Oxidative Stress Response in Gill and Liver of *Liza saliens*, from the Esmoriz-Paramos Coastal Lagoon, Portugal

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Abstract Tissue-specific responses against oxidative stress and lipid peroxidation were analyzed in wild adult mullet (*Liza saliens*) caught in the Portuguese coastal lagoon Esmoriz-Paramos. Parameters measured were catalase (CAT), superoxide dismutase (SOD), and glutathione-*S*-transferase (GST) activities in liver and gill tissues and lipid peroxidation. The enzyme activities were related to gill histopathological alterations, as well as to heavy metals (Cu and Zn) concentrations in these tissues. Gill epithelium of *L. saliens* showed histological alterations, such as epithelial hyperplasia resulting in lamellar fusion, epithelial lifting, vasodilatation, and lamellar aneurisms, with a prevalence ranging from 62% to 92%. The highest Cu content was found in liver (379 mg·kg^{-1}), while the highest Zn content was observed in gill (119 mg·kg^{-1}). SOD and CAT activities showed differences between gill and liver. The highest activities found were SOD in gill (10.1 U/mg protein) and CAT in liver (39.2 mmol/min/mg protein). In gill, CAT activity was negatively related to both Cu levels and gill lifting, while a positive relationship was found between SOD activity and fish age. The positive relationship between Cu and CAT activity in liver suggests that an increase in metabolic level is related to Cu-induced oxidative stress. The decrease in gill CAT activity can be due to osmotic stress caused by damaged gill epithelium. CAT activity in liver is an appropriate biomarker of oxidative stress in the Esmoriz-Paramos lagoon.

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The effects of pollutants in fish can be properly evaluated if bioaccumulation is complemented with other biomarkers. Contaminants usually appear in the environment as very complex mixtures that can cause interactive effects, thus biomarkers offer an integrated measurement of these effects (Orbea et al. 2002; Ferreira et al. 2005). Among pollutants that can accumulate in fish, heavy metals are of great interest because they could trigger oxidative stress in fish (Bláha et al. 2004; Deviller et al. 2005), by reactive oxygen species (ROS) generation (Durmaz et al. 2006; Lesser 2006).

Several studies revealed that exposure to contaminants in aquatic ecosystems can enhance intracellular formation of ROS, which could cause oxidative damage to biological systems (Livingstone 2003; Ferreira et al. 2005). ROS can be detoxified by an enzymatic defense system, which includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Halliwell and Gutteridge 1989). SOD is the enzyme that catalyses dismutation of the superoxide anion to O_2 and H_2O_2, and CAT reacts with H_2O_2 to form water and molecular oxygen (Livingstone

2001; Lushchak et al. 2001; Ozmen et al. 2004). Gluta-thione-*S*-transferases form a family of multifunctional phase II biotransformation enzymes, present in cytosol of most cells, that catalyze the conjugation of glutathione to a variety of compounds (Livingstone 2003).

Recent data indicate that changes in the levels of anti-oxidant enzyme activities can be used as contamination biomarkers in different aquatic organisms (Livingstone 2003; Regoli et al. 2004). Lipid peroxidation is one of the main manifestations of oxidative damage induced by var-ious compounds, including metals (Ercal et al. 2001; Livingstone 2003), and it has also been used as a biomarker of pollution (Sayeed et al. 2003; Bláha et al. 2004; Alm-roth et al. 2005).

The Esmoriz-Paramos is a coastal lagoon on the Northwest coast of Portugal, which receives untreated industrial and domestic sewage that promotes a decline in water and sediment quality and a decrease in biodiversity. Heavy metals can be up-taken by fish from water, sedi-ments, and suspended particulate material (Hardersen and Wratten 1998) and have been an important source of contamination in this ecosystem.

The most used vertebrate model in ecotoxicological studies is fish, which allows the evaluation of antioxidant responses in tissues and the hepatic oxidative damage caused by metal contamination (Orbea et al. 2002). The liver plays a primary role in the metabolism of xenobiotic compounds and it is a detoxification organ essential for excretion of toxic substances in fish (Hinton and Laurén 1990; Figueiredo-Fernandes et al. 2006a). Gill is the osmoregulatory surface tissue and it is the primary site of uptake of waterborne pollutants (McDonald and Wood 1993; Monteiro et al. 2005). The leaping grey mullet (*Liza saliens*) is the dominant species in the lagoon that comes into contact with water and sediment pollutants, through its detritus feeding behavior. Previous studies have shown Cu and Zn bioaccumulation in liver and gill of *L. saliens* from the lagoon and an increase in Zn in gill and Cu in both tissues that were age dependent (Fernandes et al. 2007a).

The aim of the present study was to determine the antioxidant enzyme activities in liver and gill of *L. saliens* from the Esmoriz-Paramos coastal lagoon and lipid per-oxidation as a measurement of liver oxidative damage. Relationships among enzyme activities, gill histopatholo-gical alterations, and metal levels were also investigated.

Materials and Methods

Study Area

The Esmoriz-Paramos lagoon, 1500 m long (N–S), 700 m wide (W–E), and 2.5 m in maximum depth, with a catchment area of 78 km^2, is located on the northwest coast of Portugal (Almeida 1998). It receives two main water inflows, one from the north and another from the south, and occasional effluents from local housing and small industry. The lagoon communication with the sea is established through a nonpermanent channel with small dimensions, particularly on the shoreline. Major inputs of contaminants into the lagoon are industrial or municipal, mostly untreated sewage from its tributaries and runoff from contaminated soils and surfaces (SIMRIA 2002).

Fish Sampling

Liza saliens were collected during April 2004 in the Esmoriz-Paramos lagoon, using a gill net. Mullets from the sea, 14 km northward from the lagoon, were also caught during the same period, and CAT liver and gill activities were measured and compared with equivalent activities in lagoon mullets. Fish were anesthetized, and gill and liver samples were removed, frozen in liquid nitrogen, and stored at –80°C, until biochemical assay. Gill samples were also randomly taken for histopathological examination and metal analyses. Fish age was determined by reading the annual ring structure of scales removed from the opercular region (Muir and Den Haas 2003).

Biochemical Analysis

Livers were homogenized in ice-cold 50 mM sodium phosphate buffer, 0.1 mM Na$_2$EDTA, pH 7.8. Gills were homogenized in ice-cold 50 mM imidazol buffer, 150 mM sucrose, 10 mM Na$_2$EDTA, pH 7.3 (homogenizer T 1500; Ystral GmH). Mitochondrial fractions were obtained after centrifugation at 15,000 g for 20 min at 4°C. SOD (EC 1.15.1.1) activity was determined by an indirect method involving the inhibition of cytochrome c reduction and spectrophotometric reading at 550 nm (McCord and Fridovich 1969). The concentrations of the reactives were 50 mM buffer, pH 7.8, 50 μM hypoxanthine, 1.98 mU/mL xanthine oxidase, and 10 μM cytochrome c (Ferreira et al. 2005). Enzyme activity is expressed as units per milligram of protein (U/mg pr), where 1 U corresponds to 50% inhibition of the xantine oxidase reaction.

GST (EC. 2.5.1.18) activity was determined according to Habig et al. (1974) adapted to microplate by Frasco and Guilhermino (2002). Concentrations of the reactive were as follows: glutathione (GSH), 10 mM in 0.1 M buffer, pH 6.5, and 1-chloro-2,4-dinitrobenzene (CDNB), 60 mM in ethanol, prepared just before the assay. The reaction mix-ture was in proportions of 4.95 mL (buffer):0.9 mL (GSH solution):0.15 mL (CDNB solution) (Ferreira et al. 2005).

GST activity was measured every 20 s in a spectrophotometer at 340 nm during the first 5 min and quantified using the period of linear change in absorbance. Enzyme activity is expressed as nanomoles per minute per milligram of protein.

CAT (EC 1.11.1.6) activity was determined by measuring the consumption of H_2O_2 monitored spectrophotometrically at 240 nm, according to Aebi (1974). The reaction volume was 1 mL and contained 67.5 mM potassium phosphate buffer, pH 7.5, and 12.5 mM H_2O_2 (Ferreira et al. 2005). CAT activity is expressed as millimoles of decomposed hydrogen peroxide per minute per milligram of protein.

Oxidative Damage

The peroxidative damage of lipids creates free radicals that result in malondialdehyde (MDA) production, which was assessed by the thiobarbituric acid method (TBARS) adapted to microplate (Ferreira et al. 2005). Absorbance was measured at 532 nm and the concentration of MDA is expressed as nanomoles of MDA per gram of liver. Total protein was measured by the Lowry method adapted to microplate (Ferreira et al. 2005).

Tissue Metal Content

Gill soft tissue was lyophilized and digested overnight with nitric acid (superpure grade) at 60°C. The digested samples were analyzed in a graphite furnace atomic absorption spectrometer (UNICAMP 939 AA-GF90). Blank determinations were done using the same procedure with Milli-Q50 water. Results are expressed as milligrams per kilogram dry weight. Analytical accuracy and precision were checked using certified reference materials, i.e., DOLT-3 and DORM-2 (National Research Council of Canada).

Light Microscopy

Gill tissue previously fixed in buffered formalin (10%) fluid for 48 h was dehydrated in graded ethanol concentrations and embedded in paraffin wax. Sagittal sections (5 μm thick) were stained with hematoxylin/eosin (H&E). Changes observed in gill tissue were analyzed under a Nikon light microscope.

A score system was used to rank the severity and extension of the gill lesions, according to Fernandes et al. (2007b). The severity of the lesions was scored as follows: 0 = no pathological alterations, 1 = focal mild pathological alterations, 2 = moderate pathological alterations, 3 = severe pathological alterations. The extent of affected lamellae was scored as follows: 0 = 0%, 1 = ≤ 10%, 2 = 11%–49%, 3 = 50%–69%, 4 = ≥70%. For each fish and lesion, the product severity × extension was calculated to establish an assessment value per gill filament varying between 1 and 12.

Statistical Analysis

Data are presented as mean ± standard deviation. Statistical calculations were performed with SPSS software. Differences among metals in tissues, enzymatic activities, and assessment values of gill lesions were tested using the Mann-Whitney U-test and the relationships between them were tested with Spearman correlations. A 5% significance level was employed throughout.

Results

The ranges of age and length of fish collected from the lagoon were 7 to 13 years and 25 to 49 cm, respectively. Gill lesion prevalence and gill histopathological assessment are presented in Table 1. Gill epithelium of *L. saliens* showed several histopathologic alterations; 77% of the fish presented three or four lesions. The main lesions observed were vasodilatation and epithelial hyperplasia, occasionally resulting in lamellar fusion, both present in 92% of fish. Epithelial lifting and lamellar capillary aneurisms were observed in 69% and 62% of fish, respectively. These histological alterations were observed at varying degrees of extension and severity. Hyperplasia and vasodilatation were scored as the maximum severity (grade 3), whereas the maximum extent (grade 4) was scored for hyperplasia and lifting. The highest mean assessment value of lesion was found for hyperplasia, followed by lifting, while the lowest was found for aneurisms and vasodilatation.

Gill and liver metal contents in *L. saliens* from the lagoon are summarized in Table 2. The highest Cu content was found in liver ($p < 0.01$), ranging from 125 to 547 mg·kg^{-1}, and the highest Zn content was observed in

Table 1 Lesion prevalence and histopathologic assessment in gill of *Liza saliens* from Esmoriz-Paramos lagoon

Lesion	Prevalence (%)	Lesion assessment[a]
Aneurysm	62	1.23 ± 1.73
Hyperplasia	92	3.17 ± 2.72
Lifting	69	2.37 ± 3.66
Vasodilation	92	1.76 ± 1.54

[a] Mean ± SD

Table 2 Copper and zinc concentrations in liver and gill of *Liza saliens* from Esmoriz-Paramos lagoon

Metal and tissue	mg·kg^{-1} dry wt[a]
Cu	
Liver	378.9 ± 122.5
Gill	9.6 ± 1.6
Zn	
Liver	99.9 ± 30.9
Gill	118.8 ± 26.5

[a] Mean ± SD

gill ($p < 0.05$), ranging from 88 to 134 mg·kg^{-1}. Branchial and hepatic enzyme activities and lipid peroxidation in fish from the lagoon are presented in Table 3. This table also reports gill and liver CAT activities in fish from the sea. Mullets collected in the lagoon showed similar GST activity levels in liver and in gill, a higher CAT activity in liver than in gill ($p < 0.01$), and a higher SOD activity in gill than in liver ($p < 0.01$). Lipid peroxidation and total protein content in liver ranged from 9 to 38 nmol MDA/g and from 90 to 266 mg/g, respectively.

Fish from the sea (22–35 cm long) showed lower liver CAT activity compared with lagoon fish ($p = 0.03$). In contrast, gill CAT activity was higher in fish from the sea.

A negative correlation between gill CAT activity and both copper levels and lifting assessment value was found in fish from the lagoon (Fig. 1). Similar results were found between gill CAT activity and severity of lifting ($r = -0.618$, $p = 0.032$). Also, in gill, a positive correlation was found between SOD and GST activities, as well as between SOD activity and fish age (Fig. 1). Figure 2 shows the two significant positive correlations between liver Cu and CAT activity and between liver Cu and lipid peroxidation. Negative correlations between GST activity and lipid peroxidation and between total liver protein content and fish length (Fig. 2) and fish age ($r = -0.572$, $p = 0.04$) were observed. No significant relationship was observed with Zn.

Table 3 Enzymatic activities, liver lipid peroxidation, and liver protein levels in *Liza saliens* from Esmoriz-Paramos lagoon and the sea

	Parameter	Liver	Gill
Enzyme activity	GST (nmol/min/mg pr)	85.8 ± 33.3	91.4 ± 33.00
	SOD (U/mg pr)	3.8 ± 0.9	10.1 ± 4.3
	CAT (mmol/min/mg pr)		
	Lagoon	39.2 ± 16.6	2.9 ± 2.6
	Sea	[21.6 ± 5.3]	[9.8 ± 4.5]
Lipid peroxidation (nmol MDA/g liver)		18.5 ± 7.5	
Protein (mg/g liver)		146.1 ± 49.7	

Note. pr, protein. Data are presented as mean ± SD

Discussion

Oxidative stress is defined as an adverse reaction resulting from the exposure of molecules, cells, or tissues to excess levels of free radical oxidants, especially ROS (Li et al. 2005; Lesser 2006). ROS produced in biological systems are detoxified by antioxidant defenses. One of the features of these antioxidant enzymes is their induction under conditions of oxidative stress, and such induction can be an important adaptation to pollutant-induced stress (Livingstone 2001). It is generally recognized that ROS production is associated with exposure to several metals (Ercal et al. 2001; Livingstone 2003), which can lead to induction of certain antioxidant enzymes (Ozmen et al. 2004).

This study reveals tissue-specific changes in SOD and CAT activities. However, no significant differences were observed between gill and liver GST activities. Induction of antioxidant enzymes is a common mechanism of adaptive response in fish that vary among tissues (Oruc et al. 2004). CAT activity was high in liver, where the Cu content was high. Paris-Palacios et al. (2000) reported an increase in hepatic CAT activity in fish, *Brachydanio rerio*, exposed to sublethal concentrations of Cu. The hepatic CAT activity in fish from the Esmoriz-Paramos lagoon was higher than in fish from the sea and higher than that reported in *Mugil cephalus* caught in polluted environments (Orbea et al. 2002; Ferreira, et al. 2005). The high CAT activity may be a response to increased H_2O_2 production (Ritola et al. 2002), to protect biological systems against ROS (Romeo et al. 2000). The high hepatic CAT activity found in this work suggests that a metabolic increase was triggered to cope with Cu-induced oxidative stress. In contrast, CAT activity in gill was lower in fish from the lagoon compared with fish from the sea and was negatively correlated with Cu concentrations and gill lifting. The lifting of filamentar and lamellar epithelium constitutes a typical defense mechanism that increases the diffusion distance between blood and waterborne pollutants. Previous studies revealed osmoregulatory disturbances as a consequence of gill permeability and cell integrity changes (Fernandes et al. 2007c) that could have affected CAT activity.

SODs are a group of metalloenzymes that plays a crucial antioxidant role and constitutes a defense system against the natural or chemically induced production of ROS (Roche and Bogé 1996; Livingstone 2001). Several studies using different species exposed to several pollutants showed an increase in SOD activity (Palace et al. 1996; Figueiredo-Fernandes et al. 2006b), namely, when fish were exposed to Cu (Sanchez et al. 2005). The induction of hepatic SOD activity was also described in some studies carried out in field polluted sites (Deviller et al. 2005; Ferreira et al. 2005). Liver SOD activity in

Fig. 1 Relationships between SOD and GST activities (**a**), SOD and fish age (**b**), CAT and gill copper content (**c**), and CAT and gill alterations (**d**) in *Liza saliens* from Esmoriz-Paramos lagoon
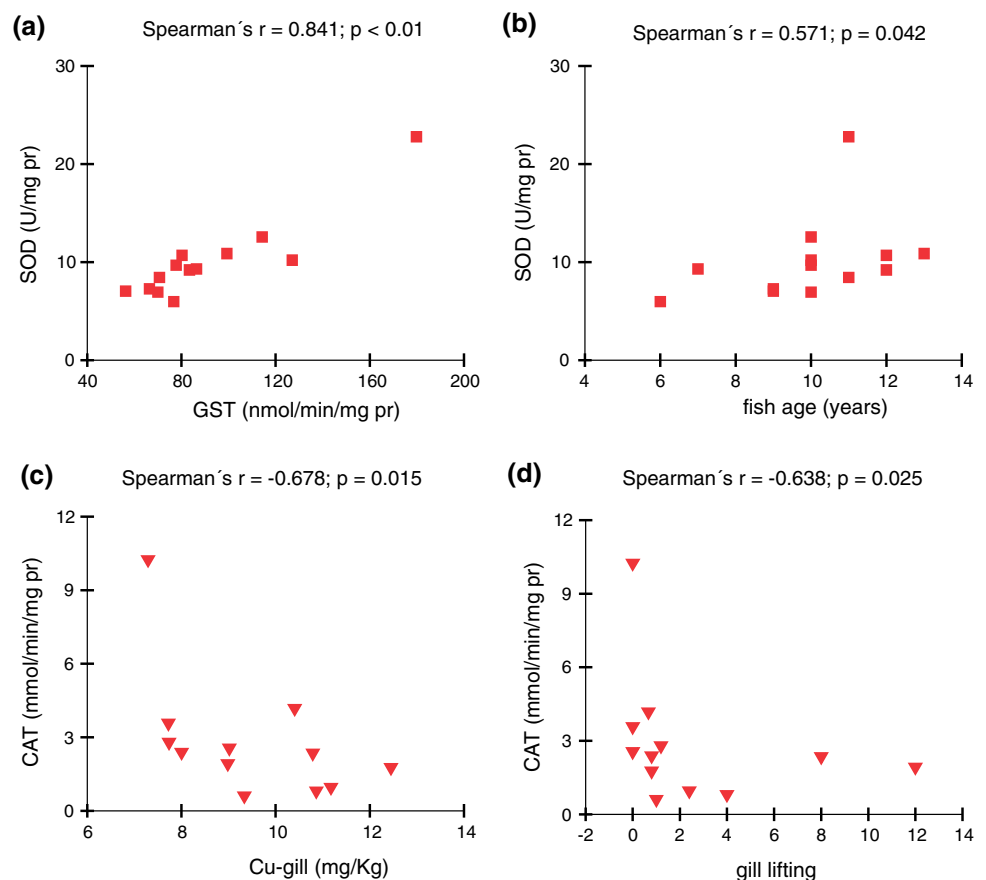

our study was low compared to that in *M. cephalus* caught in polluted environments (Orbea et al. 2002; Ferreira et al. 2005). These results suggest that the decrease in hepatic SOD activity could be due to the high copper content found in livers of fish from the lagoon. Previous experimental studies in *Oreochromis niloticus* also reported decreased SOD activity in liver (Peixoto et al. 2006). The increase in hepatic CAT activity and the decrease in hepatic SOD activity agreed with the results observed in *Geophagus brasiliensis* caught in a polluted area (Filho et al. 2001). Porte et al. (2002) also reported an increase in CAT activity and that there is no relationship between pollutants and hepatic SOD activity in wild mullet, *Mullus barbatus*.

A positive relationship between heavy metals (Cu and Zn) in gill and fish age in *L. saliens* caught in the Esmoriz-Paramos lagoon was demonstrated in a previous study (Fernandes et al. 2007a). An increase in SOD gill activity with fish age was found in the present work, and the higher SOD activity in gill than in liver could be an indicator of compensatory tissue response to face metal exposure. Oruc et al. (2004) also showed higher SOD activity in gill compared with other tissues as a result of pollutant exposure.

The conjugation of phase I metabolites with GSH is catalyzed by GST, one of the most widely studied conjugation enzymes in vertebrates. GST is involved in detoxification and excretion of foreign compounds and it may also show peroxidase activity (Paris-Palacios et al. 2000; Chung et al. 2004). This might explain the negative relationship between hepatic GST activity and lipid peroxidation found in the present study. The effects of pollutants on GST activity have been somewhat inconclusive, showing induction, no change, or inhibition of this enzyme (Stephensen et al. 2000). Some studies showed that exposure to pollutants can lead to an increase of hepatic GST activity (Sen and Kirikbakan 2004; Camargo and Martinez 2006), whereas others reported no variation (Porte et al. 2002) with respect to copper (Sanchez et al. 2005) and even decreased activity (Filho et al. 2001). Martinez-Lara et al. (1996) found a decrease in GST activity in gilthead seabream (*Sparus aurata*) exposed to pollutants and suggested that these results could be due to inactivation by ROS generated by pollutants. The values of hepatic GST activity of *L. saliens* caught in a reference site in Turkey (Sen and Kirikbakan 2004) are similar to those found in the present work. In addition, hepatic GST activities in *M. cephalus* collected

Fig. 2 Relationships between hepatic CAT and liver Cu (**a**), lipid peroxidation and GST activity (**b**), lipid peroxidation and liver Cu (**c**), and liver protein and fish length (**d**) in *Liza saliens* from Esmoriz-Paramos lagoon
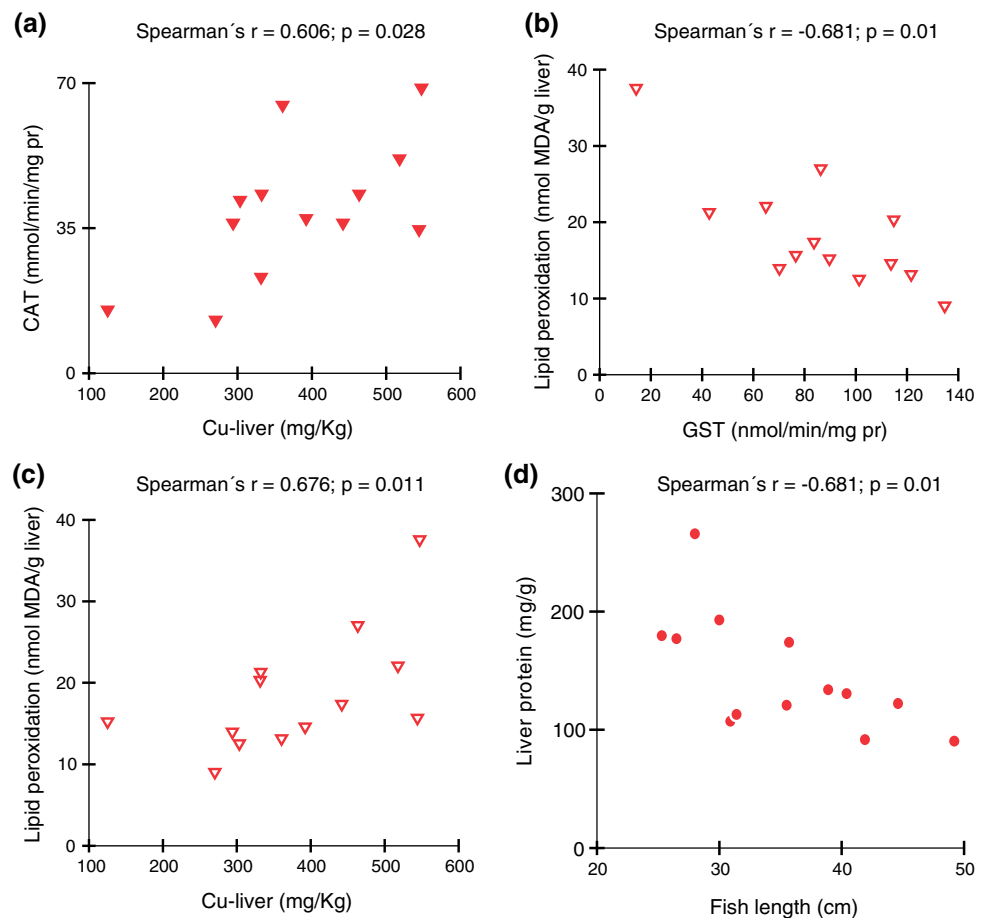
in a polluted site (Ferreira et al. 2006) were high compared to our data. A positive relationship between gill SOD and gill GST activities was found, which may reflect a reinforced response against oxidative stress in this organ.

No relationship between Zn content and oxidative stress enzymes activities was found in gill or liver of *L. saliens*. This could be related to the fact that Zn is apparently regulated in liver and its increase in gill over time is kept within a range (Fernandes et al. 2007a).

Malondialdehyde (MDA) production is a well-known oxidation product of polyunsaturated fatty acids, influencing cell membrane fluidity as well as the integrity of biomolecules (Ercal et al. 2001; Almroth et al. 2005), and is an important indicator of lipid peroxidation (Freeman and Crapo 1981). The present study revealed a positive relationship between lipid peroxidation measured as MDA and Cu liver content. The induction of hepatic lipid peroxidation caused by chronic dietary exposure to Cu was confirmed in grey mullet *Chelon labrosus* (Baker et al. 1998). Our results suggest that antioxidant enzymes were not able to prevent the hepatic lipid peroxidation induced by chronic metal exposure.

The decrease in hepatic total protein was related to fish age and fish length, suggesting that it is age dependent. These data can be related to a metabolic activity reduction in older fish, as well as protein degradation due to ROS production.

In conclusion, the present study revealed that fish developed tissue-specific enzyme responses, such as increase in CAT activity in liver and SOD activity in gill, to cope with pollution exposure. CAT activity in liver is an appropriate biomarker of oxidative stress against copper exposure in the Esmoriz/Paramos lagoon. The fact that *L. saliens* lives in the lagoon for its entire life span enabled long-term evaluation of the stress responses, which are more realistic than acute laboratory toxicity tests. Furthermore, data obtained in this study may be useful to compare biomarker fish responses from other polluted sites.

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