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Chronic Effects of Realistic Concentrations of Non-essential and Essential Metals (Lead and Zinc) on Oxidative Stress Biomarkers of the Mosquitofish, *Gambusia holbrooki*

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Abstract Metallic contamination is widespread, particularly in areas impacted by human activities. Human activities result in high loads of metals being discarded into the aquatic compartment, reinforcing the need to evaluate their toxic effects especially on exposed fish. The purpose of this study was to determine the toxic response (namely, antioxidant levels and lipoperoxidative damage) in both liver and gills of the freshwater fish species *Gambusia holbrooki*, exposed to lead and zinc. Fish were exposed for 28 days (chronic exposure) to ecologically relevant concentrations of the selected compounds. The following oxidative stress/damage biomarkers were evaluated: glutathione-*S*-transferases (GSTs), glutathione reductase (GR), and thiobarbituric acid reactive substances (TBARS). The results indicate that lead caused a significant oxidative response, with significant increase of the enzymatic antioxidant defense (GSTs activity in hepatic tissue, and GR activity in branchial tissue) of exposed organisms. On the other hand, zinc caused a significant inhibition of *G. holbrooki* hepatic GR, a biological response that may be related to the antioxidant activity exhibited by this metal. The obtained results are of high importance, especially if one considers that the obtained toxic responses occurred at low, albeit ecologically relevant, levels of exposure.

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Metal-containing waste (from domestic, agricultural and industrial sources) that is continuously released can have significant impacts on the environment, particularly aquatic ecosystems (López-Galindo et al. 2010). In recent years, the effects of metallic pollution in fish were demonstrated, showing that these organisms are able to bioaccumulate these pollutants (Greco et al. 2010). In addition, metals can induce severe alterations, including an oxidative stress response in various cell types, and also significant alterations of the elimination profile of foreign molecules from the body (Wang et al. 2009; Johnston et al. 2010). Thus, oxidative stress biomarkers, and others (especially those implicated in the metabolism of toxic molecules), have become an important assessment tool in aquatic toxicology (Livingstone 2001; Wang et al. 2009; Jin et al. 2010; Johnston et al. 2010) to monitor the occurrence of pollutants, and deleterious effects potentially exerted in aquatic organisms.

The use of molecular oxygen in normal respiratory processes in mitochondria results in the physiological production and release of reactive oxygen species (ROS; Chance et al. 1979; Wallace 1999); exposure to xenobiotics (including metals) can increase the production of ROS through several mechanisms, such as interference in the electron transport within the mitochondrial membrane and subsequent accumulation of reactive intermediates (Herrero et al. 2008). This may result in cellular damage, namely by inactivation of antioxidant enzymes, depletion of nonenzymatic antioxidants, and membrane lipid peroxidation (Modesto and Martinez 2010). The review by Franco et al. (2009) referenced that some of the most

common environmental contaminants, including several metallic species (e.g., iron, copper, chromium, cobalt, vanadium, cadmium, arsenic, nickel), could trigger apoptosis through the interference with regulating cellular mechanisms. Metals are known to interfere at several subcellular levels, such as the mitochondria (oxidation of mitochondrial RNA, and activation of the intrinsic apoptotic pathway), the endoplasmatic reticulum, and nuclear DNA. Moreover, established oxidative imbalance can cause irreversible oxidative damage in DNA and other macromolecules, or even death of organisms (Jin et al. 2010; Li et al. 2010; Modesto and Martinez 2010). ROS are removed or inactivated by antioxidant defenses, and the balance between these radicals and the antioxidant defense of living organisms is fundamental for their protection against oxidative stress and its deleterious consequences (Li et al. 2010; Modesto and Martinez 2010). Nevertheless, the exposure to chemical pollutants also may contribute to oxidative stress, by altering this equilibrium, and consequently inducing a decrease in the antioxidant defense system efficiency (Solé et al. 1996; Livingstone 2001).

Fish cope with the harmful effects of oxidative stress through adaptive responses, namely by increased activity of enzymes involved in the biotransformation and metabolism of a wide range of environmental contaminants and their metabolites (Ognjanovic et al. 2008; Modesto and Martinez 2010). The antioxidant defense system of the majority of organisms is composed by a multitude of enzymes, among which it is possible to identify glutathione reductase (GR), and glutathione-S-transferases (GSTs). These are the most frequently studied biomarkers of oxidative stress in fish (Jin et al. 2010; Modesto and Martinez 2010; Pereira et al. 2010). Lipoperoxidation (LPO) estimation also has been found to have a high value as a biomarker of toxic effects, because this parameter reflects the onset of cellular damage, as a result of oxidation of membrane lipids (Ognjanovic et al. 2008; Pereira et al. 2010).

Amongst all compounds present in the environment, in growing amounts and with evident human origin, metals are particularly important. Metals are widely dispersed in the aquatic environment, being released by anthropogenic activities (mining, release of domestic products into sewage; plumbing degradation; emissions from nuclear plants, from smelters and from burning fossil fuels) but also by natural sources (e.g., volcanoes) (Hozhina et al. 2001; Thompson et al. 2005; Rose and Shea 2007; Connan and Tack 2010; Aktar et al. 2011). Metallic species are fundamental for life, being part of macromolecules and enzymes (Liu and Thiele 1997). However, metals also are prone to establish redox cycles if in the presence of molecular oxygen, giving rise to the production of ROS

(Herrero et al. 2008). Consequently, metals are dual in their effects: albeit vital, they are eminently toxic (Liu and Thiele 1997).

Metals (especially transition metals) are toxic and capable of exerting important deleterious effects of oxidative nature in exposed organisms, as reviewed by Valavanidis et al. (2006). Metals, such as arsenic, cadmium, lead, mercury, chromium, nickel, manganese, and iron, especially in their waterborne form, can indeed increase the production of reactive oxygen species (Jadhav et al. 2007). However, exposure to metals conducing to oxidative stress is commonly followed by a set of immediate physiological adaptations (e.g., through antioxidant defenses) to prevent their adverse effects. For example, the study conducted by Grinevicius et al. (2009) on textile effluents rich in metallic species evidenced antioxidant responses in the freshwater fish *Danio rerio*. Despite the activation of antioxidant mechanisms following chemical insults by metals, damage (e.g., lipid peroxidation) is likely to occur, as reported by Siddique et al. (2008) after exposing *Drosophila melanogaster* to metals present in tannery effluents. Mining effluents also were proven to induce oxidative stress responses in fish (Kelly and Janz 2009), anurans (Marques et al. 2011), and mammals (Reglero et al. 2009).

The purpose of the present study was to evaluate the chronic effects induced by exposure to environmentally realistic concentrations of two metals (nonessential lead and essential zinc) on oxidative stress parameters of the freshwater fish *Gambusia holbrooki*. Being distinct in nature and biological effects, these two metals are dispersed widely in Portuguese estuaries (Mucha et al. 2003; Fernandes et al. 2008). However, it is important to know in detail their toxic effects when in ecologically relevant levels to ascertain about the putative common toxic mechanisms and to know the biological responses elicited by fish to cope with these two different compounds.

The oxidative stress parameters GSTs, GR, and thiobarbituric acid reactive substances (TBARS) were measured in hepatic and gill tissues to serve as putative biomarkers of effect in this study, provided their role in key biological processes determinant for the survival of the individuals: the enzymes are involved in detoxification by phase II metabolism (conjugation with glutathione) and antioxidant activity, and TBARS are indicative of lipoperoxidative damage (Nunes et al. 2008, 2015a, b). As target organs, we chose liver and gills because the liver is the main organ of xenobiotic metabolism in fish, and the gills are the primary barrier against the entrance of xenobiotics into the body and also are the first line of detoxification and elimination of deleterious compounds (Wood and Soivio 1991; Evans et al. 2005).

Materials and Methods

Fish Capture, Quarantine, Acclimation, Depuration, and Exposure

G. holbrooki, commonly known as mosquitofish, was the species selected for this study. This fish is a euryhaline organism widely distributed in both freshwater systems and estuaries of temperate regions. *G. holbrooki* possesses high fecundity and holds an intermediate position as a secondary consumer in the aquatic food web. Furthermore, this species is abundant, easy to catch and also easy to maintain in the laboratory. Because it is not native to Portugal, ethical issues related to the ecological consequences of capturing these animals are negligible. These characteristics suggest this organism as a suitable animal model in ecotoxicology (Nunes et al. 2004, 2008).

The experiments were carried out with *G. holbrooki* specimens, collected in the natural lake of Pateira de Fermentelos, located in the center region of Portugal. This lake is characterized by low levels of anthropogenic pollution (Ferreira et al. 2003). Fish were captured using hand nets and immediately transported to the laboratory. Prior to the toxicity tests, specimens were kept in plastic tanks during 5–7 days for acclimation. The tanks were supplied with dechlorinated tap water, and kept under continuous aeration and constant temperature ($20 \pm 1\ °C$). Inspections were conducted twice a day in order to discard diseased and dead specimens. After this period of quarantine, fish were maintained in tanks under laboratory-controlled conditions before being exposed to the metals. The specimens were fed daily with commercially available fish food (Sera Vipan® flakes). The water was renewed once a week.

The experimental design was performed according to the adequate OECD guidelines (OECD 2000). Specimens were individually exposed in plastic bottles (for human consumption of water), previously rinsed with distilled water. The containers were filled with 200 mL of dechlorinated tap water. Each treatment had 10 replicates (1 fish per replicate), which were randomly distributed into the experimental containers and submitted to the following treatments: 156.25, 312.5, 625, 1250, and 2500 $\mu g\ L^{-1}$ for lead and 9.375, 18.75, 37.5, 75, and 150 $\mu g\ L^{-1}$ for zinc. All assays included one control with blank dechlorinated tap water. Stock solutions of the tested compounds were prepared in ultrapure water immediately before dosing. Concentration ranges were established by combining previously calculated LC_{50} values (for the higher concentrations) and levels already described in Portuguese estuaries (for the lower levels: Mucha et al. 2003; Fernandes et al. 2008). This allowed covering a low-dose chronic exposure

regime that is likely to occur under real environmental scenarios.

Fish were exposed for 28 days in conditions (temperature and aeration) similar to those described above for the maintenance routine. Food was provided once every 2 days, ad libitum, following renewal of test solutions. Parameters, such as mortality, pH, temperature, oxygen, and conductivity, were measured daily during the experimental period; all measurements were within the validation ranges of the adopted testing guideline (pH $\pm$ 0.5 pH units; temperature $20 \pm 2\ °C$; oxygen $\geq 60\ \%$ of saturation levels).

All procedures were performed in compliance with the EU and Portuguese laws and institutional guidelines regarding animal welfare and experimentation, and were approved by our institutional committee.

Chemicals and Stock Solutions

Stock solutions of both tested metals were obtained by dissolving high-purity salts [zinc sulphate ($ZnSO_4.7H_2O$, Sigma–Aldrich 204986, 99.0 %) and lead chloride ($PbCl_2$, Sigma–Aldrich 268690, 98.0 %)] in dechlorinated tap water. To confirm contaminant levels, water analysis were made by Atomic Absorption Spectrophotometry-Flame Atomization (AAS-FA) using a Varian SpectrAA 220FS (Varian Inc., California, USA). Water samples were collected, stored in plastic vials, previously decontaminated by washing with 10 % nitric acid (HNO_3), and frozen for further analysis. Measured water metallic contents were within the expected ranges of concentrations.

Enzymatic Assays

After the exposure period, fish were sacrificed by decapitation and gills and liver were removed. Both gills and livers were homogenized in ice-cold phosphate buffer (50 mM, pH 7.0, with 0.1 % Triton X-100), and then the homogenized tissues were centrifuged at 15,000 g for 10 min at 4 °C. Supernatants were divided into aliquots, which were used for the different enzymatic determinations. Each homogenate sample was composed by 10 livers or 10 gills. Samples were stored at −80 °C until the enzymatic determinations.

Enzymatic activity was determined for GST and GR. LPO extent was assessed by the quantification of TBARS. GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioether whose formation can be followed by the increment of absorbance at 340 nm. Thereby, GST activity was determined by spectrophotometry, according to Habig et al. (1974), and the results were expressed as nanomols of

thioether produced per minute, per milligram of protein. The activity of GR was determined by spectrophotometry, according to the protocol by Carlberg and Mannervik (1985). In this assay, the GR activity can be monitored by the NADPH oxidation, by following the decrease in absorbance values at 340 nm. Enzymatic activity was expressed as micromoles of NADPH oxidized per minute and per milligram of protein. The extent of lipid peroxidation was measured by the quantification of TBARS, according to the protocol described by Buege and Aust (1978). Absorbance readings of each sample were performed at 535 nm. This methodology is based on the reaction of compounds such as malondialdehyde (MDA) formed by degradation of initial products of free radical attack, with 2-thiobarbituric acid (TBA). TBARS concentrations were expressed as MDA equivalents. Protein quantification of samples was determined according to the Bradford method (Bradford 1976), adapted to microplate, in order to express enzymatic activity or TBARS concentration relatively to protein content of the analyzed tissues.

Statistical Analysis

The biomarker responses were analyzed (with the software Statistica 5.0) to detect statistically significant changes in treated groups (exposed to Pb or Zn) relative to the corresponding control group. Data were checked for normality and homoscedasticity prior to statistical analysis using one-way ANOVA followed by the Dunnet multicomparison test to discriminate significantly different treatments. The significance level (α) adopted was 0.05. Data were expressed as mean $\pm$ standard error (SE).

Results

Lead Exposure

Chronic exposure to lead did not induce significant alterations on gill GST activity ($F = 1.81$; $df = 5$, 10; $p > 0.05$; Fig. 1a). However, statistically significant differences were observed in hepatic GST activity ($F = 7.63$; $df = 5$, 12; $p < 0.05$; Fig. 1b). Lead caused a gradual dose-dependent increase in the GST activity in the liver and an extremely high activity was observed in fish exposed to 2500 µg L^{-1}. For the gill tissue, exposure to lead resulted in a significant increase of GR activity. Despite this increase, no coherent pattern was observed: a maximum level was attained by specimens exposed to 312.5 µg L^{-1}, followed by a gradual decrease of activity in fish exposed to the highest dose of lead ($F = 5,75$; $df = 5$, 12; $p < 0.01$; Fig. 1c). The liver of exposed fish showed a rise in the activity of GR at the higher concentrations (1250 and

2500 µg L^{-1}). However, no statistically significant differences were found between control and treated fish ($F = 1.08$; $df = 5$, 11; $p > 0.05$; Fig. 1d). TBARS concentrations in exposed fish were not significantly different from those found for control animals (Fig. 1e): livers of exposed organisms showed lower, albeit not significant, TBARS concentrations than controls ($F = 0.71$; $df = 5$, 12; $p > 0.05$ and $F = 1.34$; $df = 5$, 12; $p > 0.05$, respectively; Fig. 1f).

Zinc Exposure

Individuals chronically exposed to zinc at 75 µg L^{-1} showed an increase in gill GST activity; however, this was a nonsignificant modification compared to the control treatment ($F = 0.78$; $df = 5$, 11; $p > 0.05$; Fig. 2a). Figure 2b shows a rise in the hepatic GST activity for the lowest concentration (9.375 µg L^{-1}), despite the absence of statistical significant differences between treatments ($F = 9.49$; $df = 5$, 11; $p < 0.01$). GR activity evidenced a gradual, but not significant, increase of activity values in gills of treated specimens, accompanying the increase in the metal concentration ($F = 0.22$; $df = 5$, 10; $p > 0.05$; Fig. 2c). Zinc exposure caused a reduction of GR activity in the liver of mosquitofish, showing significant differences between control and exposed fish ($F = 2.87$; $df = 5$, 9; $p < 0.05$; Fig. 2d). In treated fish, both gills and liver presented a mild, nonsignificant tendency for a rise of TBARS concentration ($F = 0.63$; $df = 5$, 12; $p > 0.05$ and $F = 1.40$; $df = 5$, 12; $p > 0.05$, respectively; Fig. 2e, f).

Discussion

Our results showed that both metals—lead and zinc—could cause significant alterations in *G. holbrooki* physiological regulation of the antioxidant defense mechanism. The meaning of these modifications must be separately discussed, due to the difference between lead, a known toxicant, and the essential metal zinc.

Lead is a toxic metal, capable of inducing severe impairments, derived from oxidative stress (Liu et al. 2010). Our results confirm that lead was generically the most toxic compound tested, with significant alterations in GSTs and GR activities at comparatively lower concentrations. The results showed a somewhat similar response to the patterns previously described, with significant increases of the enzymes GST and GR, especially in liver of *G. holbrooki*. A similar pattern was not observed for gill tissue, suggesting a tissue-specific modality of toxicity.

The presented data (namely those reflecting effects in terms of glutathione metabolism, such as increased GR activity) suggest an association of exposure to lead and
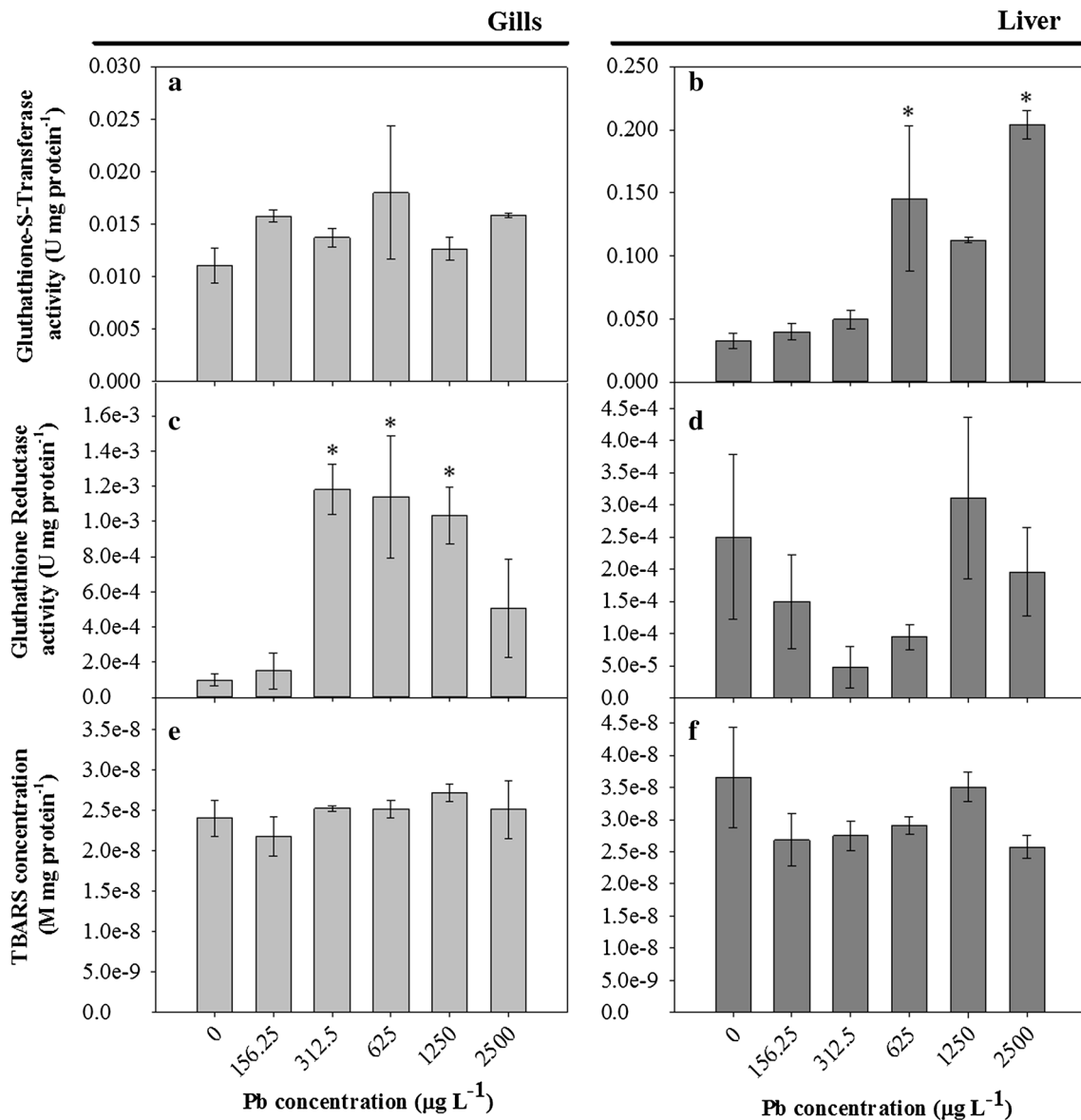
Fig. 1 Chronic effects of lead on biomarkers of *Gambusia holbrooki* **a** gills GSTs activity, **b** liver GSTs activity, **c** gills glutathione reductase activity, **d** liver glutathione reductase activity, **e** gills TBARS concentrations, **f** liver TBARS concentrations. Results are expressed as means of 10 replicate assays, and correspondent standard error bars. *Asterisk* significant differences, $p < 0.05$

oxidative damage in *G. holbrooki*. This marked elevation of GR activity indicates that the oxidative use of GSH as a scavenger occurred, and the organism required higher rates of conversion of reduced glutathione, from its oxidized form back to the biological active status.

The rise of liver GST activity indicates that conjugation with reduced glutathione may be a major pathway of lead detoxification. Modulation of GSTs activity by lead exposure is not surprising: the study conducted by Daggett et al. (1997) showed that lead could simultaneously induce and decrease the GSTs activity in exposed organisms, depending on the analyzed tissue. In fact, lead metabolism seems to be dependent on conjugation with GSH, as shown

by a large number of studies in distinct animal models (Roomi et al. 1987; Di Ilio et al. 1989; Garçon et al. 2007). The work conducted by Thomas and Juedes (1992) showed that the acute exposure of the marine fish *Micropogonias undulates* to lead induced a strong augment of GSH content in liver tissue. This was a short-term response to lead exposure that was not followed by a sustained increase in GSH following chronic exposures—reinforcing the notion that lead metabolism is clearly GSH-dependent, at least during an initial phase. The involvement of lead in the glutathione metabolism was also shown by Spokas et al. (2006), after exposing the fish *Pimephales promelas* to increasing levels of lead. Lead was causative of a
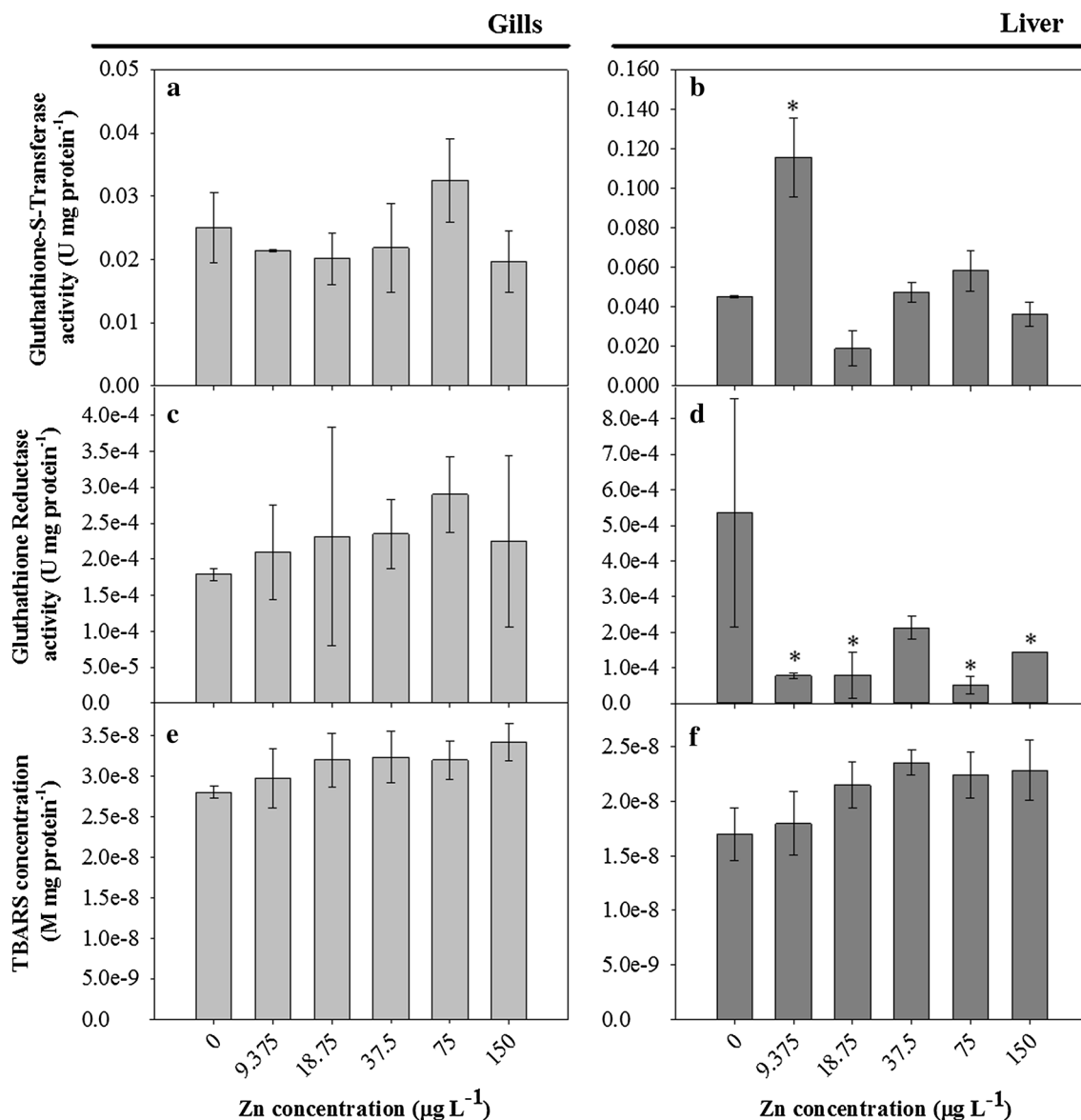
Fig. 2 Chronic effects of zinc on biomarkers of *Gambusia holbrooki* **a** gills GSTs activity, **b** liver GSTs activity, **c** gills glutathione reductase activity, **d** liver glutathione reductase activity, **e** gills TBARS concentrations, **f** liver TBARS concentrations. Results are expressed as means of 10 replicate assays, and correspondent standard error bars. *Asterisk* significant differences, $p < 0.05$

significant reduction of the ratio 'reduced glutathione/oxidized glutathione', suggesting not only that GSH was been used for the scavenging of lead, but also that lead was responsible for a pro-oxidative imbalance, corroborating our data. So, the marked increase observed in GST activity may indicate that the organisms are facilitating excretion of lead through conjugation with GSH.

Despite the increase in the antioxidant efficacy, it is not possible to conclude definitively that oxidative stress was installed after exposure to lead. In fact, considering the indicative marker of lipid peroxidation, MDA concentrations measured by the TBARS assay in exposed organisms were not comparatively higher than those reported for

control organisms. This suggests that the global antioxidant response was satisfactory to prevent the onset of oxidative damage, which hence never occurred during the time course of the intoxication.

Despite the absence of a noticeable response reflected by increased MDA concentrations, this entire set of results suggests the possibility of an oxidative based response, which is in agreement with earlier published data. Lead also was the causative agent of redox modifications in a fish species, namely white seabream (*Diplodus sargus*), with increased levels of catalase and superoxide dismutase, as described by Ferreira et al. (2008). Oxidative stress was the main outcome of exposure of the snail

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species *Theba pisana* to lead, as shown by Radwan et al. (2010), with increased lipoperoxidation, reduction of GSH content, enhancement of antioxidant enzymes (e.g., GSTs, catalase, and glutathione peroxidase). The work conducted by Daggett et al. (1998) showed that massive acute administration of lead to rodents could cause pronounced disturbances in the antioxidant defense, as evidenced by a significant depletion of hepatic GSH; consequently, per-oxidative damage occurred in the liver tissue of exposed animals, shown by an increase in the amounts of MDA. The study by Liu et al. (2010) showed that lead was cap-able of exerting severe modification in liver homeostasis of rodents, made clear by an increase in lipid peroxidation, an increase in hydrogen peroxide levels, the inhibition of both Cu/Zn and Mn superoxide dismutase, the decrease of glu-tathione peroxidase and catalase activities, and histological damage (structure damage, hepatocellular necrosis, and leukocyte infiltration).

Zinc is an essential metal, and its toxicity should only be expected following high-dose exposure. Our results evi-denced a significant decrease in GR of liver of exposed fish, which despite being an oxidative-related effect, is not consistent with a clear oxidative stress scenario. Partially similar results were already attained by Franco et al. (2008), when exposing the freshwater fish species *Cyprinus carpio* to zinc. These authors found that zinc was capable of reducing in a dose dependent manner the activity of GR in brain, liver, and gill tissues. A significant reduction of GR following zinc exposure was also reported in the uni-cellular algal species *Scenedesmus* sp. by Tripathi et al. (2006), accompanied by several other oxidative-related responses. A significant inhibition of an antioxidant defense, glutathione peroxidase, was previously reported by Radwan et al. (2010), following exposure of *Theba pisana* to zinc. This same study showed that zinc elicited an induction of GST activity, a finding that is not in agreement with our results.

It is clear that physiological effects may arise after exposure of living organisms to an essential metal, such as zinc. The study conducted by Loro et al. (2012) evidenced the pro-oxidative effects of zinc in the fish species *Fun-dulus heteroclitus*. Exposure to high levels of zinc was causative of significant effects, in terms of the antioxidant defense mechanisms (superoxide dismutase and GSTs activities) and peroxidative damage (increased TBARS levels). In our case, GST activities (both in liver and in gills) remained unchanged after zinc exposure, showing that the conjugation pathway was not favored; the already referred study conducted by Loro et al. showed that con-jugation with glutathione was possible, but only after massive exposures to zinc (500 μg L^{-1}).

The pro-oxidative effects of zinc exposure in fish are not always of straightforward characterization. The study published by Qu et al. (2014) showed that the effects of short-term exposure to zinc in *Carassius auratus* were reflected by a transient failure of the antioxidant system; the activities of some of the hepatic antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxi-dase) were significantly decreased, a pattern that also was followed by reduced glutathione levels. However, it is not licit to suggest that the alterations observed in our study may be consequent to an oxidative stress scenario, since no other modifications in the selected biomarkers were noticed. Even if literature data show the involvement of GSTs as part of the zinc-derived response, it is more likely that the metabolism of this metal is dependent on a phase II reaction, with the involvement of conjugation with glu-tathione, rather than being an antioxidant response. In fact, zinc may act as a protective chemical in cases of chemi-cally derived oxidative stress. The experiments by Koty-zová et al. (2009) showed that administration of zinc could prevent the oxidative damage elicited by a primary aggression (mainly nephrotoxicity and hepatotoxicity) caused by ferric nitrilotriacetate in rats. Similarly, Brzoska et al. (2009) showed that zinc could also prevent oxidative stress caused by cadmium in bone tissue of rats. Simulta-neous exposure of the aquatic duckweed *Spirodela poly-rhiza* to copper and zinc resulted in a significantly reduced toxic response, by interference/competition with the uptake mechanism (Upadhyay and Panda 2010). Toxic effects caused by copper exposure were reflected by significant increases in oxidative indicators, such as augmented levels of lipid peroxidation, total peroxide, superoxide anion, and lipoxygenase activity. Zinc concomitant exposure caused a significant increase in the activity of antioxidant enzymes (e.g., catalase, ascorbate peroxidase, and peroxidase), which acted to prevent the occurrence of damage charac-teristic to copper exposure.

Metal toxic effects of oxidative nature are not simple to devise and depend on other variables, such as species used, type of exposure, simultaneous presence of other metallic species, and levels to which organisms are exposed. Pad-mini and Rani (2009) showed that metallic pollution in an Indian stream induced oxidative stress in hepatic tissue of the fish *Mugil cephalus*, translated into an increase in oxidative damage markers (lipids and proteins), a signifi-cant decrease in the GSH content, and a sustained decrease in enzymatic activities, namely of the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, GSTs, and GR. Similar results were obtained by Ruas et al. (2008), when assessing the physiological impairment caused by exposure of cichlid species (*Oreochromis niloticus, Tilapia rendalli,* and *Geophagus brasiliensis*) to metal pollution. The toxic challenge was causative of severe changes in the activity of antioxidant defense mechanisms, such as blood enzymes (glutathione

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peroxidase, catalase, and superoxide dismutase), and also at the level of peroxidative damage (increase lipoperoxidation). Metal-derived oxidative stress is not limited to animals, as described by Cuypers et al. (1999) and by Yadav et al. (2010), with consequent response in terms of antioxidant enzymes in autotrophic organisms. Algal species are also prone to suffer oxidative effects following exposure to metallic contaminants, as demonstrated by Tripathi et al. (2006); this study showed that zinc (among other metals) was causative of oxidative effects in *Scenedesmus* sp., with increases in the antioxidant enzymatic defense (catalase, superoxide dismutase, glutathione reductase), and other antioxidant mechanisms (such as total thiolic content). The work described by Dazy et al. (2009) evidenced that the response of the bryophyte *Fontinalis antipyretica* to metals (Cd, Cu, Pb, and Zn) resulted in the establishment of an oxidative stress scenario, highlighted by a significant up-regulation of physiological levels of defenses, such as antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, ascorbate, and guaiacol peroxidases) and increased lipid peroxidation. *Ulva* sp. also was shown to be responsive to high concentrations of metals (Pereira et al. 2009), with considerable modifications in the normal activities of enzymatic antioxidant defenses, such as catalase, glutathione peroxidase, glutathione reductase; lipid peroxidation caused by metals was also a significant result drawn from this study, showing a direct linkage between metals and oxidative damage. Dimkpa et al. (2008) chose exposure to several metallic species (including Al, Cu, Fe, Mn, Ni, and U) to study the antioxidant response in cowpea (*Vigna unguiculata*) and observed severe alterations in terms of lipid peroxidation, and an enhanced superoxide dismutase activity.

Conclusions

It is reasonable to sustain that lead caused a significant, oxidative related stress response, in agreement with the already established patterns of pro-oxidative deleterious effects observed in other organisms, namely in aquatic species. The fact that these modifications occurred following exposure to environmentally relevant concentrations of this metal is noteworthy. The importance of having reported significant alterations after exposure to lead concentrations that are likely to occur under natural conditions may mean that fish species are particularly susceptible to oxidative damage in the wild; in real scenarios of contamination, exposures to low concentrations generally occur along longer periods of time, a factor that increases the probability of long-term, irreversible, oxidative alterations. On the other hand, zinc did not elicit substantial

toxic alterations, which is an observation in line with previously obtained data. In fact, and despite the potential for being a protective agent against oxidative damage elicited by other compounds, zinc may also generate oxidative alterations, as observed in our study. Additionally, the assessed biomarkers are of limited value to fully characterize an oxidative stress scenario. These differences in response to zinc, which may be ultimately modulated by the levels and durations of exposure, indicate a set of toxic effects that still require to be addressed.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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