 doi:10.20944/preprints202007.0235.v1

Article

Non-Enzymatic Antioxidant Responses of *Mytilus galloprovincialis* under Cadmium-Induced Oxidative Stress Risk

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Abstract: Exposure to metals is known to generate oxidative stress in living organisms, which are able to respond with the induction of antioxidant defenses, both enzymatic and non-enzymatic. The aim of this work is to study the correlation among several non-enzymatic component of the antioxidant system, that are physiologically related to both metal sequestration and defense against metal-induced oxidative stress, using the blue mussels (*Mytilus galloprovincialis*) as model organism. Specimens of this marine bivalve were experimentally exposed to cadmium (Cd), used as oxidative stress risk inducer. Cd, metallothionein (MT), glutathione (GSH), malondialdehyde (MDA) contents, and glutathione reductase (GR) activity in gills and in digestive glands were assessed at 0, 12, 24, 48, 72 and 96 h. The obtained results provide new data about the relationships among the non-enzymatic antioxidant cellular components considered in this study. These constitute the prompt physiological responses to the risk of oxidative stress in blue mussels exposed to Cd in controlled laboratory conditions.

Keywords: cadmium; glutathione; glutathione reductase; malondialdehyde; metallothionein; *Mytilus galloprovincialis*; oxidative stress; ROS scavenging

1. Introduction

About 3% of oxygen utilized by cells may form reactive oxygen species (ROS) [1], but this rate of formation can easily increase in both physiological and non-physiological conditions. One of the latter conditions is exposure to xenobiotic substances, such as metals such as cadmium (Cd).

Cd presence in the environment is constantly increasing due to its extensive industrial use either alone or mixed with different chemical compounds [2]. Although Cd is a redox-stable metal, ROS are often implicated in Cd toxicology, as shown both in a number of cell-culture systems [3,4], and in intact animals through different exposure routes [5-7]. Therefore, the production of ROS by Cd must be mediated through indirect mechanisms.

The cytotoxic effects of ROS include peroxidation of membrane lipids with increasing malondialdehyde (MDA) levels through oxidation of polyunsaturated fatty acids, alteration of redox balance, enzyme inactivation, and DNA damage [8-10].

Organisms can respond to a high presence of ROS by activating their antioxidant defense system, consisting of both antioxidant enzymes and small molecules and proteins that act as non-enzymatic antioxidants, such as glutathione (GSH) and metallothioneins (MTs) [11,12].

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The tripeptide GSH (γ-glutamyl-cysteinyl-glycine) is considered one of the most important antioxidant agents, involved in the protection of cell membranes from lipid peroxidation. GSH is present in the cell at millimolar concentrations and functions as a redox buffer to maintain the overall cellular redox state, but it also acts as cofactor of glutathione peroxidase in the conversion of hydroperoxides to their corresponding alcohols [13,14].

Increased synthesis of MTs occurs after metal exposure and in the presence of oxyradical-generating compounds. MTs are a class of cysteine-rich proteins with high metal affinity and are closely associated with the detoxification of toxic metal ions such as Cd^{2+}, thus protecting from oxidative stress [15-19]. MTs have also function as non-enzymatic scavenger of ROS [20] and are involved in interaction mechanisms with GSH.

MTs have therefore been widely used as biomarkers of metal contamination exposure [21-23]. To understand the physiological mechanisms in front of the Cd influx in the cells it is also important to determine the metal subcellular partition between the insoluble and soluble fractions because MTs, GR and GSH are present in the soluble fraction. Various biomarkers can be used to better understand the physiological responses of organisms to pollution [24-26] and use of a lipid peroxidation indicator coupled with chemical analysis of xenobiotic accumulation is often recommended [27,28].

The mussels' ability to readily bioaccumulate metal pollutants makes them more accurate *in situ* bioindicators than fish [29-31]. Thus, bivalve mollusks, and in particular *Mytilus* spp., are among the most widely used monitoring organisms to assess the impact of marine pollution, despite the disadvantage that some physiological parameters exhibit seasonal changes which may alter results [32-34]. Pollutants are usually complex mixtures whose biological effects on organisms are difficult to predict by means of chemical analyses alone. A number of these biological responses - biochemical, histological and physiological - are actually indexes of either exposure or stress at molecular, cellular or organism levels. They can therefore be considered stress biomarkers, as their assessment usefully integrates the most common chemical measures and provides additional information about insult *versus* biota, and possibly its nature [30]. Since different cellular components contrast the negative effects produced by contaminants, these biological parameters are widely used as biomarkers to evaluate the effects of pollutants on organisms [24,35].

Hemocytes are the main components of the bivalves' immune system [36,37]. As such, they carry out important immune functions and phagocytic activity against alterations induced by toxic metals like Cd, because they can accumulate metals in their endolysosomal system. Hemocytes then die and are eliminated from these organisms together with their contaminant [38-40].

Many researchers analyzed the involvement of single elements of the antioxidant defense system in marine organisms exposed to chemicals, metals in particular, but it is well known that the oxidative networks are very complex and several oxidative and antioxidant mechanisms still remain to be elucidated [41]. In particular, it is necessary to study the integrative activities of various components of the antioxidant system against prooxidant stressors and/or environmental pollutants. The present study focused on the non-enzymatic antioxidant physiological response of mussels in oxidative stress condition experimentally induced by short-term (96h) exposure to sublethal Cd exposure, and on some molecules specifically involved in metal chelation and ROS scavenging.

The aim of this work is to study the correlation among several non-enzymatic component of the antioxidant system, that are physiologically related to both metal sequestration and defense against metal-induced oxidative stress risk. In particular, the relationships between GSH and MT were analyzed, also considering the efficiency of the GR activity, which keeps the GSH/GSSG ratio constant.

2. Materials and Methods

2.1. Animals and treatments

Specimens of *M. galloprovincialis* were collected at the beginning of summer (June) at the end of the reproductive period in the Venice lagoon in a site near the mouth of the Chioggia harbor, which has tidal exchange with marine waters, and which resulted free of Cd. Mussels of similar size (4.5 cm ± 0.25 maximum diameter) were chosen for the experiment, kept in 18 liters aerated aquaria, filled

 doi:10.20944/preprints202007.0235.v1

with filtered seawater (FSW; salinity 32.5‰) at a temperature of 21 ± 0.5°C, and fed on Liquifry Marine (Interpet Ltd, Dorking, United Kingdom). After 5 days of acclimatization, the animals were randomly divided into 2 groups. One group (30 specimens) was used as control, the other (100 specimens) was exposed to a sublethal concentration of 0.350 ppm Cd. We choose this dose according to our previous results (under revision) and literature data [42,43]. This dose is not acutely toxic as in our experience it does not produce mortality of specimens at least up to 15 days but induces physiological responses. It is therefore a suitable concentration for subacute treatment. The water Cd concentration, obtained from appropriate dilution of a $CdCl_2$ solution in FSW, was monitored daily and, when needed, restored. However, the water of aquaria was totally changed every 2 days.

One aquarium for the controls (30 individuals) and four for the treated (100 individuals, 25/tank) organisms were used, containing everyone 12.5 liter of water.

After 0 (only for controls), 12, 24, 48, 72 and 96 h, 5 specimens from the controls and 10 specimens from the treated ones were collected. Their hemolymph was drawn out with a 1 ml syringe containing 0.5 ml of marine water with 5 mM EDTA to prevent cell aggregation and was immediately used to assess cell mortality. Gills and digestive glands were dissected, frozen in liquid nitrogen and stored at −80°C until they were used for Cd, MT, GSH and MDA levels and GR activity.

2.2. Cell mortality

Hemolymph cell mortality was determined with the Trypan blue exclusion test. Briefly, 10 µl of 0.4% Trypan blue solution, filtered before use, were added to 10 µl of hemolymph cell suspension. After 2 minutes of incubation at room temperature, the cells were counted under a microscope with a hemocytometer (four 1x1 mm squares, with 20-50 cells/square) and the percentage of dead cells calculated.

2.3. Cd bioaccumulation

Aliquots of about 100 mg for each tissue sample were dried in an oven at 60°C. Subsequently, acid digestion was performed on weighed dry samples using concentrated AristaR HNO_3 (1 ml) in Teflon vessels in a model MDS-2000 microwave Digestion System (CEM S.R.L, Cologno al Serio, Italy). This microwave system is controlled by a computerized program specially designed according to the type of sample, with specific pressure and temperature conditions for optimal dissolution. Cd concentrations were determined by atomic absorption spectrophotometry using a Perkin Elmer 4000 flame furnace atomic absorption spectrometer (Perkin-Elmer, Waltham, MA, USA). The instrument was calibrated by standard addition methods [19,44], and by reference to fresh standard salt solutions. Control blank solutions on reagents and equipment revealed negligible sample contamination. Values were finally expressed as µg of Cd/g dw. Cd was also determined in cell-free extracts and expressed as µg of Cd/g ww and subsequently was evaluated the metal partitioning between insoluble tissue and cell-free fractions. The ratio between wet and dry weight was calculated and resulted equal to 0.244±0.017 in digestive glands and to 0.140±0.031 in gills. Bioconcentration factors, i.e. the Cd ratio between body and water concentration (BCF=CB/CW) were also determined in the two organs [45]. BCF is expressed in units of liter per kilogram (ratio of mg of chemical per kg of organism to mg of chemical per liter of water).

2.4. Preparation of cell-free extract tissue

The digestive gland and gills of each specimen (n = 5 for control and n = 10 for treated each time) were dissected. Portions of the two organs were homogenized in 4vol/g of tissue of 20 mM Tris–HCl buffer pH 7.5 with 0.006 mM leupeptine, 0.5 mM phenylmethylsulphonylfluoride (PMSF) as antiproteolytic agents, and 0.01% β-mercaptoethanol as a reducing agent for MT assay and Cd content determination. Each homogenate was centrifuged at 16,000×g at 4°C for 30 mins [46].

For GSH determination, tissues were homogenized with 5 vol of 5% sulfosalicylic acid/g of tissue and centrifuged at 16,000×g for 30 mins at 4°C.

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To test GR activity and MDA levels, tissues were homogenized in 5 vol/g of tissue of 50 mM sodium phosphate buffer pH 7.4, 1 mM EDTA, and centrifuged at 16,000x*g* at 4°C for 30 mins.

To prevent sample oxidation, 10 µl of BHT in acetonitrile were added to 1ml of homogenate before centrifugation.

After centrifugation, supernatants were collected and immediately utilized for analyses. All assays were performed in duplicate.

Soluble protein concentrations were determined in cell-free extracts according to Lowry method [47] using bovine serum albumin (BSA) as a standard. Protein concentrations were expressed as mg/ml.

2.5. Metallothionein

MT contents were measured in the resulting cell-free extracts with the silver-saturation method [48]. Briefly, samples were incubated with 1 ml glycine buffer (0.5 M, pH 8.5) and 1 mL of 20 mg/L silver solution for 20 mi. Excess of the metal was removed by adding 0.2 mL bovine red blood cell hemolysate, followed by heat treatment in a 100°C water bath for 10 min. The heat treatment caused precipitation of Ag-bound hemoglobin and other proteins, except for heat-stable MTs. The denatured proteins were removed by centrifugation at 4,000xg for 10 min. The hemolysate addition, heat treatment, and centrifugation were repeated two times. Finally, the solution was centrifuged at 20,000xg for 20 min. The content of Ag in the supernatant, which was proportional to the MT level, was estimated by atomic absorption spectrophotometry using a Perkin Elmer 4000 flame furnace atomic absorption spectrometer (Perkin-Elmer). The amount of MTs was normalized against total soluble cell protein content. The results were expressed as µg MT/mg total protein.

2.6. Glutathione quantification

To assess total glutathione (GSH+GSSG), tissues were sonicated twice for 30 secs with 5% sulfosalicylic acid (5 ml of acid/g ww of tissue), and centrifuged at 16,000×g for 30 mins at 4°C. Total glutathione was determined by enzymatic recycling following Anderson [48], which is based on GSH oxidation by 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to issue GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG is reduced to GSH by the action of highly specific glutathione reductase and NADPH. The rate of TNB formation was recorded at 412 nm and was proportional to the nmol of GSH and GSSG in the samples. This rate was compared with a GSH standard curve. Values were expressed as nmol/g ww.

2.7. Glutathione Reductase determination

GR activity was determined according to Williams [50] and Goel [51] methods which are based on NADPH oxidation during the reduction of oxidized glutathione, GSSG (λ = 340 nm, ε = 6.22 mM^{-1} cm^{-1}). The final assay conditions were 100 mM Na-phosphate buffer pH 7.0, 1 mM GSSG, and 60 µM NADPH. Enzyme activity was expressed as U GR and normalized against total soluble cell protein content (= nmol NADPH oxidized $min^{-1}mg^{-1}$ of total protein).

2.8. Malondialdehyde determination

As a marker of lipid peroxidation, MDA concentration was analyzed with the BioAssay™ Lipid Peroxidation Colorimetric Assay Kit (United States Biological, Salem, MA, USA) following the manufacturer's instructions. MDA concentrations were calculated by a standard plot of MDA, provided with the kit, using the same procedure employed for the tissue samples. Determination was done with a spectrophotometer at 586 nm as reported on data sheet. The MDA levels were reported as MDA nmoles/mg total protein.

2.9. Statistical analyses

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Values are reported as mean ± standard deviation (SD). Variations of biological parameters were investigated by the one-way analysis of variance (ANOVA) and mean comparisons were performed using the Tukey's test. The level of significance was set at $p < 0.05$. Correlations among variables were determined using the one-tailed Bravais Pearson correlation method.

3. Results

As reported in Materials and methods, time-control parameters were determined for each sample. Since the results were not significantly different at different times, they are reported as a single value, which is the total samples mean ± SD, at T_0 in each graph.

3.1. Cell mortality in the hemolymph

Mortality in the hemolymph soared with Cd exposure - up to 35% after 24 h, with a final 48% mortality rate. The difference between treated and control organisms was already statistically significant ($p < 0.001$) after 12 h exposure (Figure 1).

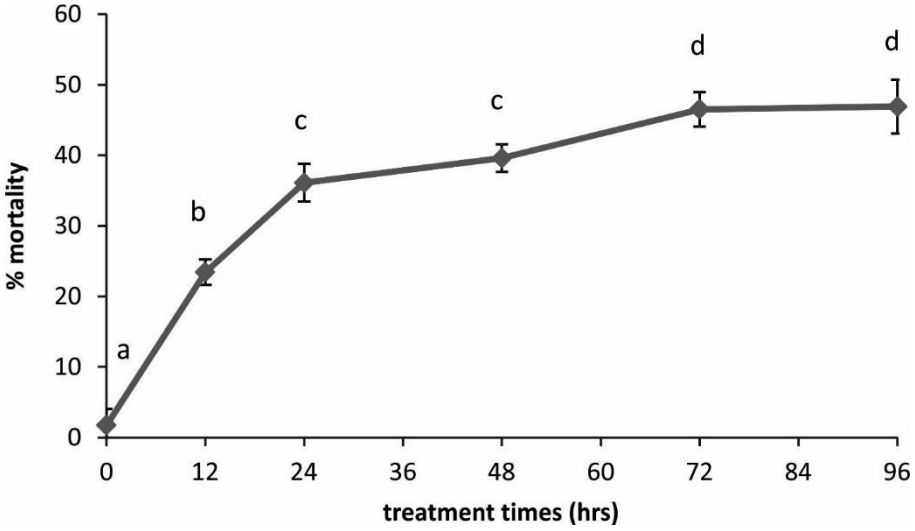

Figure 1. Haemocyte mortality percentage (Trypan blue test) of control (0 h) and treated *M. galloprovincialis* experimentally exposed to Cd for up to 96 h. Values are indicated as mean ± SD. n for each time = 10. n control = 30 Different letters correspond to significant statistical differences ($p < 0.05$) among different treatment times. n for each time = 10. n control = 30.

3.2. Cd levels

Cd levels in controls resulted extremely low (0.244 and 0.120 µg/g dry wt in digestive gland and gills respectively), confirming that the mussel collection zone was not polluted with this element. The Cd content (µg/g dw) in gills increased significantly for 48 h during the treatment ($p < 0.001$), and for 72 h ($p < 0.001$) in digestive glands (Figure 2). From the beginning of exposure up to 48 h, gills accumulated more Cd than digestive glands ($p < 0.001$), while at 72 and 96 h the Cd content was higher in digestive glands ($p < 0.05$).

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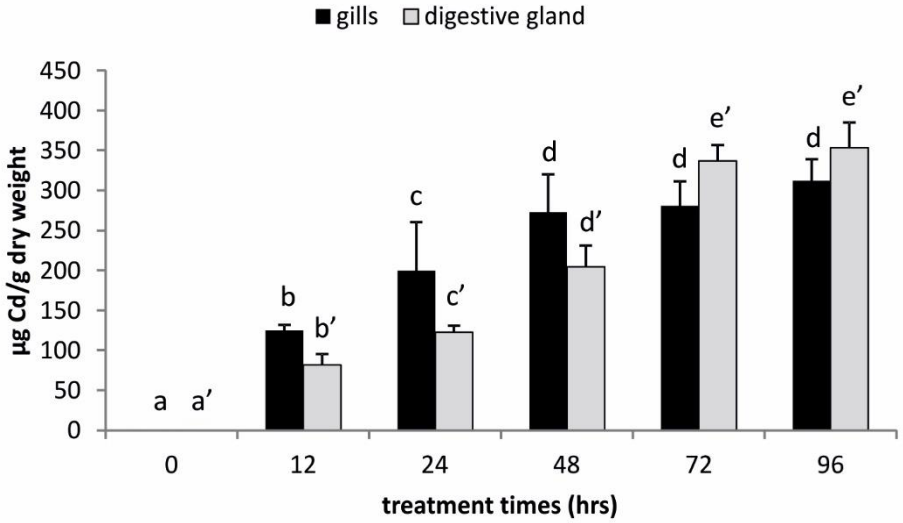

Figure 2. Cd concentrations (µg/g dw) in gills and digestive glands of control (0 h) and treated *M. galloprovincialis* experimentally exposed to Cd for up to 96 h. Values are indicated as mean ± SD. Different letters correspond to significant statistical differences ($p < 0.05$) among different treatment times. n for each time = 10. n control = 30.

The maximum Cd concentration in both organs was observed after 96 h with 353.5 µg/g dw in digestive glands, and 312.2 µg/g dw in gills. The Bioconcentration Factor was equal to 1,019 l/kg in digestive glands and 900 l/kg in gills.

The two tissues showed different Cd partitioning behaviors between cell-free extracts and insoluble fractions. In gills, a higher partitioning coefficient was measured in the insoluble fraction from the beginning of the treatment to up to 48 h of exposure. At 72 and 96 h, most Cd was recovered in the cell-free extract; the shift of Cd content toward the cell-free fraction started to occur between 24 and 48 h of treatment. Conversely, in digestive glands, Cd was found in greater amounts within the cell-free extract at every time lapse except at 72 h, when Cd partitioning was equal in the two fractions (Figure 3).

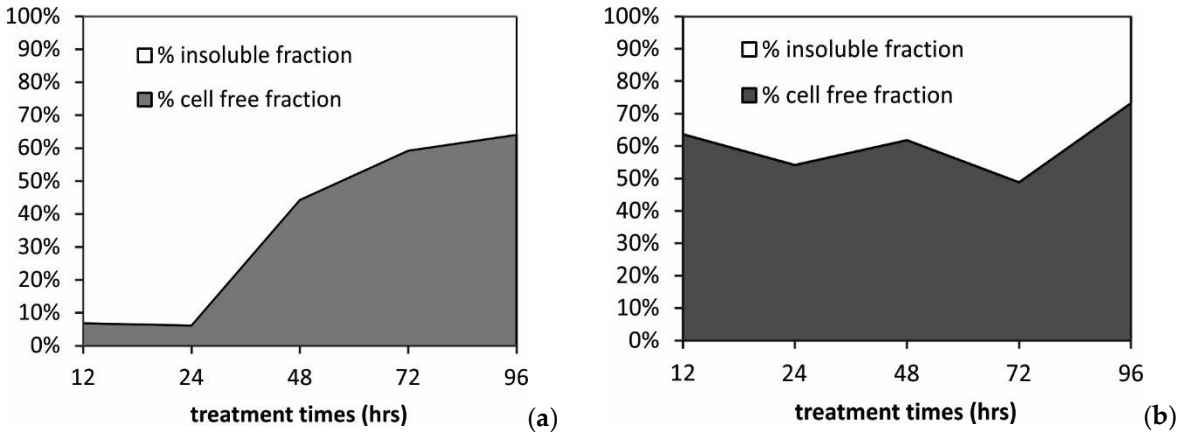

Figure 3. Cd partitioning percentages between cell-free extract and insoluble fraction in (a) gills and (b) digestive glands of control (0 h) and treated *M. galloprovincialis* experimentally exposed to Cd for up 96 h. n for each time = 10. n control = 30.

3.3. MT

Figure 4 shows MT levels during Cd treatment in gills and digestive glands. The MT induction trend is very similar in both organs. A steady MT increase associated with metal exposure duration was observed up to 96 h, when the highest value was measured in both tissues, but with different starting times. In digestive glands, the increase occurred at 12 h of treatment and went up to about 7.5 μg/mg total protein at the end, which is approximately seven times the value detected in controls ($p < 0.001$). In gills, MT induction began only at 24 h of Cd exposure. After that, MT levels increased in relation to exposure time up to approximately 6.5 μg/mg total protein at 96 h, which is about 5 times the value found in controls ($p < 0.001$).

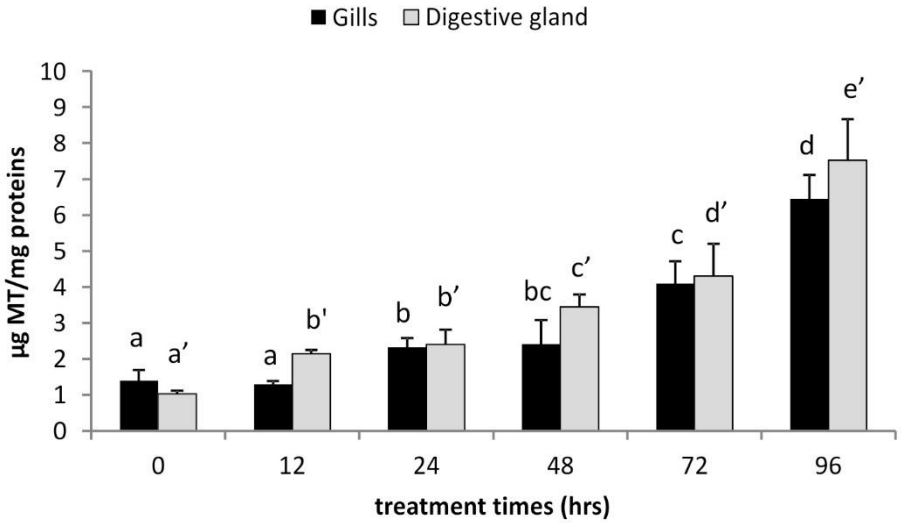

Figure 4. MT concentrations (μg/mg total protein) in gills and digestive glands of control (0 h) and treated *M. galloprovincialis* experimentally exposed to Cd for up to 96 h. Values are indicated as mean ± SD. Different letters correspond to significant statistical differences ($p < 0.05$) among different treatment times. n for each time = 10. n control = 30.

3.4. GSH

GSH levels were very different in the two tissues (Figure 5): in controls, concentration was more than double in digestive glands (about 320 nmoles/g ww) than in gills (about 120 nmoles/g ww). In gills, a GSH time-dependent increase was observed starting from 12 h of exposure up to a 96-h value of 225 nmol/g ww, almost double the control value ($p < 0.001$). In digestive glands, GSH content significantly decreased at 12 h ($p < 0.001$) and went back to control values after 24 h of exposure. At 48 and 72 h, GSH levels reached their maximum value of about 900 nmoles/g ww, 3-4 times the value of controls ($p < 0.001$), then dropped at 96 h, but remained higher than in controls ($p < 0.001$).

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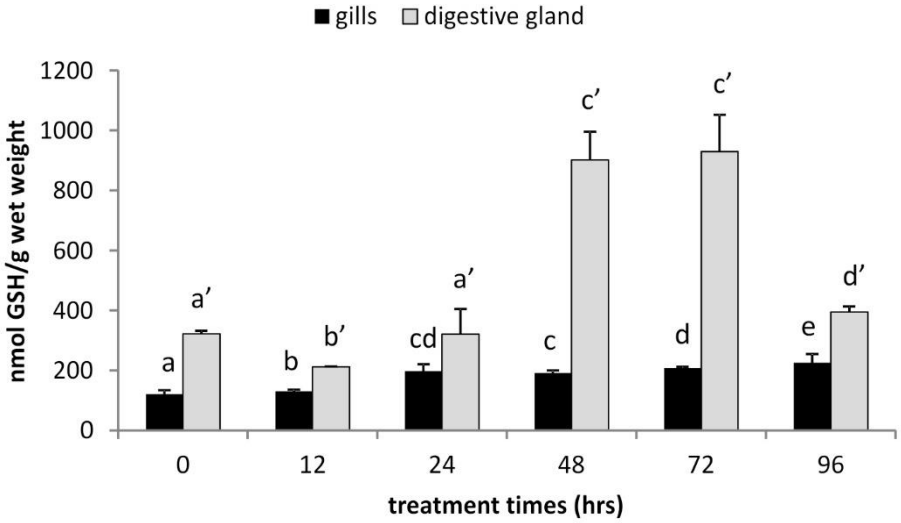

Figure 5. GSH content (nmol/g wet weight) in gills and digestive glands of control (0 h) and treated *M. galloprovincialis* experimentally exposed to Cd for up to 96 h. Values are indicated as mean ± SD Different letters correspond to significant statistical differences ($p < 0.05$) among different treatment times. n for each time = 10. n control = 30.

3.5. GR activity

As Figure 6 shows, GR activity was higher in gills than in digestive glands ($p < 0.001$).

In gills, GR activity increased significantly for up to 24 h ($p < 0.001$) compared with controls; at 48 h remained unchanged, and increased again at 72 h, issuing the highest value - about 30 UGR. At 96 h, GR activity significantly decreased compared with the 24, 48 and 72 h values, but was nonetheless higher than in controls ($p < 0.001$).

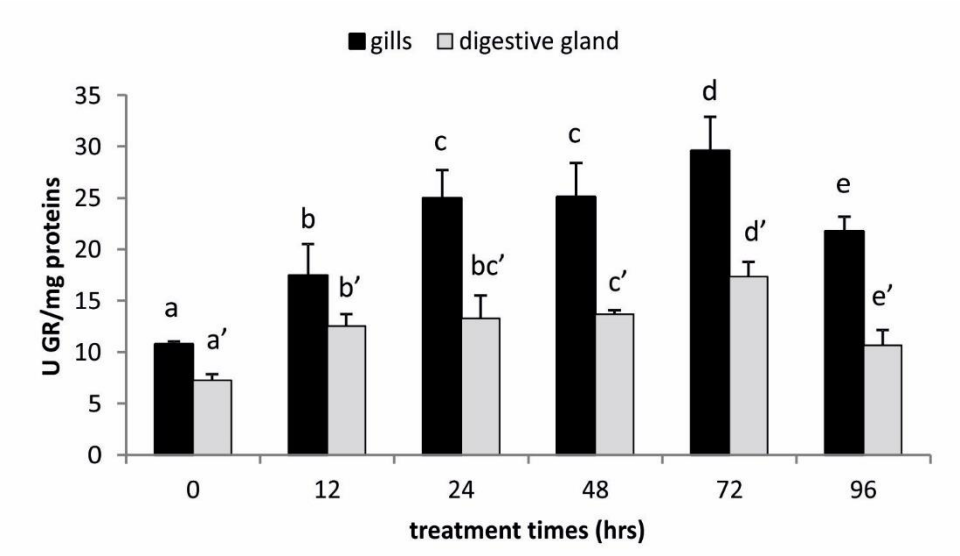

Figure 6. GR activity U GR/mg total protein in gills and digestive glands of control (0 h) and treated *M. galloprovincialis* experimentally exposed to Cd for up to 96 h. Values are indicated as mean ± SD. Different letters correspond to significant statistical differences ($p < 0.05$) among different treatment times. n for each time = 10. n control = 30.

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In digestive glands, the GR activity trend associated with exposure times was like the one observed in gills. After 12 h exposure, GR activity almost doubled the control value ($p < 0.001$). Then it slowly but gradually increased to a maximum value of about 17 UGR after 72 h. After 96 h, the value of GR activity was still higher than in controls ($p < 0.001$), but lower than that measured at any other exposure time.

3.6. MDA levels

MDA levels increased in both tissues after Cd exposure (Figure 7). MDA levels in gills were at least double those of digestive glands ($p < 0.001$).

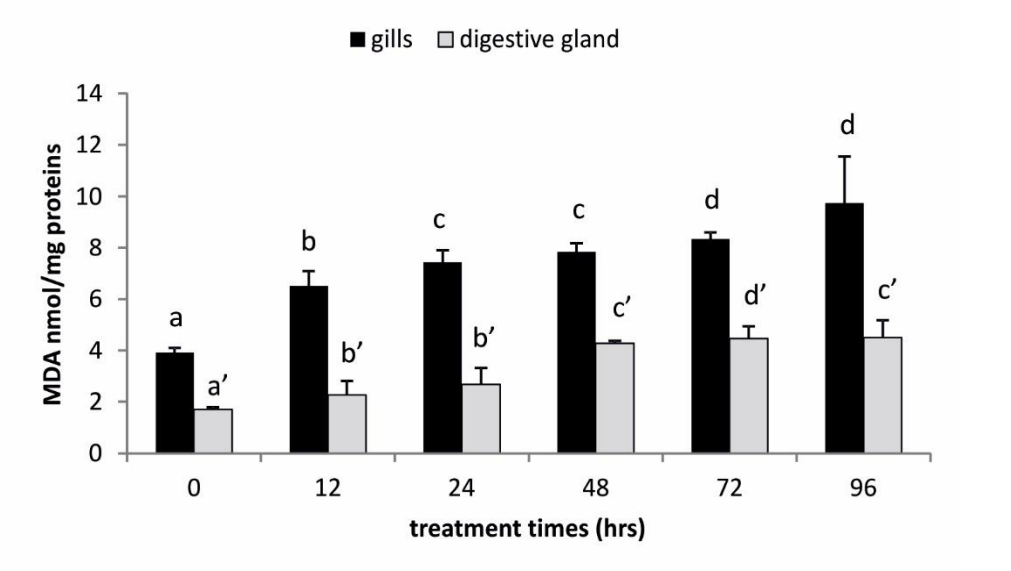

Figure 7. MDA content (nmol/mg total protein) in gills and digestive glands of control (0 h) and treated *M. galloprovincialis* experimentally exposed to Cd for up to 96h. Values are indicated as mean ± SD. Different letters correspond to significant statistical differences ($p < 0.05$) among different treatment times. n for each time = 10. n control = 30.

In gills, MDA content more than doubled after Cd exposure. The increase was not significantly different between 24 and 48 h and between 72 and 96 h. The highest MDA level was reached at 96 h ($p < 0.001$) with a value of about 10 nmol/mg total proteins.

In digestive glands, MDA content increased almost threefold after Cd exposure. However, the increase was not steady - between 12 and 24 h there was no significant difference, for instance - after 48 h there was a further rise, and then the level remained substantially unchanged in the next 48h. Comparison with controls revealed that the highest MDA level was reached at the end of the experiment, with a value of 4.50 nmol/mg total protein ($p < 0.001$).

3.7. Correlation analyses

Correlations between Cd concentrations and the tested biomarkers for both digestive glands and gills were assessed. The results are shown in Table 1.

Table 1. Correlation coefficients between the variables tested in gills and digestive gland of mussels exposed to Cd

	Cd (total)	Cd (cell-free extract)	MT	GSH	GR	MDA
gills						

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Cd (tot)	1.000		0.686	****	0.753	****	0.848	****	0.798	****
Cd (cfe)		1.000	0.745	****	0.696	****	0.518	***	0.551	****
MT			1.000		0.707	****	0.548	****	0.752	****
GSH					1.000		0.776	****	0.604	****
GR							1.000		0.728	****
MDA									1.000	
digestive gland										
Cd (tot)	1.000		0.944	****	0.571	***	0.612	****	0.652	****
Cd (cfe)		1.000	0.926	****	0.561	****	0.510	***	0.737	****
MT			1.000		0.276	*	0.335	**	0.677	****
GSH					1.000		0.570	****	0.367	**
GR							1.000		0.463	***
MDA									1.000	

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$

Cd accumulation always resulted positively and significantly (at least $p < 0.01$) associated with all biomarkers.

Correlation analyses between all the different biomarkers were positive and significant (at least $p < 0.05$) both in gills and digestive glands.

4. Discussion

This study aims to investigate the antioxidant physiological responses of mussels to oxidative stress risk induced by short-term sublethal Cd exposure, in relation to the tissue expression of some molecules and proteins involved in non-enzymatic ROS scavenging; these parameters have been associated with lipid peroxidation as a cell damage index.

After a 96-h exposure period, none of the specimens died; despite this, however, mortality rates were relevant in hemolymph cells (up to 48% at the end of exposure) (Fig 1). Hemocytes are the main components of bivalve immune system and represent the main defense mechanism against xenobiotics [36,37,52]. The presence of foreign materials stimulates their phagocytic activity. In that way they carry out important immune functions even against alterations induced by toxic metals such as Cd that they can accumulate in the endolysosomal system [38]. So it is believable that, in front of high, but not lethal to the organism, Cd exposures, the immune system activates a sudden full response with hemocytes assuming the metal. In that way the cells are subjected to severe alterations then die and are eliminated from the organisms together with their contaminant [38-40]. These results are consistent with other literature data [39,43] and could explain even, at least in part, the decrease in Cd accumulation rate in gills and digestive gland during the treatment.

A conspicuous Cd content increase during the exposure was observed both in gills and in digestive gland. The two organs displayed also similar Bioconcentration Factors with the metal reaching quite similar final levels in both at the end of the experiment. However, a markedly different behavior in subcellular Cd partition between the insoluble and the soluble fractions was observed in the two organs during the exposure time. In digestive gland, Cd partitioning remained in favor of the soluble fraction accounting for about the 60% of the total Cd accumulated. At the contrary in the gill most of the metal, up to 95%, was initially recovered in the insoluble fraction at 12 and 24 hours, but at 48 hours the Cd partition, changed in favor of the cell free fraction.

Many studies have focused on the subcellular partition of Cd in bivalves. However, the subcellular distribution of the metal seems to behave in different ways depending on the species, the life history of the organism, the exposure level of the metal, as reported in the venerid clam *Ruditapes philippinarum* [53], in marine bivalves such as oysters and clams [54], in the scallop *Chlamys varia* [55], in the clam *Mya arenaria*, in mussel [56] and in the Antarctic bivalve *Laternula elliptica* [57].

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The Cd subcellular partition shift that we observed in the gill after 24 hours is possibly linked to the MT synthesis activated, to obtain a Cd detoxification. This hypothesis is supported by several studies showing that Cd scavenging and storage is a dynamic process [58-61] and that when the metal influx rate is higher more Cd is present in the soluble fraction, associated with the MTLP Metallothionein Like Proteins [61]. A continuous induction of MTs was observed in the two organs as a consequence of Cd exposure.

The different Cd partitioning found between the insoluble and cell-free fractions in the two organs could be related with the different times of MTs biosynthesis in gills and digestive glands. In digestive glands, where MT was early synthesized, Cd partitioning was always in favor of cell-free fraction. In gills, on the contrary, most (up to 95%) of the metal was recovered in the insoluble fraction up to 24 h. In this condition, it is very likely that most of the metal present in this tissue has not yet entered into cells but is simply adsorbed to the cell surface, thus not being able to interact with the molecular systems that induce a significant increase in the biosynthesis of new MTs. A sudden Cd shift towards cell-free fraction occurred starting at 24 h, at the same time as MT induction.

The increase in MTs is a typical defense response to metal exposure in almost all organisms [62-64]. The detoxification role of MT in *Mytilus* against the threat caused by Cd is confirmed by the highly significant correlation between MT and Cd levels in both the metal target organs. MT function in Cd detoxification is primarily carried out through the high affinity binding the metal to the protein, thus sequestering Cd and making the metal bio-unavailable, i.e., unable to interact with cell macromolecules. Other proposed functions of MTs, such as maintaining essential metal (e.g. Zn) homeostasis, scavenging ROS, regulating gene expression and tissue regeneration [65], could all contribute towards protection against Cd toxicity.

MTs could also function as non-enzymatic antioxidant defense and as amplification systems of reducing power through the induction of thionein and/or GSH. In fact, MT oxidation leads to Zn release, implementing first the Zn-dependent induction of the genes encoding MTs (MTF-1), and then glutamyl cysteine synthetase [66], which is the first enzyme involved in GSH biosynthesis.

Indeed, after gills and digestive glands were exposed to Cd, GSH levels are varied, albeit with different behavior. Actually, an initial and temporary decrease was observed in digestive glands. This early GSH content decrease could have different causes, all dependent on increased ROS production such as: a) GSH oxidation and GSSG depletion, b) reduction of GSH biosynthesis in favor of MT induction, and c) (even partial) inactivation of the enzymes involved in GSH synthesis. The second hypothesis is not coherent with the previously hypothesized MT amplification system in response to Cd exposure. The third hypothesis is associated with Cd toxicity, though the temporary nature of this phenomenon makes this hypothesis unlikely. Moreover, it is known that Cd is one of the major inducers of γ-glutamyl-cysteine ligase, the enzyme that catalyze of GSH biosynthesis [67].

The first hypothesis is the most probable and could indicate a GSSG depletion effect as a mechanism to keep the GSH/GSSG ratio (an index of cell redox status) constant. It is well known that a decrease in this ratio leads to oxidative stress conditions [68]. GSSG depletion is a well-known mechanism based on membrane transport in which the Multidrug Resistance Protein 1 (MRP1) acts [69]. This phenomenon is more evident in digestive glands probably because of their higher metabolism, higher oxygen consumption and ROS formation than gills. The rapid increment of GSH content that occurred at 48 h perhaps shows an initial toxic effect on the enzymes of the antioxidant system, which gradually become ineffective in fighting increased ROS production.

The GSH drop observed in digestive glands at 96 h could be associated with a further increase of ROS formation, not completely scavenged by antioxidant defense mechanisms, and the concomitant increase of cell structure damage, as suggested by the rise in MDA content.

MDA is the main degradation product of lipid peroxidation, and its levels show the extent of oxidative cell damage [70], perhaps caused by Cd-induced hydroxyl radicals, as suggested by the positive correlation between MDA values and Cd concentrations. These data provide strong evidence that ROS oxidative damage is produced despite the activation of the antioxidant defense system. In particular, in the experimental conditions to which mussels were subjected in the present study, MT

and GSH contents were insufficient in preventing ROS damage, probably as a result of the concurrent inactivation of antioxidant enzymes such as GR, which is one of the first lines of defense against ROS.

GR recycles reduced GSH from its oxidized form, which is essential for the maintenance of cellular homeostasis and the scavenging of nucleophilic compounds [71]. GR induction, because of Cd treatment, was observed in various organisms and in different conditions. GR activity in the freshwater mollusc *Dreissena polymorpha* increased selectively by Cd exposure and not by other pollutants [72]. Because of Cd exposure, the hepatic transcript level of the GR gene in the fish *Takifugu obscurus* showed the highest expression in all antioxidant enzymes. Incidentally, the GR gene expression pathway was very similar to the behavior of GR activity levels observed in this study [73]. Here, during Cd treatment, GR activity had a very similar time evolution both in gills and in digestive glands, with the highest values at 72 h of exposure followed by a partial decline at 96 h. The GR decline at the end of the experiment could probably be associated with the toxic ROS effect on antioxidant enzymes as hypothesized above. From this point of view, it would be interesting to verify if any similar activity reduction is also found in other enzymes that are known to play an important role in the antioxidant defense system, such as superoxide dismutases, catalase, glutathione peroxidases and peroxiredoxins. The expression and the activity of these enzymes are functional correlated in organisms, as demonstrated in both physiological conditions and during environmental perturbations [74-83], representing a complex defense system whose effective regulation is still being studied [84-96].

However, the obtained data show that, although the non-enzymatic antioxidant defenses not play by itself a protective role that prevents lipid peroxidation, they certainly limit it. In fact, although the increase in Cd accumulation is conspicuous between 12 h and 96 h (about 170% in the gills and 340% in the digestive gland), the increase in lipid peroxidation, although present, is much more limited (about 50 % in the gills and 80% in the digestive gland).

Based on the collected results and on the considerations previously made, we can hypothesize the following theoretical framework that explains the mechanisms of the observed phenomena, different but partly superimposable for the two considered organs.

In the digestive gland, Cd enters the cytoplasm early, probably increasing the formation of ROS, which have an oxidizing effect against biological macromolecules, as shown by the MDA data. This negative effect is initially limited by GSH which is oxidized to GSSG: part of this is reduced to GSH by GR, of which we see increased tissue activity; part is instead eliminated from the cell leading to a reduction in total GSH levels at 12 h. Subsequently, the induction of MTs has a protective role against the oxidation of GSH and cellular macromolecules, and in fact the levels of MDA stabilize, as well as those of GR. However, Cd continues to enter the intracellular environment, further implementing the induction of MTs and also the biosynthesis of GSH, a response that is however insufficient to avoid oxidative damage, at least in this phase. The ex-novo biosynthesis of GSH compensates the oxidation of this tripeptide by ROS produced by the presence of Cd, and therefore limits the need for an increase in the GR activity, which begins to rise only at 72 h, when a limit is reached in the increasing of GSH biosynthesis. At 96 h the situation stabilized, as the oxidative damage to biological macromolecules is under control, especially thanks to the high presence of MTs. This severely limits the need for high cellular levels of GSH which in fact drop, probably in relation to the stop of GSH biosynthesis and the elimination of GSSG (also the GR activity decreases).

In the gills, the Cd begins to enter consistently in the intracellular environment only at 48 h, and therefore the initial oxidation of membrane lipids is attributable to the ROS that are formed at the level of membrane itself, where the Cd adsorption takes place. In this first phase, GSH rather than MTs plays a central role in facing the risk of oxidative stress and this is due both to the ex-novo biosynthesis of the tripeptide, and to the reduction of GSSG by GR. Subsequently, when the Cd begins to accumulate in the intracellular environment, an induction of MTs occurs, which makes an increase in GSH biosynthesis unnecessary, protecting this molecule from oxidation, with a consequent limited induction of GR. At 96 h, the situation is under control, similarly to what happens in digestive gland, with a stabilization of the risk of oxidative stress especially thanks to the high levels of MTs.

 doi:10.20944/preprints202007.0235.v1

It goes without saying that the proposed model is partially speculative, as a finer reconstruction would demand a considerably larger number of analyses; nevertheless, we believe that, even in the present form, our work can represent a relevant contribution to the field, providing new data about the relationships among the non-enzymatic antioxidant cellular components considered in this study. These constitute the prompt physiological responses to the risk of oxidative stress in blue mussels exposed to Cd in controlled laboratory conditions.

The main importance of this work is the new information on correlation of several physiological parameters related to both metal sequestration and defense against metal-induced oxidative stress. This picture, although partial, represents an example of how biosynthesis of small molecules, such as GSH, and expression of proteins such as MTs and GR, put in place an integrated response against the toxic effect of a not essential metal, such as Cd, with reference to ROS production. In this respect our results highlighted the double function of GSH and MTs, both as chelating molecules and ROS scavengers. The peculiar features of the experimental conditions show, however, that under heavy Cd exposure the integrity of this defense system becomes compromised, increasing the sensitivity of *M. galloprovincialis* to the oxidative stress.

Funding: The work was supported by a grant from the Italian MIUR (DOR2018) to P.I. and G.S.

Acknowledgments: We thank Luigi Pivotti for his support in the discussion of statistical analyses, Franco Cattalini for lab support and metal analyses, Vito Sabia and Andrea Sambo of the zoological station of the Department of Biology (Chioggia) for their help in retrieving mussels and maintaining the station.

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

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