

Mercury distribution in target organs and biochemical responses after subchronic and trophic exposure to Neotropical fish *Hoplias malabaricus*

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Abstract In the present study, we investigated the mercury distribution, mercury bioaccumulation, and oxidative parameters in the Neotropical fish *Hoplias malabaricus* after trophic exposure. Forty-three individuals were distributed into three groups (two exposed and one control) and trophically exposed to fourteen doses of methylmercury each 5 days, totalizing the doses of $1.05 \mu\text{g g}^{-1}$ (M1.05) and $10.5 \mu\text{g g}^{-1}$ (M10.5 group). Autometallography technique revealed the presence of mercury in the intestinal epithelia, hepatocytes, and renal tubule cells. Mercury distribution was dose-dependent in the three organs: intestine, liver, and kidney. Reduced

glutathione concentration, glutathione peroxidase, catalase, and glutathione *S*-transferase significantly decreased in the liver of M1.05, but glutathione reductase increased and lipid peroxidation levels were not altered. In the M10.5, most biomarkers were not altered; only catalase activity decreased. Hepatic and muscle mercury bioaccumulation was dose-dependent, but was not influenced by fish sex. The mercury localization and bioaccumulation corroborates some histopathological findings in this fish species (previously verified by Mela et al. in *Ecotoxicol Environ Saf* 68:426–435, 2007). However, the results of redox biomarkers did not explain histopathological findings previously reported in M10.5. Thus, fish accommodation to the stressor may reestablish antioxidant status at the highest dose, but not avoid cell injury.

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Introduction

Mercury (Hg) contamination has been recognized as an important issue since the early 1970s, and nowadays, there is plenty of evidence supporting that this ubiquitous metal is one of the most toxic to living organisms. There are several Hg chemical forms in

natural environment, and from these mercurial compounds, methylmercury (MeHg) is the most important in terms of toxicity and health effects (Qiu et al. 2009). MeHg easily accumulates along the aquatic food chain with a biomagnification ratio in the order of 10,000–100,000 (WHO 1990; Bidone et al. 1997), where dietary exposure is the major route of uptake in fish (Oliveira Ribeiro et al. 1999, 2002; Scudder et al. 2009). Consequently, top predator fish represent important links between mercury contamination and human health, being the main route of mercury uptake by human populations (Lebel et al. 1998; Aschner 2002).

The critical methylmercury target organs following trophic exposure are the nervous system, liver, intestinal mucosa, kidney, blood, and muscle (Baatrup 1991; Oliveira Ribeiro et al. 1999, 2002, 2006; Mela et al. 2007, 2010; Rabbito et al. 2011). A previous study with *Hoplias malabaricus* (Mela et al. 2007) revealed important histopathological changes, including increased number of macrophages aggregation in the liver, hepatic lesions, cytoskeleton disarrange, alteration in hepatocytes' nuclear shape and heterochromatin distribution, and morphological disorders. Recently, Silva et al. (2012) showed that this organ is an important target tissue to this species even if naturally exposed to MeHg, and biochemical and morphological parameters are important tools to evaluate the exposure. However, some aspects remained obscure, such as the mercury localization related to the effects in target tissues.

The development of the autometallographic (AMG) method was reported as efficient to investigate the localization of mercury deposits in histological slides (Danscher and Möller-Madsen 1985; Soto et al. 1998), but even today mercury localization data in fish tissue are scarce (Baatrup et al. 1986; Baatrup and Danscher 1987; Mela et al. 2010, 2012). AMG is very sensitive since it requires the presence of few atoms of a metal such as gold, silver, mercury, metal sulfides, or metal selenides in the tissue to catalyze the deposition of metallic silver around them (Alvarado et al. 2005; Mela et al. 2012), which appear as black points aggregated visualized by light microscopic.

Environmental pollutants' exposure can result in increased reactive oxygen species (ROS) generation and alteration of antioxidant mechanisms (Monserrat et al. 2007). Mercury, in particular, has a large number of potential molecular targets. This metal

can react with sulfhydryls of antioxidant molecules such as glutathione (GSH) or other proteins, inhibiting protein synthesis, DNA repair, or disrupting cytoskeleton array and intracellular Ca^{2+} balance (De Flora et al. 1994; Sanfeliu et al. 2003; Bridges and Zalups 2005). Mercury can uncouple the mitochondrial electron transport chain, promoting the hydrogen peroxide formation and if enhancing the subsequent iron- and copper-induced production of the highly reactive hydroxyl radical (Yee and Choi 1996; Zaman and Pardini 1996; Konigsberg et al. 2001). If established, this condition may lead to a redox unbalance/oxidative stress within the cell (Berntssen et al. 2003). Such condition could increase lipid peroxidation ratios that with other parameters are indicators of pollutant-mediated oxidative stress.

Data about the toxic effects of mercury on Neotropical fish in the Amazon basin are still insufficient, even though the largest and the richest hydrographic basin when concerning the biodiversity on Earth. According to Silva et al. (2012), even today, the levels of mercury in muscle of *H. malabaricus* is exceeding the safety limit levels for human consumption (WHO 1990). This species is an important consumed fish by Amazon riverine human populations (Boischio and Henshel 1996). Over the last decade, the Brazilian Neotropical predator fish *H. malabaricus* has been utilized as a fish model for toxicology and ecotoxicology investigations by our research group (Alves Costa et al. 2007; Filipak Neto et al. 2008; Silva et al. 2012), including the study on the effects of methylmercury (Mela et al. 2007, 2012).

The present study was conducted in the context of a larger research initiative that has been looking at factors that influence Hg exposure and toxicity. In this part of the study, methylmercury localization was determined in the intestine, liver, and kidney of the fish *H. malabaricus* after subchronic trophic exposure. Biochemical biomarkers were evaluated to examine the liver redox milieu and comprehend whether oxidative stress was involved with the results of the previous investigation (Mela et al. 2007) and of the recent study of Silva et al. (2012). Additionally verify whether the liver defense systems could deal with MeHg pro-oxidant effects in long-term exposure and support biochemical data in the current study or in other previously published.

Materials and methods

Animals

Forty-three mature freshwater fish *H. malabaricus* were obtained from a fish farm station located in Paraná State, Southern Brazil, and transported to Federal University of Paraná. Before the experiment, fish were acclimated to experimental conditions for 30 days (one fish for each 30-L aquarium in dechlorinated tap water, $T = 21 \pm 2$ °C, 12:12 h photoperiod). The food supply provided to each fish was one young live *Astyanax* sp, a natural freshwater prey fish species, from the same fish farm station without direct polluting sources. Fish were fed once every 5 days. All procedures using animals were performed according to the NIH guidelines and Federal University of Paraná commission for studies involving human or animal subjects (<http://www.bio.ufpr.br/ceea/html/index.html>).

Experimental design: MeHg exposure

After acclimation to laboratory conditions, fish were randomly separated into three groups. Two groups of fish ($n = 15$ each, 156 ± 9.2 g of wet weight and 23 ± 0.5 cm of total length) were fed with young live specimens of *Astyanax* sp. previously intraperitoneally injected with an aqueous solution of MeHg (CH_3HgCl , Sigma[®], in HCl 0.1 M), so that every experimental fish *H. malabaricus* received fourteen individual doses of either $0.075 \mu\text{g g}^{-1}$ (total: $14 \times 0.075 = 1.05 \mu\text{g g}^{-1}$; M1.05 group) or $0.75 \mu\text{g g}^{-1}$ of MeHg (total: $14 \times 0.75 = 10.5 \mu\text{g g}^{-1}$; M10.5 group). Since each experimental fish was kept in one individual aquarium, the ingested dose of MeHg was controlled and estimated assuming that the injected MeHg in the prey was entirely transferred to the predator, as the time for prey ingestion was about few seconds due to the voracious habits of the *H. malabaricus*. The dose of $0.075 \mu\text{g g}^{-1}$ used in this work was very close to real conditions found in prey fish from Amazonian rivers impacted by mercury (Mela et al. 2007). The tenfold higher dose ($0.75 \mu\text{g g}^{-1}$) was used for comparison purposes. A control group ($n = 13$, 128 ± 84 g of wet weight and 22.4 ± 4.1 cm of total length) was maintained used as control where live *Astyanax* sp. were injected only with distilled water. After 14 doses and 70 days, fish were

anesthetized with 0.02 % MS222 (ethyl-ester-3-aminobenzoic acid, Sigma[®]) and killed by spinal cord section. Biological samples were removed and immediately fixed for autometallography or stored at -76 °C for biochemical assays and mercury quantification.

Autometallography (AMG)

Liver, kidney, and intestine samples were fixed with 3 % glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h at 4 °C and rinsed with buffer (0.1 M sodium cacodylate buffer, 2 % NaCl, pH 7.4), dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus[®] (Sigma). Then, autometallography was performed according to the protocol proposed by Danscher and Möller-Madsen (1985). For the autometallography development, tissue sections (5 μm) were coated with a thin film of gelatin by dipping the slides in 0.5 % of gelatin and then AMG developed for 60 min at 26 °C in a water bath. The process was stopped by replacing the AMG developer with thiosulphate solution for 10 min and rinsing the slides in 40 °C running tap water to remove the gelatin. Then, the slides were dipped in a 2 % Farmer's solution as described by Danscher and Nørgaard (1983). The sections were stained with Hematoxylin and Eosin (Woods and Ellis 1994), dehydrated with ethanol and xylene series, mounted with Entellan (Merck), and observed under the Leica[®] DME light microscope.

Biochemical assays

For biochemical assays, tissues were thawed on ice, homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.2), centrifuged at 9,000g for 30 min at 4 °C, and stored at -76 °C. Catalase (CAT) activity (Aebi 1984), glutathione peroxidase (GPx) activity (Takahashi 1994), glutathione S-transferase (GST) activity (Keen et al. 1976), superoxide dismutase (SOD) activity (Crouch et al. 1981), glutathione (GSH) measurement (Sedlak and Lindsay 1968), lipid peroxidation (LPO) (Jiang et al. 1991), and protein content (Bradford 1976) were measured according to modified protocols previously published (Moura Costa et al. 2010). Glutathione disulfide reductase (GR) activity (Sies et al. 1979): 50 μl of supernatant or PBS (blank) and reaction medium (170 μl , 0.5 mM β -NADPH, 5.0 mM glutathione disulfide (GSSG),

5.0 mM EDTA, 0.1 M potassium phosphate buffer, pH 7.6, and 25 °C) were mixed in a 96-well microplate. Absorbances were measured at 340 nm for 10 min. Delta-aminolevulinic acid dehydratase (δ -ALAd) activity (Sassa 1982): 50 μ l of supernatant was mixed with 550 μ l of reaction solution (4.0 mM δ -aminolevulinic acid hydrochloride (ALA-HCl), 0.5 % Triton X-100 in 100 mM sodium phosphate buffer, pH 6.3) and incubated at 25 °C for 1 h. The reaction was stopped with 400 μ l of 4 % trichloroacetic acid and 99.45 mM HgCl₂ in water and ice bath. For blanks, the reaction was stopped before incubation for 1 h. Then, tubes were centrifuged at 5,000g for 5 min at 4 °C. A volume of 150 μ l of supernatant and 150 μ l of Ehrlich reagent solution (18.18 mg ml⁻¹ of *p*-dimethylamino benzaldehyde, 3.18 mg ml⁻¹ HgCl₂, 76.36 % glacial acetic acid, and 18.18 % perchloric acid in water) were placed in a 96-well microplate. After 15 mins of incubation, absorbances were measured at 570 nm and enzymatic activity was calculated after comparison with a porphobilinogen standard curve.

Mercury quantification

Samples of liver and muscle were thawed and weighted, and chemical extraction was performed according to Bastos et al. (1998). Total mercury was quantified by atomic absorption spectrophotometer coupled with cold vapor generation FIMS-400 (Flow Injection Mercury System, PerkinElmer). Analytical control was accompanied by the analysis of reagent blanks and reference certified samples (AFPX 5130).

Statistical procedures

Linear regression and correlation analyses were performed for mercury bioaccumulation levels in the liver and muscle, and fish sex or biometric parameters length and mass. The biochemical assays were performed with livers of every fish, and the mean of three to four replicates per sample was calculated. These means, in a total of 13 for control, 15 for M1.05, and 15 for M10.5 (equal to the number of fish per group), were utilized for one-way analysis of variance (ANOVA) followed by Tukey's posttest when appropriate. Pearson's correlation coefficient was utilized to compare the relationship between variables (biological and biochemical parameters) and liver mercury concentration.

Results

Experimental conditions

No mortality or changes of fish mobility, skin coloring, and integrity were observed in the control and experimental groups throughout the experiment.

Mercury distribution

The liver, kidney, and intestine from control fish did not reveal the presence of mercury (silver deposits) observed by autometallography analysis, but were visualized in those organs of exposed individuals, particularly in the higher doses (M10.5 group).

Intestine

In the intestine of control individuals, columnar epithelial cells, goblet cells, and the extracellular matrix were visualized without any silver deposition (Fig. 1a), while in exposed individuals silver deposits were found inside the epithelial cells and at the extracellular matrix, but not inside the goblet cells. Under the highest mercury dose (Fig. 1c) was observed a visible increase in the silver deposits comparatively to the lowest dose (Fig. 1b).

Liver

Individuals from control fish showed no silver deposit in the liver as expected (Fig. 1d). Conversely, silver deposits were found inside hepatocyte cytoplasm of exposed groups. As observed in intestine, mercury dose influenced silver deposition (Fig. 1e, f) with M10.5 group presenting the highest deposition (Fig. 1f).

Posterior kidney

No silver deposits were observed in individuals from control group (Fig. 1g), but the animals exposed to MeHg showed a dose-dependent mercury distribution, almost exclusively in the renal tubules (Fig. 1h, i) was found.

Biochemical assays

From the eight biomarkers considered in the present study, five had significant differences ($p < 0.05$) when

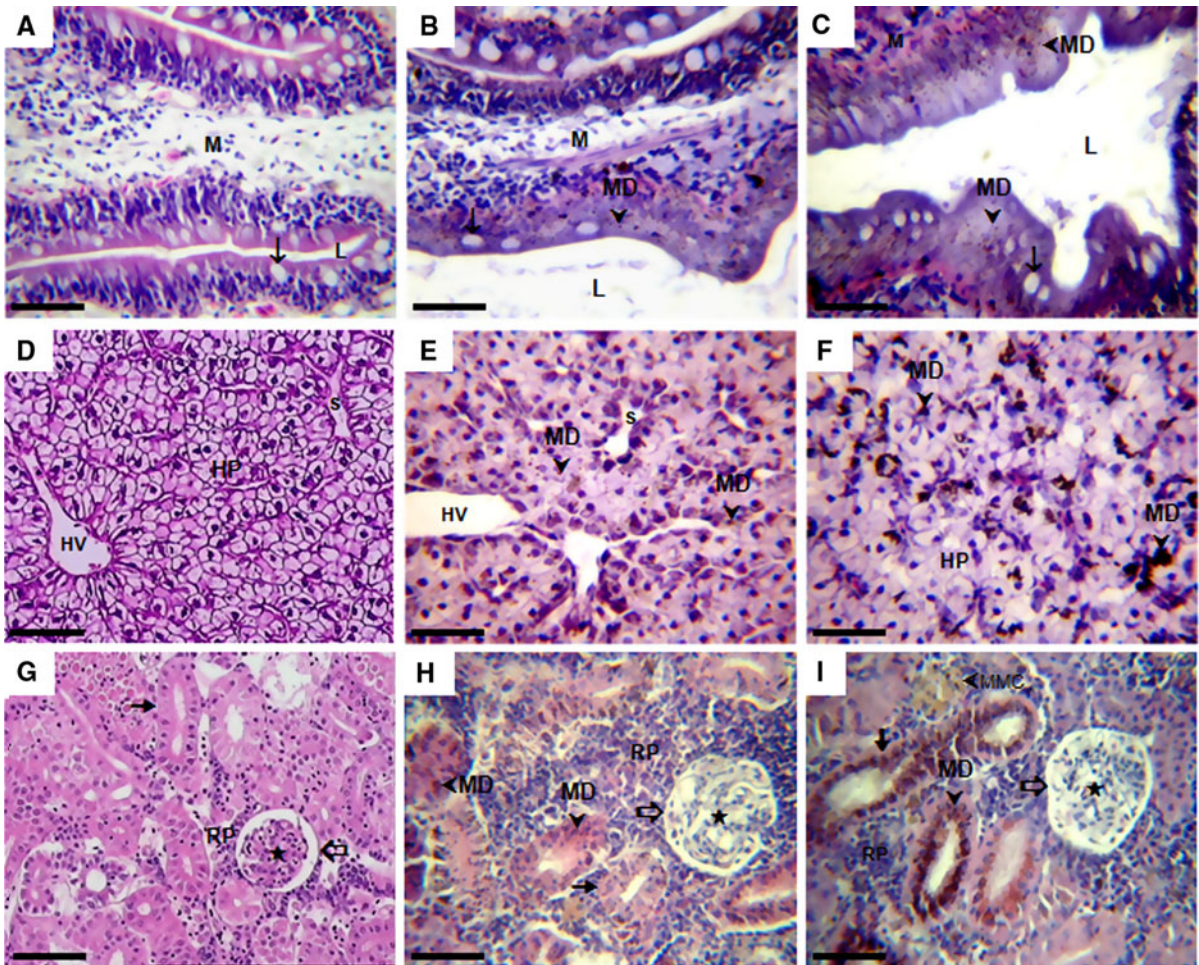


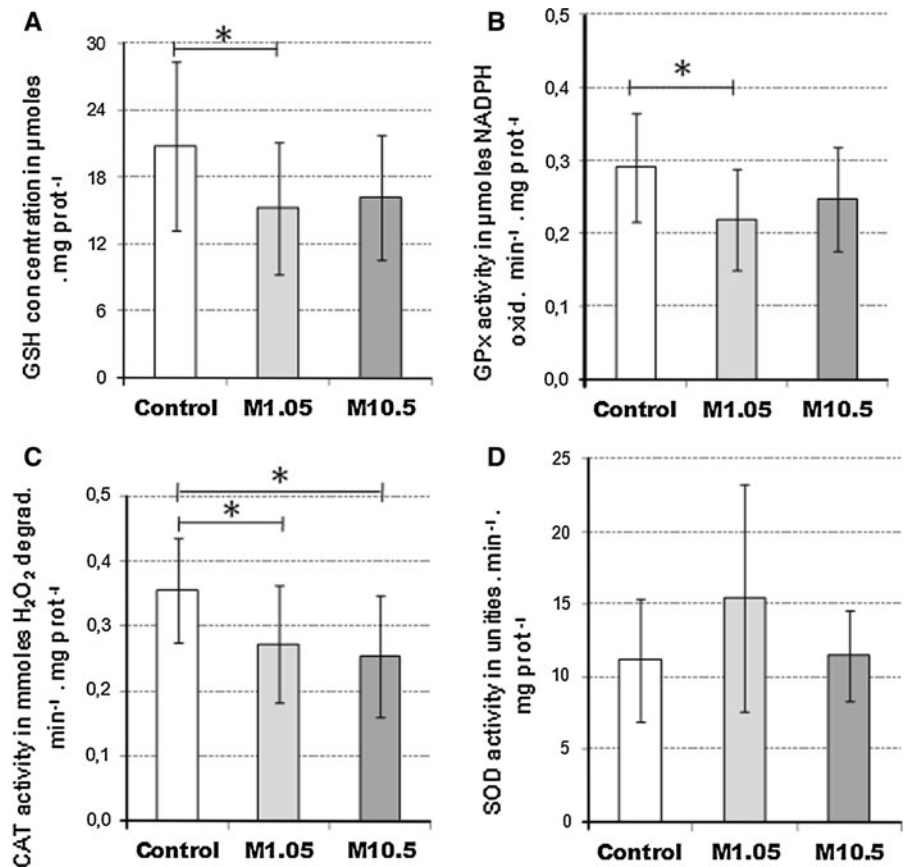
Fig. 1 Autometallography of the intestine, liver, and kidney of *H. malabaricus* counterstained with Hematoxylin–Eosin. Intestine of control group (a), M1.05 group (b), and M10.5 group (c). Goblet cells (downwards arrow), extracellular matrix (m), and lumen (l). Liver of control group (d), M1.05 group (e), and M10.5 group (f). Hepatocyte (HP), hepatic vein (HV), sinusoids (s), and mercury deposits (MD black rightwards arrowhead).

Kidney of control group (g), M1.05 group (h), and M10.5 group. Renal parenchyma (RP), glomerulus (black star), Bowman's capsule (left shaded white rightwards arrow), kidney ducts (heavy triangle headed rightwards arrow), melanomacrophage centers (MMC), and mercury deposits (MD black rightwards arrowhead). Scale bars 40 μ m

compared with the control group. The antioxidant molecule GSH, which is also substrate for GST conjugation activity, had its concentration decreased by 27 % in the M1.05 group (Fig. 2a). The hydrogen peroxide-degrading enzymes such as GPx and CAT had 25 and 26 % of decreases in activity in the M1.05 group in comparison with the control group (Figs. 2b, c). For CAT, a decrease of 29 % in its activity was also observed in the M10.5 group, when compared to the control group (Fig. 2c). However, M1.05 and M10.5 groups had similar CAT activities, meaning the absence of dose–response curve (Fig. 2c). MeHg

exposure did not alter SOD activity (Fig. 2d). The xenobiotic-conjugating phase II enzyme GST had a decrease of 26 % in its activity in the M1.05 group (Fig. 3a). Conversely, GR activity increased in the M1.05 group (88 %) when compared to the control group (Fig. 3b). Both enzymatic activities were not altered in the M10.5 group (Fig. 3a, b). The δ -ALAd activity was not altered in either MeHg-exposed groups (Fig. 3c). Likewise, lipid peroxidation levels were similar in the control, M1.05, and M10.5 groups, indicating the absence of MeHg-induced hydroperoxides accumulation (Fig. 3d).

Fig. 2 Biochemical biomarkers in the liver of *H. malabaricus*. **a** GSH concentration, **b** GPx activity, **c** CAT activity, **d** SOD activity. Data are expressed as mean \pm standard deviation. * $p < 0.05$



Mercury quantification

The ingested doses of MeHg resulted in hepatic concentrations of 1.09 ± 0.88 (M1.05) and $8.26 \pm 6.45 \mu\text{g Hg g}^{-1}$ (M10.5) (Table 1). However, since the control group had low but detectable liver mercury levels of $0.05 \pm 0.06 \mu\text{g Hg } \mu\text{g g}^{-1}$ resulting from natural exposure (background levels), the differences between exposed and control groups, i.e., the hepatic bioaccumulation during the experiment, were of $1.04 \mu\text{g Hg g}^{-1}$ and $8.21 \mu\text{g g}^{-1}$, respectively, for the M1.05 and M10.5 groups. Data about mercury bioaccumulation in the liver and muscle revealed variable bioaccumulation ratios per individual fish as indicated by the standard deviation and minimum and maximum values (Table 1). In addition, from the fish in the M1.05 group, exposed to the concentrations of mercury equivalent to those found in natural preys of *H. malabaricus* from impacted areas, 100 % of fish bioaccumulated Hg levels exceeding the safe limit established for human consumption (WHO 1990) of $0.5 \mu\text{g Hg g}^{-1}$ (Table 1). The administration of the

highest dose of MeHg resulted about eightfold more bioaccumulation for the liver and about fourfold for the muscle than the lowest dose, meaning a dose-dependent uptake and bioaccumulation in both organs (Table 1). Curiously, the bioaccumulation of Hg in the liver was close to the concentration of methyl mercury administered, confirming that this organ is an important site of methyl mercury accumulation under dietary exposure. However, the quantified mercury may be as inorganic mercury, once the liver shows demethylation process. Liver mercury levels and fish biometric or biochemical parameters had no correlations; males and females had statistically similar mercury levels.

Discussion

Results of mercury localization as demonstrated in the current study are important data to understand physiological events concerns to methylmercury exposure to fish, but few studies of mercury localization in

Fig. 3 Biochemical biomarkers in the liver of *H. malabaricus*. **a** GST activity, **b** GR activity, **c** δ -ALAd activity, **d** lipid peroxidation. Data are expressed as mean \pm standard deviation. * $p < 0.05$

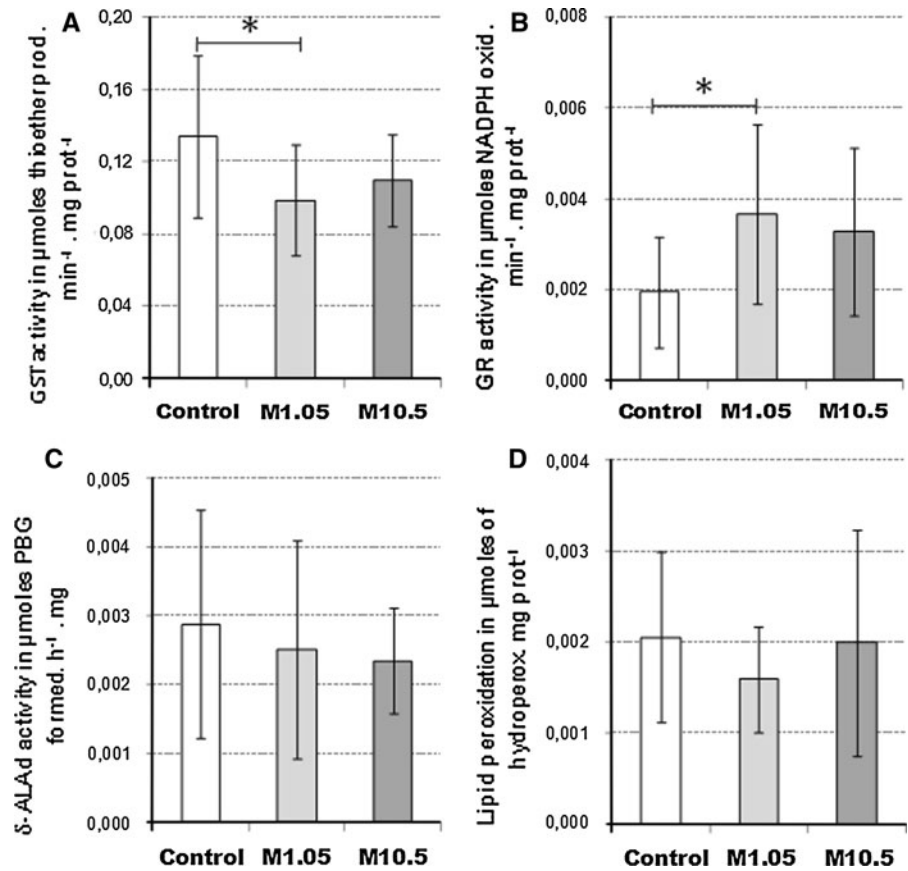


Table 1 Biometric and Hg bioaccumulation data on *H. malabaricus* after 70 days of dietary exposure to methylmercury

	Control	M1.05	M10.5
Number of fish (Males/females)	13 (9/4)	15 (6/9)	15(7/8)
Standard length (cm)	22.4 \pm 4.1	24.1 \pm 5.4	23.7 \pm 4.5
Weight (g)	128 \pm 84	161 \pm 107	152 \pm 78
Hg measured in the muscle	0.09 \pm 0.06 (0.03–0.21)	1.07 \pm 0.26 (0.63–1.53)	4.77 \pm 1.99 (2.71–8.16)
Hg bioaccumulation in the muscle during the experiment	0.0	0.98 \pm 0.20	4.68 \pm 1.93
Hg measured in the liver	0.05 \pm 0.06 (0.00–0.19)	1.09 \pm 0.88 (0.13–3.44)	8.26 \pm 6.45 (2.55–26.36)
Hg bioaccumulation in the liver during the experiment	0.0	1.04 \pm 0.83	8.21 \pm 6.41

Values: mean \pm standard deviation (minimum – maximum); w.w. (wet weight). Mercury bioaccumulation represents the values of total mercury measured in the fish tissues, whereas bioaccumulation in the experiment refers to the amount of mercury measured at the end of experiment subtracted by the mean of mercury levels in the control group, which represents the background mercury levels

target tissues been determined for Brazilian fish species. These studies are emerging but still insufficient to establish a model permitting the evaluation of risk of exposure to Amazon species, where mercury is a regional problem due to gold mining in the past and by the more recent burn forest (Rabitto et al. 2011).

Due to the high trophic position of *H. malabaricus*, high levels of mercury in target organs have been recently reported in Amazon region (Dorea et al. 2006; Silva et al. 2012), showing that this species is an important biosensor and target to mercury exposure in natural conditions. The identification of mercury

localization through silver deposits by autometallography is a consolidated method (Danscher and Nørgaard 1983; Danscher and Möller-Madsen 1985; Danscher et al. 2004). In the current study, it was explored to demonstrate the mercury distribution in tissues of a native species of Amazon region. This method was already successfully used by Mela et al. (2012) in retina of *Hoplias malabaricus* also exposed to methylmercury.

The presence of mercury in the epithelial cells of intestine of *H. malabaricus* and, at a minor extent, in the extracellular matrix represents the main tissue targets of the organ. Although the intestine presents a strong regulatory capacity to deal with dietary metals (Handy 1993), there are few information about mercury localization in fish intestine. The absorption of methylmercury from the gastrointestinal tract has been reviewed by Nordberg and Skerfving (1972), and Japanese studies were summarized by Kojima and Fujita (1973). These revisions suggest that methylmercury is almost completely absorbed in the intestinal tract and was confirmed by Oliveira Ribeiro et al. (1999). Epithelial cells from intestinal mucosa represent a biological barrier that selects the entrance of essential nutrients as well as contaminants. But MeHg absorption can occur by passive diffusion through neutral amino acids carrier proteins (Leaner and Mason 2004) and accumulate in the epithelial cells or toward the connective tissue and transported via bloodstream to other target organs.

After intestine naturally the absorbed MeHg will first to liver. The chemical analysis showed a bioaccumulation of eightfold more in liver when compare the higher and lower doses of MeHg in a dose-dependent manner. The results of mercury localization corroborated this finding showing a higher incidence of silver deposits in hepatocytes of *H. malabaricus*, confirming the physiological route of MeHg after uptake in intestine. According to Loumbourdis and Danscher (2004), MeHg is delivered to the liver through portal system naturally indicating this organ as important target tissues to evaluate the effects of mercury. This statement has been also reported by Ung et al. (2010), and the bioaccumulation in liver was confirmed by Berntssen et al. (2003) and Maury-Brachet et al. (2006). According to Alvarado et al. (2005), MeHg crosses the hepatocyte plasma membrane, is sequestered in the lissome, and binds to thiol groups anywhere before to be eliminated by the bile and or redistributed by blood stream to other target

tissues (Ballatori 1991). The intestinal reabsorption, if eliminated by bile, decreases the capacity of the organism to eliminate the mercury, whereas in case of redistribution, the kidney may accumulate the metal. The current results confirm these findings once both liver and kidney of *H. malabaricus* showed silver deposits in a dose-dependent manner.

The kidneys of teleosts receive a large portion of the cardiac output because of their extensive portal system. A large volume of blood flow to renal tissues causes the kidney to be exposed to high levels of circulating compounds and may lead to xenobiotic accumulation in the kidneys (Pritchard and Bend 1984). The role of kidney on mercury elimination depends on the mercurial form, preferably inorganic form by urine leading to the hypothesis that the mercury presence in the kidney of *H. malabaricus* is the inorganic form, derived from the molecular modifications of MeHg, probably in liver. Previous studies showed that after a 4-week exposure to dietary MeHg, the kidneys of sturgeon species showed prominent renal tubules degeneration (Won Lee et al. 2012). Similar changes were reported in *Poecilia reticulata* (Wester and Canton 1992) and *Clarias batrachus* (Kirubakaran and Joy 1988) exposed to waterborne MeHg. Thus, in MeHg-treated animals, severe renal tubule damage is evident and now we demonstrated also the Hg accumulation in these cells. The major target organ for HgCl_2 is the kidney and for MeHg is the brain (Clarkson 2002; Klaassen 2006). However, according to Shi et al. (2011), in kidney of rats, the MeHg produce more severe nephrotoxicity than HgCl_2 , indicating that kidney is also a target organ for MeHg following long-term exposures, and further investigations are warranted. It is certain that the kidney plays an important role in the excretion of metallic ions and that the tubular epithelium is involved in this excretion (Suzuki 1977). So, the occurrence of silver deposits in cells from tubes of posterior kidney in *H. malabaricus* revealed at least the potential effects of mercury on important physiological aspects as osmoregulation and reabsorption of essential macromolecules and ions to the organism.

Most biochemical biomarkers in the M10.5 group were unaltered, even though some of these biomarkers were altered in the M1.05 group, indicating that these biomarkers are not well suitable for long-term MeHg toxicity evaluation in *H. malabaricus*. According to Velisek et al. (2011), the oxidative stress is a cause of

exposure to different classes of pollutants, including toxic metals as mercury. Elia et al. (2003) reported similar findings, with reduced hepatic GSH levels in the fish exposed to the lowest dose of mercury and recovery of GSH levels in the fish exposed to the highest dose. In the present study, not only GSH levels were recovered or simply unaltered at the highest dose, but also GPx and GST activities. Only CAT activity remained altered.

Mercury is broadly accepted as a pro-oxidant that exerts oxidative stress, induces decrease in GSH levels, and causes lipid peroxidation (Stoys and Bagchi 1995). The decrease in CAT and GPx activities described in the current data, as well as GSH levels, revealed the potential of increases in the ROS levels in liver cells of *H. malabaricus* due to MeHg exposure. However, there are several molecules involved in the antioxidant defense mechanisms acting in concert and liver defenses are “strong” in comparison with other tissues such as kidney and brain. Lipid peroxidation levels were therefore not altered. The absence of lipid peroxidation was an indication that the MeHg liver toxicity previously reported for *H. malabaricus* (Mela et al. 2007) was not entirely due to oxidative stress per se, even though molecules with antioxidant properties such as CAT, GPx, GSH, and GST might be involved.

In particular, decreases in GSH levels can lead to toxicity due to the decrease in GST-conjugating activities that are physiologically important, disruption of the reducing environment of cytosol or many other processes that require normal GSH levels. One important factor that contributed to the decreased GSH levels was probably the conjugation with mercury (Cookson and Pentreath 1996). The GR increase helped avoiding further GSH decrease, but it was not completely efficient since GSH levels do decreased in the M1.05 group. GR increase was an important indication of liver defense mechanisms working during long-term exposure, but some key enzymatic systems such as CAT and GPx continued being partially impaired by MeHg even after 70 days of exposure in the M1.05 group. This impairment, however, was completely eliminated in the M10.5 group and, on both groups, did not lead to measurable lipid oxidative damage.

Decreased GPx and GST activities could mean worst abilities to deal with hydrogen and lipid peroxides. Long-term mercury exposure may give enough time for defense systems to control lipid peroxidation levels not only in tropical fish such as *H. malabaricus*, but also in

fish from temperate zones (Berntssen et al. 2003). The absence of abnormal lipid peroxidation, however, does not mean the absence of abnormal biomolecules damage. Lipids, proteins, and nucleic acids have different sensitivities to different kinds of damage, and mercury could damage the two latter molecules without eliciting lipid peroxidation and oxidative stress.

Mela et al. (2007) reported increased histopathological alterations, such as increased liver injury index, macrophage aggregates and necrosis, the presence of atypical cytoplasmic electron dense granules within hepatocytes, and high vacuolization of the endothelial cells in *H. malabaricus* liver from the M1.05 group. Although we have confirmed the presence of MeHg in tissues of *H. malabaricus*, the results indicate that MeHg had induced these alterations by mechanisms other than oxidative stress, since lipid peroxidation, which is an excellent biomarker of oxidative damage, was not altered in the M10.5 group. Conversely, oxidative stress was present in the M1.05 group as indicated by the reductions in the enzymatic activities and GSH levels, although the intensity was not enough to lead to lipids damage. Furthermore, reduction in GSH concentration and GST, CAT, and GPx enzymatic activities could contribute to MeHg toxicity, since these molecules also participate other processes that not only redox regulation. Based on the presence of alterations in most biochemical biomarkers in the M1.05 group, but not in the M10.5 group, these biomarkers cannot be considered appropriated for long-term evaluation of MeHg toxicity in *H. malabaricus*, since the biochemical results did not indicate harmful stress, which was clearly observed by morphological analysis (Mela et al. 2007).

High levels of mercury were detected in the liver and muscle of *H. malabaricus* after long-term dietary exposure. Mercury concentrations in fish liver were frequently higher than those in muscle, as previously reported (Thompson 1990). Bioaccumulation occurred in a dose-dependent, but sex-independent manner, and the accumulation levels confirmed the high mercury bioavailability from food and fast gastrointestinal absorption in the order of 90–95 % previously reported (Oliveira Ribeiro et al. 1999; Berntssen et al. 2003; Mela et al. 2007). In the M1.05 group, fish were exposed to doses of mercury considered environmentally relevant or realistic, since *H. malabaricus* feeds from fish that bioaccumulate similar mercury levels in nature. Furthermore, mercury levels in *H. malabaricus*

from Suriname (Mol et al. 2001) and in Amazon basin (Dorea et al. 2006) are in the range of mercury levels after laboratory exposure, and both laboratory and field mercury levels were higher than the safe limit of 0.5 ppm established for human consumption (WHO 1990). Piscivorous fish such as *H. malabaricus* usually have higher mercury contents than non-piscivorous species due to biomagnification (Mol et al. 2001; Dorea et al. 2006). For the M10.5 group, the dose utilized cannot be considered realistic, because Hg levels in the order of $8.26 \mu\text{g g}^{-1}$ w.w. had never been reported for *H. malabaricus* liver, even though muscle mercury levels greater than those observed for the species ($4.68 \mu\text{g g}^{-1}$ w.w.) had been reported for other fish species from Amazon basin (Dorea et al. 2006).

The present study provided useful information on the MeHg distribution in the intestine, liver, and kidney of *H. malabaricus* as base to understand some physiological disturbs related by literature in the few decades. Additionally, the mercury localization in these tissues corroborates many effects previously described for this species after subchronic exposure to MeHg (Mela et al. 2007, 2012) or naturally exposed (Miranda et al. 2008; Silva et al. 2012). Oxidative stress biomarkers were not suitable to confirm previously observed liver lesions. The present data with other related results showed that the oxidative stress has not been the main mechanism of MeHg hepatic toxicity or fish accommodation during subchronic exposure had masked it. In conclusion, the biochemical biomarkers as utilized in the present study are not the best indication to evaluate subchronic or chronic exposure to mercury, validating these methods at least to corroborate acute exposures. Finally, the main gain of the current data is the possibility of discussion together the localization and possible mechanism of effect of mercury in target tissues as intestine and posterior kidney, related, respectively, with the uptake and elimination of this toxic metal in fish.

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