performed with 8M LiCl [43] to remove glucidic contaminants. The RNA quantification was performed using the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). RNA integrity was assessed by running an aliquot of RNA (1000 ng μL^{-1}) on a denaturing gel stained [44]. The cDNA synthesis was performed using a Biotechrabbit™ cDNA Synthesis Kit (Berlin, Germany) at 50°C for 1 h + 99°C for 5 minutes, from 1 μg of total RNA in a 20 μL reaction mixture, containing 2 μL of dNTP Mix (10 mM each), 0,5 μL of RNase Inhibitor, 40 U μL^{-1}, 0,5 μL of Oligo (dT) 12-18 (10 μL), 4 μL of 5x Reverse Transcriptase Buffer, 1 μL of RNA Template, 1 μL of RevertUPTM II Reverse Transcriptase and PCR Grade Water up to 20 μL. PCR reactions were performed with 50 ng of cDNA and GRS Taq DNA polymerase (Grisp, Porto, Portugal). The PCR program was the following: 95°C for 5 minutes and 40 × (95° for 30 s, Ta for 30 s, 72°C for 30 s), final elongation at 72°C for 5 minutes.

4.4. qRT-PCR analysis

To evaluate the expression of *mt-1* and *mt-2* mRNAs, real-time qRT-PCR analysis was performed. cDNAs for both target genes were amplified with the specific primers reported in Table S1. To control for variation in the efficiency of cDNA synthesis and PCR amplification reactions, the housekeeping gene *gapdh* was amplified with species-specific primers (Table S1). qRT-PCR amplifications were carried out using the qPCRBIO SyGreen Mix Separate-ROX kit (PCR Biosystems,Wayne, PA, USA) and the following program: 95°C for 2 minutes, 40 × (95°C for 20 seconds and 60°C for 60 seconds) and then the dissociation stage 95°C for 15 s, 60°C for 1 minute, 95° for 15 s and 60°C for 15 s.

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4.5. Estimation of metal and metallothionein concentrations

Portions of the tissues were homogenized by Polytron in 4 vol g^{-1} of tissue of 0.5 M sucrose, 20 mM Tris–HCl buffer pH 8.6, supplemented with 0.006 mM leupeptin, 0.5 mM PMSF (phenylmethylsulfonyl fluoride) as antiproteolytic agents, and 0.01% β-mercaptoethanol as reducing agent. The homogenates were centrifuged at 48,000×g for 60 min at 4 °C.

Cu and Cd contents were determined by atomic absorption spectroscopy using a PerkinElmer (Waltham, MA, USA) 5100 graphite furnace atomic absorption spectrometer. The instrument for metal analysis was calibrated by standard addition methods and by reference to fresh standard salt solution. Control blank solution on reagents and equipment revealed insignificant contamination of samples. Values were expressed as ng of single metal/mg of total proteins assayed by the Folin phenol reagent method [45] using bovine serum albumin as standard.

MT concentration was determined in supernatant by the silver saturation method [25]. In order to discriminate reduced and oxidized MTs, the Santovito et al.'s method [26] was applied. The amount of MTs was normalized against total soluble cell proteins assayed as previously described.

4.6. Statistical Analyses

Statistical analyses were performed with the PRIMER statistical program (PRIMER-e, Auckland, New Zealand). One-way ANOVA was followed by the Student–Newman–Keuls test to assess significant differences ($p < 0.05$). The data were expressed as the average of five analyzed specimens ± standard deviation (SD).

4.7. Western blot Analysis

Western blot analysis was performed using the SuperSignal™ West Pico Chemiluminescent Substrate kit (ThermoFisher Scientific), with mouse anti-metallothionein IgG1 (ThermoFisher Scientific) as primary antibody and HRP labeled anti-mouse IgG as secondary antibody (Promega, Madison, WI, USA).

5. Conclusions

The obtained data highlighted the phenotypic plasticity of *T. hansoni*, a species evolved in an environment characterized by natural high concentrations of Cu and Cd, and suggested the possibility for the Antarctic fish to face the challenges of a world that is becoming every day more toxic. In this physiological characteristic, metallothioneins certainly play a fundamental role, performing the function of both metal-chelating molecules and non-enzymatic antioxidant. Certainly, this latter function is integrated with other proteins that play a role of protection against oxidative stress such as antioxidant enzymes, whose expression in Antarctic fish also has peculiar adaptive characteristics.

In addition, our results also provide an important contribution to achieve the objectives for the establishment of the Marine Protected Area (MPA) of the Ross Sea, proposing the escribed toxicological evaluations a functional instrument for the previous and initial monitoring of the ecosystem of the new MPA.

Obviously, it will be necessary to implement the knowledge of the physiological responses against environmental stress that other species, belonging to all kingdoms, are able to carry out against the toxicity of xenobiotic substances. This will certainly be the main goal to have a more detailed overview, not only within the group of Antarctic fish but also in the entire food web of the Antarctic ecosystem.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Primer pairs used for qRT-PCR. Amplicon sizes and annealing temperatures (Ta) are al-so indicated.

Author Contributions: Conceptualization, G.S.; methodology, P.I. and G.S.; validation, R.B.; formal analysis, E.P., S.S. and R.B.; investigation, E.P., S.S., R.B., P.I., and G.S.; resources, P.I. and G.S.; data curation, G.S.; writing—original draft preparation, G.S.; writing—review and editing, E.P., S.S., R.B.,

P.I. and G.S.; visualization, G.S.; supervision, G.S.; project administration, G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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