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Tissue-specific bioaccumulation and oxidative stress responses in juvenile Japanese flounder (*Paralichthys olivaceus*) exposed to mercury*

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Abstract To understand mercury (Hg) toxicity in marine fish, we measured Hg accumulation in juvenile Japanese flounder (*Paralichthys olivaceus*) and assessed the effects on growth and antioxidant responses. After Hg exposure (control, 5, 40, and 160 μg/L Hg) for 28 d, fish growth was significantly reduced. The accumulation of Hg in fish was dose-dependent and tissue-specific, with the maximum accumulation in kidney and liver, followed by gills, bone, and muscle. Different antioxidants responded differently to Hg exposure to cope with the induction of lipid peroxidation (LPO), which was also tissue-specific and dose-dependent. As Hg concentration increased, superoxide dismutase (SOD) and catalase (CAT) activities increased significantly, whereas glutathione *S*-transferase (GST) activity and glutathione (GSH) levels decreased significantly in the gills. SOD and glutathione peroxidase (GPx) activities and the GSH level increased significantly in the liver. SOD activity and GSH levels increased significantly, but CAT activity decreased significantly with an increase in Hg concentration in the kidney. LPO was induced significantly by elevated Hg in the gills and kidney but was least affected in the liver. Therefore, oxidative stress biomarkers in gills were more sensitive than those in the liver and kidney to Hg exposure. Thus, the gills have potential as bioindicators for evaluating Hg toxicity in juvenile flounder.

Keyword: mercury (Hg); antioxidants; lipid peroxidation; bioaccumulation of metal

1 INTRODUCTION

Mercury (Hg) is a ubiquitous environmental contaminant that poses serious risks to organisms and ecosystems (Yang et al., 2002; Chen et al., 2009; Wang et al., 2009). In aquatic ecosystems, inorganic Hg can be methylated by bacterial processes to form a more toxic substance, methylmercury (MeHg). However, because less than 10% of total Hg (mean: 1%–5% in the water column) exists in the MeHg form, inorganic Hg is believed to have a more significant effect on aquatic animals (Gonzalez et al., 2005; Zhang and Wong, 2007). Aquatic animals such as fish take up Hg either by direct exposure through their body or by ingestion. Hg can then bioaccumulate and biomagnify through the food chain (Alvarez et al., 2006). Uptake and elimination pathways differ substantially among tissues (e.g., liver, kidney, gills, and muscle), thus, Hg accumulation is tissue-specific

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(Olson et al., 1973; Niimi and Kissoon, 1994; Yediler and Jacobs, 1995; Rothschild and Duffy, 2005). The accumulation of Hg above certain levels in fish can result in serious biological disturbances or individual death (Jezierska and Witeska, 2001). To date, a variety of adverse effects of Hg exposure have been observed in fish, including neurological, respiratory, immune, renal, dermatologic, reproductive, and developmental problems (Friedmann et al., 1996; Carta et al., 2003; Risher and Amler, 2005).

The toxic effects of Hg are commonly associated with the formation of reactive oxygen species (ROS) in cells (Larose et al., 2008; Verlecar et al., 2008). Xenobiotics like Hg generate ROS such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), alkoxyl radicals ($RO^{\bullet}$), and singlet oxygen ($^1O_2$), which in turn react indiscriminately with various organic chemicals and induce protein degradation, enzyme inactivation, and lipid peroxidation (LPO). Together, these processes may lead ultimately to cell and tissue damage in fish (Winston and Di Giulio, 1991; Livingstone, 2001). However, cells can neutralize the harmful effects of ROS and detoxify themselves via a variety of cellular antioxidative defense systems that employ enzymatic and non-enzymatic components to prevent LPO damage (Di Giulio et al., 1989; Verlecar et al., 2007). These antioxidants are characterized by their inducibility under conditions of oxidative stress, which serves as an important adaptation in unfit conditions (Livingstone, 2001). Antioxidants such as glutathione (GSH), glutathione peroxidase (GPx), glutathione *S*-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) are widely used as antioxidative biomarkers to investigate the toxic effect of metals (including Hg) to fish (Rana et al., 1995; Guilherme et al., 2008; Vieira et al., 2009; Monteiro et al., 2010).

A number of researchers believe that pollution is one of the primary factors contributing to the collapse of marine fisheries. However, the toxic effects of marine pollutants to the fish (especially to their early life stages, ELSs) have not been thoroughly investigated in Chinese coastal waters. For many commercial fishery species, the Bohai Sea is a traditional spawning and nursery area. In recent decades, pollution has degraded the aquatic environment thereby reducing fishery productivity and decreasing the populations of a number of commercial fish species, such as Japanese flounder (*Paralichthys olivaceus*) (Jin et al., 2005). The average Hg concentration in sea water in the Bohai Sea ranges from 0.002 μg/L to 0.15 μg/L. However, levels as high as 2.59 μg/L to 32.00 μg/L in certain locations have been detected (e. g., near industrial waste-water outlets) (Zhang, 2001; Liu et al., 2003). Hg exposure can cause developmental problems such as low hatching success, low survival, and high levels of morphological malformation in flounder embryos and larvae (Huang et al., 2010). Thus, Hg is suspected to have a negative impact in the spawning and nursery areas of wild flounder populations. The toxic effects of Hg on juvenile flounder, however, remain poorly understood.

With the general goal of understanding the toxicological effects of Hg exposure on juvenile flounder, we exposed fish to sub-lethal concentrations of Hg for 28 d. Our specific objectives were: (1) to measure the bioaccumulation of waterborne Hg in the liver, kidney, gills, muscle, and bone and document the effect of accumulation on flounder growth; (2) to determine the tissue specific (gills, liver, and kidney) response of antioxidants (GSH, GPx, GST, SOD, and CAT) to oxidative stress induced by Hg exposure; (3) to evaluate the effectiveness of using tissue-specific antioxidants and Hg accumulation to assess Hg toxicity in juvenile flounder.

2 MATERIAL AND METHOD

2.1 Test fish and test design

We obtained juvenile flounder (11.5±0.6 cm in total length, L_T; 9.8±1.8 g in body weight, W_B) from the Shunyuan Fish Hatchery Station, Rizhao, China. The fish were acclimatized in a flow-through pond with filtered seawater (pH, 8.1±0.1; salinity, 33±1; dissolved oxygen, 7.5±0.2 mg/L) for one week before they were used in the experiments. During acclimation, the fish were fed sand lance (*Ammodytes personatus*) twice a day with a light regime of 14L:10D. A thermostat-controlled water bath system was used to maintain the water temperature at 17±1°C.

After acclimation, the fish were anesthetized with 2-phenoxy ethanol, weighed (to the nearest 0.1 g) and measured (to 0.1 cm). Fifteen fish of similar size were transferred into each of the 60-L experimental tanks, which were spaced randomly in an indoor pool. Fish size did not differ significantly between replicates or concentrations (ANOVA, $P>0.05$ in all cases; Table 1). The fish were acclimated for 24 h in the experimental tanks, without feeding. We then exposed the fish to either a blank control (0 μg/L) solution or

Table 1 The initial weight (W_0), final weight (W_T), final total length (L_T), condition factor (CF), and specific growth rate (SGR) of flounder juveniles exposed to $HgCl_2$ for 28 d

Growth index	Nominal concentration (μg/L Hg)			
	Control	5	40	160
W_0 (g)	9.7±0.7	9.8±0.8	9.7±0.9	9.8±0.9
W_T (g)	17.2±1.2	17.4±1.2	15.1±3.7	12.7±2.1*
L_T (cm)	12.4±0.6	12.7±0.3	12.2±0.4	11.8±0.2*
CF (g/cm^3)	0.91±0.15	0.84±0.10	0.82±0.14	0.76±0.10
SGR (%/d)	2.04±0.26	2.09±0.25	1.48±0.87	0.91±0.57*

Data are expressed as mean±S.D.; * indicates significant difference compared to the control at $P<0.05$.

an Hg solution of 5, 40, or 160 μg/L. Four replicates were performed for each concentration. Each experimental tank was filled with 50 L of filtered seawater. The fish rearing conditions were identical to those for the acclimation described above. We used $HgCl_2$ (purity over 99.5%; CAS No: 7487-94-7; Sigma-Aldrich Chemical Co., USA) that was dissolved in deionized water to obtain a stock solution (1.0 g Hg/L), from which appropriate aliquots were diluted in filtered seawater to obtain the designated concentration in each experimental tank. The solution in the tanks was renewed daily with a freshly prepared solution of the same Hg concentration. Oxygen was gently provided by a continuous air-bubbling system. The fish were fed sand lance to satiation twice a day during the test. The bioassay had the duration of 28 days.

2.2 Sampling and Hg analyses of test solutions

Water samples for chemical analysis were collected from each experimental tank every other day following the start of the test. A total of 56 water samples were analyzed for each concentration. They were sampled immediately after the water was renewed and were then acidified with 1% (v/v) HNO_3 solution awaiting Hg concentration analysis. The concentration of Hg in water samples was measured using automated atomic fluorescence spectrometry (AFS; Titan AFS-610A, China) according to the Specification for Marine Monitoring—Mercury Analysis in Seawater GB 17378. 4-2007 (SAPRC, 2008). The concentration error (%), which was used to validate the accuracy of the Hg concentration in the test solution, was defined as the absolute difference between the measured and nominal concentrations divided by the nominal concentration and multiplied by 100.

2.3 Fish growth determination and Hg accumulation analysis

No experimental fish died during the test. Six fish were sampled randomly from each experimental tank at the end of test. They were weighed (W_B) and measured (L_T) to determine growth. The fish were then sacrificed by cervical dislocation, and the gills, liver, kidney, dorsal muscle, and vertebral bone tissues were separated and rinsed in ice-cold physiological salt solution (0.9% NaCl). The tissues were immediately transferred to vials and stored in liquid nitrogen awaiting the quantification of Hg accumulation.

Fish growth was assessed by the specific growth rate (SGR) and condition factor (CF). The SGR (%/d) of the individual fish was calculated using the formula: $SGR = (e^g-1)\times100\%$, where $g=(\ln W_T - \ln W_0)/t$, where W_0 is the mean W_B of fish at the beginning of test in each experimental tank, W_T is the W_B of fish sampled at the end of the test, and t is the duration of the test in days. CF was calculated according to the formula: $CF= (W_B/L_T^3)\times100\%$. We used the average SGR and CF for each experimental tank in subsequent analyses.

Tissue samples for the quantification of Hg were thawed, washed with deionized water, and dried with absorptive paper. They were then weighed, placed in a sealed, acid-washed polytetrafluoroethylene container with concentrated HNO_3 (1 mL for 0.1 g sample) at 50°C for digestion over at least 12 h. The gills, liver, muscle, and bone samples of each individual were digested separately. The kidneys of the six fish from each tank were pooled into one sample for Hg quantification because they were too small to be analyzed individually. The solutions were then diluted with ultrapure water and Hg accumulation was determined by the AFS method, as in Liang et al. (2003).

2.4 Biochemical assays

Similar to the sampling procedure for the quantification of Hg accumulation, an additional six fish were randomly sampled from each experimental tank at the end of the test for biochemical analysis. After being sacrificed by cervical dislocation, the gills, liver, and kidney tissues of the fish were separated and then immediately transferred to acid-rinsed vials and stored in liquid nitrogen. The gills

and liver samples of each individual fish were processed and analyzed separately, whereas the kidneys of the six fish from each tank were pooled into one sample for biochemical analysis. For the biochemical analysis, the fish tissues were thawed and thoroughly washed with an ice-cold physiological salt solution, surface dried with tissue paper and weighed. Afterwards, the tissue samples were homogenized with 10 mmol/L ice-cold Tris-HCl buffer (pH 7.4; for 0.1 g of sample, 0.9 mL of buffer was added) and then centrifuged at 10 000×*g* for 10 min at 4°C in a centrifuge (Eppendorf 5804R, Hamburg, Germany). The supernatant was immediately analyzed for the activities of GPx, GST, SOD, and CAT as well as the contents of GSH, malondialdehyde (MDA), and protein.

GSH content was determined by the method of Hissin and Hilf (1976) using the fluorescent reagent *o*-phthalaldehyde (OPT) at 420 nm. GPx activity was measured using H_2O_2 and GSH as substrate following the description in Rotruck et al. (1973). One unit of GPx activity was defined as the amount of enzyme oxidizing 1 μmol GSH per min per mg protein. GST activity was assayed using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate (Habig et al., 1974). One unit of GST activity was defined as the amount of enzyme that conjugates 1 μmol of CDNB with GSH per min per mg protein. SOD activity was determined by measuring the inhibition of the auto-oxidant of pyrogallol at 325 nm according to Marklund and Marklund (1974). One unit of SOD activity was defined as the amount of enzyme exhibiting 50% inhibition of the auto-oxidation rate of pyrogallol. CAT activity was measured at 240 nm by determining the decay of hydrogen peroxide levels (Beers and Seizer, 1952). One unit of CAT activity was defined as the amount of enzyme catalyzing the degradation of 1 μmol of H_2O_2 per min, the specific activity corresponded to transformation of 1 μmol of substrate (H_2O_2) per min per mg protein. LPO level was measured in terms of malondialdehyde (MDA) content, the decomposition products of polyunsaturated fatty acid hydroperoxides were determined by the thiobarbituric acid (TBA) reaction (Ohkawa et al., 1979). Thiobarbituric acid reactive substances (TBARS) values were expressed as nmol MDA per mg protein. Protein content was determined using bovine serum albumin as the standard at 595 nm (Bradford, 1976). All measurements were conducted using an ultraviolet spectrophotometer (UNICO WFZ UV-2802PC/PCS; Shanghai, China) at 25ºC.

2.5 Statistical analyses

The data are reported as the mean±S.D. and were checked for assumptions of normality and homogeneity of variance. Both assumptions were met for all the biochemical parameters. Thus, differences between treatment and control means for these parameters were subsequently analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. However, the Hg accumulation and SGR datasets did not meet both assumptions so were subjected to a log-transformation. We pooled the kidneys of the six fish from each tank to measure Hg accumulation and perform biochemical analyses. The remaining tissues were not pooled. For these tissues, we calculated the average value for the six individuals from each tank for use in subsequent analyses statistical analyses. All analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P<0.05$.

3 RESULT

3.1 Hg concentrations of test solutions, Hg bioaccumulation and fish growth

During the test, the total Hg level in the control was 0.049±0.018 μg/L, whereas the levels in the 5, 40, and 160 μg/L tanks were 4.7±0.7, 36.5±3.1, and 156.3±2.6 μg/L, respectively. The concentration errors were 2.3%–13.5% of their nominal counterparts, which met the criteria (±20%) required for toxicity tests of this kind (OECD, 1992).

At the end of the test, the L_T, W_T, and SGR of the flounder were significantly affected by the Hg concentration (ANOVA, $P<0.05$ for all cases; Table 1). In the 160 μg/L Hg treatments, the L_T, W_T, and SGR values of the flounder were significantly lower than those of their respective controls (Dunnett's test, $P<0.05$ at all cases). However, there was no significant difference in CF between the treatments and control (ANOVA, $P>0.05$ at all cases; Table 1).

After 28-d exposure, the Hg levels increased significantly and dose dependently in all tissues (ANOVA, $P<0.05$ at all cases). The Hg levels in gills and bone (respectively) in the 5, 40, and 160 μg/L Hg treatments were significantly higher than those in the controls (Dunnett's test, $P<0.05$ at all cases; Fig.1). The Hg levels in the liver, kidney, and muscle tissues (respectively) for the 40 and 160 μg/L Hg treatments were significantly higher than those in the controls (Dunnett's test, $P<0.05$ at all cases; Fig.1).

3.2 Oxidative responses to Hg exposure

We observed significant changes in GSH levels in all three tissues after 28-d exposure to Hg (ANOVA, $P<0.05$ at all cases; Fig.2a). At 40 μg/L, the gill tissue GSH content was 38.3% lower than that in the control. However, at 5, 40, and 160 μg/L, GSH was significantly higher in the liver than those in the controls (by 234.7%, 223.7%, and 183.6%, respectively). Furthermore, GSH levels were higher in the kidney tissues of fish exposed to 160 μg/L (by 99.1% compared with the control) (Dunnett's test, $P<0.05$ in all cases; Fig.2a).

Liver GPx activity was 92.8% and 76.1% higher in fish exposed to 5 and 160 μg /L Hg, respectively, than in the controls (ANOVA Dunnett's test, $P<0.05$; Fig.2b). The concentration of Hg did not have a significant effect on GPx activity in either gills or kidney tissue (ANOVA, $P>0.05$ for both cases; Fig.2b).

Moreover, Hg concentration did not significantly affect GST activity in either liver or kidney tissue (ANOVA, $P>0.05$ at both cases; Fig.2c). However, gill GST activity was significantly inhibited (by 37.7%) at 160 μg/L Hg compared with the control (ANOVA Dunnett's test, $P<0.05$; Fig.2c).

SOD activity in all three tissues was significantly affected by the Hg exposure (ANOVA, $P<0.05$ at all cases; Fig.3a). The SOD activity in liver tissue was significantly induced relative to the control by 132.6%–147.9% at concentrations of 40–160 μg/L (Dunnett's test, $P<0.05$ at all cases; Fig.3a), whereas it was significantly induced relative to the control by 58.5% and 48.9% in gills and kidney tissues, respectively, at 160 μg/L Hg (Dunnett's test, $P<0.05$ for both cases; Fig.3a).

The Hg concentration did not have a significant effect on CAT activity in liver tissue (ANOVA, $P>0.05$; Fig.3b). CAT activity was 54.8% higher in the gills of fish exposed to 160 μg/L Hg but 35.6% lower in the kidney tissue of fish exposed to 40 μg/L

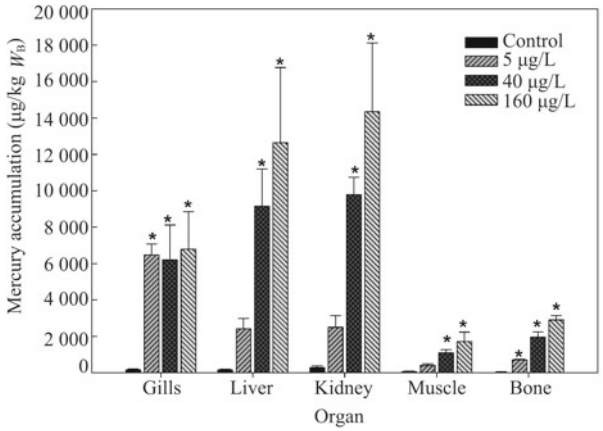

Fig.1 Mercury accumulation (μg/kg W_B; mean±S.D., n=4) in the gills, liver, kidney, muscle, and bone of flounder juveniles exposed to $HgCl_2$ for 28 d

* indicates significant difference compared to the control at $P<0.05$.

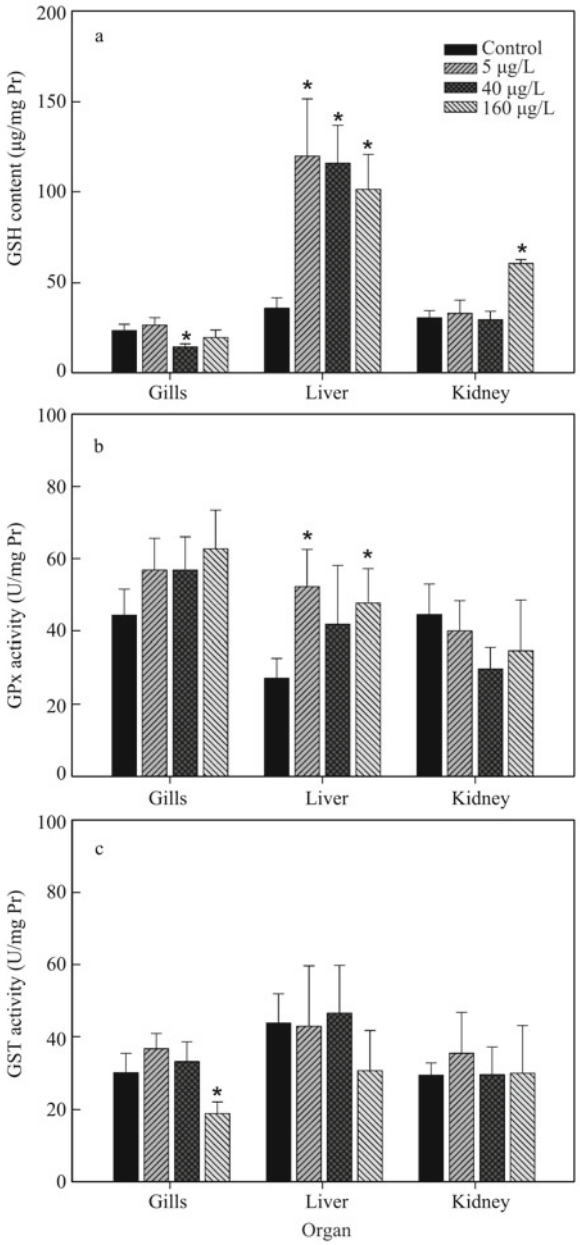

Fig.2 Changes in (a) GSH content (μg/mg Pr, mean±S.D.), (b) GPx activity (U/mg Pr, mean±S.D.), and (c) GST activity (U/mg Pr, mean±S.D.) in the gills, liver, and kidney of flounder juveniles exposed to $HgCl_2$ for 28 d

* indicates significant difference compared to the control at $P<0.05$.

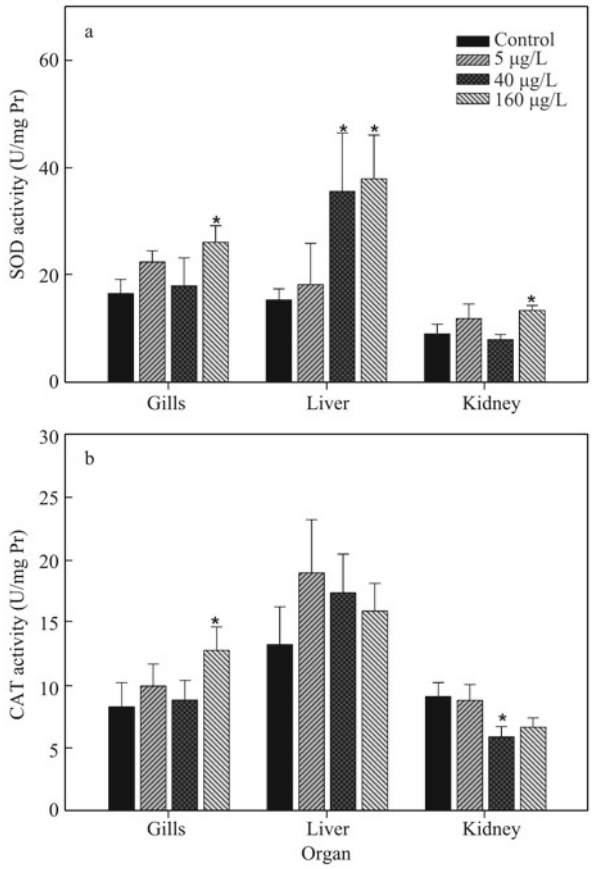

Fig.3 Changes in (a) SOD activity (U/mg Pr, mean±S.D.) and (b) CAT activity (U/mg Pr, mean±S.D.) in the gills, liver, and kidney of flounder juveniles exposed to $HgCl_2$ for 28 d

* indicates significant difference compared to the control at $P<0.05$.

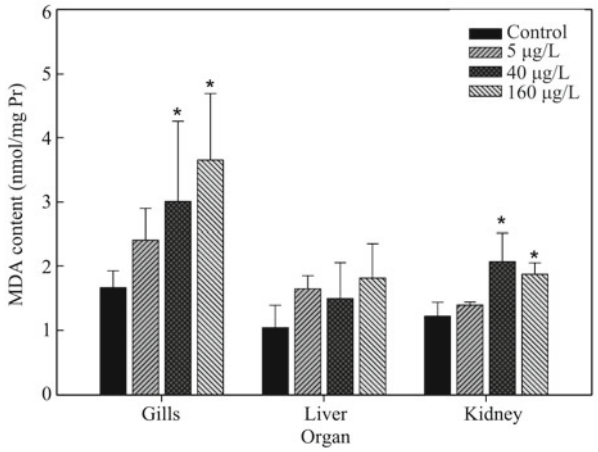

Fig.4 Changes in MDA content (nmol/mg Pr, mean±S.D.) in the gills, liver, and kidney of flounder juveniles exposed to $HgCl_2$ for 28 d

* indicates significant difference compared to the control at $P<0.05$.

Hg when compared with the controls (ANOVA Dunnett's test, $P<0.05$ at both cases; Fig.3b).

At the end of the test, MDA levels in gills and kidney tissues were significantly higher at the two highest Hg exposure concentrations (ANOVA, $P<0.05$ at both cases; Fig.4). MDA levels increased relative to the controls by 80.2%–118.6% in the gill tissue and by 53.0%–69.2% in the kidney tissue at Hg concentrations of 40–160 μg/L (Dunnett's test, $P<0.05$; Fig.4). However, MDA levels in the liver were not significantly affected by the Hg concentration (ANOVA, $P>0.05$; Fig.4).

4 DISCUSSION

4.1 Tissue-specific Hg bioaccumulation and the effect on fish growth

The level of Hg was significantly higher in all tissues after 28-d exposure. The accumulation was dose dependent and differed among the tissues as follows (highest to lowest): kidney≈liver>gills> bone>muscle. This is consistent with observations in other fish species such as rainbow trout (*Oncorhynchus mykiss*; kidney>liver>gills>muscle; Niimi and Kissoon, 1994) and mullet (*Mugil cephalus*; liver>gills>muscle; Middaugh and Rose, 1974). However, the pattern of accumulation appears to differ in some fish whereby the highest metal levels are found in the gills (e.g., Catfish *Ictalurus melas* and carp *Cyprinus carpio*; gills>kidney>liver>muscle; Yediler and Jacobs, 1995; Elia et al., 2003; Huang et al., 2007; Matrinxã *Brycon amazonicus*; gills>liver> muscle; Monteiro et al., 2010). Taken together however, these data suggest that organs such as the liver, kidney, and gills are the most metal-burdened tissues in fish, whereas muscle contains much lower levels of Hg (Niimi and Kissoon, 1994; Jezierska and Witeska, 2001; Huang et al., 2007; Monteiro et al., 2010).

In fish, the liver and kidney play important roles in the uptake, storage, detoxification and elimination of metals. These processes are generally believed to be closely related to Hg accumulation in these two organs. Like other marine fish (Berntssen et al., 2003), the flounder used in the present study likely absorb waterborne inorganic Hg in the intestinal tract as large volumes of seawater are swallowed to maintain osmotic homeostasis. The Hg subsequently transferred to other internal organs (e.g., the liver and kidney) where it accumulates. The gills also accumulate large amounts of Hg because they are the first organ that contacts waterborne Hg directly. The gills are the primary uptake site of waterborne ions and are where metal concentrations increase, especially at the

beginning of exposure before the metal enters other tissues of the organism (Jezierska and Witeska, 2001). High levels of Hg accumulation have also been reported in the gills of carp and rainbow trout exposed to aqueous Hg (Olson et al., 1973; Huang et al., 2007). In contrast, the Hg levels in the muscle tissue of flounder (395.5–1 718.1 μg/kg) were only 11.9%–16.3%, 11.1%–15.8%, 6.1%–25.3%, and 55.7%–59.2% of the levels in liver (2 420.9–12 645.8 μg/kg), kidney (2 497.6–14 347.7 μg/kg), gills (6 465.0–6 783.9 μg/kg), and bone (706.0–2 902.2 μg/kg) tissues, respectively. The muscle acted as a terminal reservoir that accumulated Hg gradually from other organs. Muscle tissue accounted for the largest proportion of the body mass of these fish. Thus, the Hg was less concentrated in the muscle than in the other tissues. Although metals like copper, cadmium, lead, and zinc are deposited at high concentrations in fish bones (Jezierska and Witeska, 2001), little is known about the accumulation of Hg in fish bones. We observed greater accumulation of Hg in the bones of flounder than in their muscle tissue. This finding is with observations in the black scoter (*Melanitta nigra*) in a field study by Rothschild and Duffy (2005).

Exposure to Hg is known to inhibit growth in a number of fish species, such as the fathead minnow (*Pimephales promelas* 0.26–3.69 μg/L $HgCl_2$; Friedmann et al., 1996). This may be due, in part to impairment of feeding behavior. For example, exposure to 10–20 μg/L Hg^{2+} adversely affected the feeding behavior of killifish (*Fundulus heterociitus*) (Weis and Khan, 1990). In addition, Hg can disrupt hormone function (e.g., thyroid and cortisol, which are related to metabolism) as well as protein synthesis, both of which may lead to decreased growth (Friedmann et al., 1996; Jezierska and Witeska, 2001; Matta et al., 2001). Thus, the accumulation of Hg in flounder over a certain concentration is likely to have detrimental effects on a variety of physiological and biochemical processes, including metabolism and antioxidative defense systems. The exposure of the flounder to a high concentration of Hg (e.g., 160 μg/L) was associated with low appetite and sluggish prey capture behavior. These potentially deleterious effects of Hg on the foraging and food ingestion of flounder likely contributed to the observed reduction in growth.

4.2 Tissue-specific oxidative responses of fish to Hg exposure

The gills play an important role in respiration, osmoregulation, and acid-base balance in fish. They are also the first organs to directly come in contact with waterborne Hg. After passing through the gills, Hg enters the blood and is transported to other organs, during which oxidative stress can often be induced in cells. Accordingly, fish commonly utilize a variety of enzymatic and/or non-enzymatic defense mechanisms to counteract the oxidative stress to protect themselves from Hg-induced LPO (Monteiro et al., 2010). In the present study, the antioxidants in the gills of the flounder responded actively to Hg exposure. Concomitant induction of both SOD and CAT activities in the gills during exposure to high concentrations of Hg (e.g., 160 μg/L) reflected the occurrence of oxidative stress. One of the most biologically important antioxidants, SOD, catalyzes $O_2^{\cdot-}$ and H^+ into H_2O_2 and O_2. Subsequently, the H_2O_2 generated in this process can be reduced to H_2O and O_2 by CAT or GPx (Winston and Di Giulio, 1991). Because an increase in the production of $O_2^{\cdot-}$ and H_2O_2 induces SOD and CAT activity, SOD and CAT are often used as biomarkers of ROS production (Monteiro et al., 2006). Our results are consistent with the responses of SOD and CAT in the gills of matrinxã that were exposed to 150 μg/L $HgCl_2$ for 4 d (Monteiro et al., 2010). The SOD-CAT system is typically the first line of defense against ROS, the induction of this system implies an adaptive response of the flounder's antioxidative defense system to cope with Hg-induced oxidative stress.

The decline in GSH levels in the gills was probably caused by GSH binding with Hg^{2+} to prevent cellular structures from interacting with Hg. Fish typically remove Hg^{2+} from their systems by direct conjugation with GSH or GST when they encounter Hg ions, and these chemical reactions decrease GSH levels (Elia et al., 2003). As with our observations, a decrease of GSH in the gills has also been reported in snakehead fish (*Channa punctatus*) following exposure to 5 μg/L $HgCl_2$ for 30 d (Rana et al., 1995). GST, a GSH-dependent enzyme, catalyzes the conjugation of xenobiotics with GSH, generating less toxic substance (Monteiro et al., 2010). GST also plays an important role in protecting tissues from toxification. We observed inhibition of GST activity following exposure to 160 μg /L Hg. Because GST utilizes GSH as a co-factor, the decreased level of GSH in the gills may be responsible for the inhibition of GST activity at higher Hg concentrations. Nevertheless, the depletion of GSH and the inhibition of GST activity may increase susceptibility to ROS attack and increase the risk of oxidative stress in cells (Thomaz et al.,

2009). MDA content increased significantly at exposure concentrations of 40 and 160 μg/L, suggesting that LPO damaged the gills of the flounder. This observation corroborates the general belief that Hg exposure commonly causes LPO in the gills of fish (Rana et al., 1995; Monteiro et al., 2010). Thus, the SOD-CAT system was induced in the gills of fish that were exposed to Hg, in addition, GSH and GST were inhibited by high Hg concentrations. When the balance between the antioxidant/pro-oxidant systems was disturbed at concentrations of 40 and 160 μg/L, LPO damage was induced.

Protein biosynthesis and carbohydrate metabolism take place in the liver. This organ is also the primary site for detoxification and excretion of toxic substances. In addition, the liver has been reported to play a critical role in redox metabolism (Oliveira et al., 2008). Therefore, it is generally believed that antioxidants in the liver actively respond to toxicity when fish are exposed to metals like Hg (Vieira et al., 2009). In the present study, SOD activity in the liver of the flounder was induced at concentrations of 40 and 160 μg/L, whereas there was no significant alteration in CAT activity in any of the groups exposed to Hg. This may be related to the "competitive effects" provided by GPx activity. GPx can scavenge H_2O_2 in cells by catalyzing the reaction of H_2O_2 and GSH to H_2O and GSSG. GPx works best at low H_2O_2 concentrations, whereas CAT detoxifies H_2O_2 when the GPx pathway reaches saturation with substrate (Łukaszewicz-Hussain and Moniuszko-Jakoniuk, 2004). Thus, GPx serves as the first line of antioxidative defense against H_2O_2 (Oliveira et al., 2008). In the present study, Hg-induced hepatic GPx activity may have fully detoxified H_2O_2 produced in the liver, and as a result, CAT remained unchanged. Furthermore, the most important detoxification function of GPx is to terminate the propagation of the radical chain, and thus it protects membranes from oxidative damage (van der Oost et al., 2003). Consistent with our results, induced hepatic GPx activity has also been reported in matrinxã (Monteiro et al., 2010) and goby (*Pomatoschistus microps*; Vieira et al., 2009) exposed to Hg.

The liver is a major site of GSH synthesis. GSH is a major non-protein thiol that can serve as both a substrate for conjugation with xenobiotics and as an antioxidant to protect cellular components from oxidative stress (Larose et al., 2008; Faria et al., 2009). As with other species such as mullet (Thomas and Wofford, 1984), snakehead (Rana et al., 1995) and matrinxã (Monteiro et al., 2010), we observed increased hepatic GSH levels at all Hg concentrations in the liver of flounder. During exposure to Hg, GSH levels in the liver are elevated to enhance the hepatic uptake of amino acid substrates and the activities of biosynthetic enzymes with the aim of protecting fish from oxidative stress (Monteiro et al., 2010). However, it can not be taken as a general rule that Hg exposure increases hepatic GSH levels in fish livers. A decreased hepatic GSH level was reported in perch (*Anabas testudineus*) exposed to 166 μg/L Hg^{2+} for 24 h (Chatterjee and Bhattacharya, 1984), whereas Elia et al. (2003) reported that hepatic GSH levels were not altered in catfish exposed to 70 or 140 μg/L Hg^{2+} for 10 d. Together, these observations suggest that the change in GSH levels in the liver in response to Hg exposure is species-specific and is affected by experimental factors (such as exposure dose and duration) and/or other environmental factors. We did not observe differences in hepatic MDA content in flounder exposed to different concentrations of Hg. This suggests that antioxidants in the liver responded to low levels of Hg exposure and effectively protected the liver from LPO damage. The effectiveness of the antioxidant defense mechanism may be partly attributed to the simultaneous induction of hepatic SOD, GPx, and GSH.

The kidney receives and filters most of the post-branchial blood flow. The primary function of the kidney is to detoxify and eliminate toxic substances (Üner et al., 2005). Our results suggest that the antioxidative response of the kidney is less sensitive than in the gills or liver. In the kidney, we observed a significant change in SOD activity (increased by 48.9%) and GSH level (increased by 99.1%) in fish exposed to 160 μg/L and in CAT activity (decreased by 35.6%) in fish exposed to 40 μg/L, relative to the controls. Similarly, increases in SOD activity and GSH content have been documented in the kidney of Atlantic salmon (*Salmo salar*; Berntssen et al., 2003) and snakehead (Rana et al., 1995). Although little is known about the changes in CAT activity in the kidney of fish during Hg exposure, other metals (e.g., cadmium, zinc, and chromium) appear to inhibit CAT activity in the kidney (Atli et al., 2006). This effect may be due to the excessive generation of H_2O_2, causing inactivation of CAT. We did not observe differences in GPx and GST activity in fish exposed to different concentrations of Hg. Despite the increases in SOD activity and GSH level (used to scavenge ROS and inhibit LPO) in the kidney, the

lack of change in other related enzymes (e.g., GPx and GST) resulted in the antioxidant response being insufficient to scavenge the excess ROS when Hg concentrations exceeded 40 and 160 μg/L. As a result, MDA content increased significantly at these concentrations. Similarly, increased LPO levels have also been observed in the kidney of snakehead and Atlantic salmon exposed to Hg. This is likely because the kidney is the primary site of systemic contamination so is associated with high levels of lipid peroxidation (Rana et al., 1995; Berntssen et al., 2003).

Our data suggest that the oxidative response is more sensitive in the gills than the other two organs. This suggests that the gills may be more susceptible to oxidative stress than are the liver and kidney in flounder that are exposed to Hg. This is likely due to the fact that gills are the first and primary target of waterborne Hg so they play a significant role in metal uptake, storage, and transfer. This is consistent with observations that the antioxidant potential of gills is weaker than that of other organs in fish (Pandey et al., 2001).

5 CONCLUSION

The accumulation of Hg in flounder was tissue-specific in the order of kidney≈ liver> gills> bone> muscle. Furthermore, the accumulation was correlated with the exposure concentration. Exposure to Hg at concentrations of 5–160 μg/L inhibited the fish growth. Antioxidants responded differently to Hg exposure to cope with LPO induction, and the response was tissue-specific and dose-dependent. The antioxidant defense system effectively protected the liver from LPO damage but failed to protect the gills and kidney from LPO damage. Oxidative stress biomarkers in the gills were more sensitive than those in the liver and kidney to Hg exposure. Thus, the gills appeared to have potential as bioindicators for evaluating Hg toxicity in juvenile flounder.

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