



Tissue-Specific Accumulation and Antioxidant Defenses in Flounder (*Paralichthys olivaceus*) Juveniles Experimentally Exposed to Methylmercury

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Abstract

Methylmercury (MeHg) is the most toxic form of mercury and can accumulate in the cells of marine organisms, such as fish, causing adverse effects on various physiological functions. This study examined MeHg accumulation and its toxicological role in antioxidant defenses in tissues, including the liver, gills, and muscle of flounder (*Paralichthys olivaceus*) juveniles. After 30 d of MeHg exposure (0, 0.1, 1.0, 10.0, and 20.0 $\mu\text{g L}^{-1}$), the accumulation of MeHg in the three tissues correlated positively with the concentration of MeHg and exhibited tissue specificity in the order of liver > gills > muscle. Among the antioxidant markers, the activities of SOD (superoxide dismutase) and GST (glutathione S-transferase) as well as the content of glutathione (GSH) in the liver and gills were induced at 0.1–10.0 $\mu\text{g L}^{-1}$ but repressed at 20.0 $\mu\text{g L}^{-1}$. The activities of SOD and GST and the content of GSH in the muscle significantly increased with increasing MeHg concentration. Catalase (CAT) activity in the liver was induced at 0.1–1.0 $\mu\text{g L}^{-1}$ but inhibited at 10.0–20.0 $\mu\text{g L}^{-1}$, whereas exposure to MeHg did not remarkably affect CAT activity in the gills and muscle. The levels of lipid peroxidation (LPO) increased dose dependently, showing tissue specificity with the highest level in the liver, then the gills, followed by muscles. Overall, higher sensitivity to oxidative stress induced by MeHg was detected in the liver than the gills and muscle. These findings improve our understanding of the tissue-specific accumulation of heavy metals and their roles in antioxidant responses in marine fish subjected to MeHg exposure.

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Mercury (Hg) is a widespread metal in aquatic and marine environments, and its primary sources are both natural (active volcanoes, forest fires, cinnabar, and fossil fuels) and anthropogenic (hydroelectric, mining, pulp and paper industries, incineration of municipal and medical waste, and emissions from coal-using power plants), although the latter are of the most concern (Zhang and Wong 2007; Pereira et al. 2019). The speciation of Hg released into the aquatic environment is related to the biogeochemical characteristics of the environment, which are affected by many factors, such as dissolved organic carbon (DOC), dissolved organic matter (DOM), soft ligands, sulfide, suspended matter, redox, and pH conditions in sediments (Pereira et al. 2019). Hg^{2+} is the most stable form in natural waters (Langston and Bebianno 1998) and is the main form methylated into MeHg by a bacterially mediated process (Fitzgerald et al. 2007; Wu et al. 2018). Usually, methylation occurs in sediments, particularly at the interface with water, which can be considered a significant source of MeHg to the water column (Kim et al. 2006; Brown et al. 2015; Cesário et al. 2017). Various forms of Hg, such as ethylmercury, MeHg, and HgCl_2 , can be absorbed

by aquatic organisms via food intake and water filtration, transferred up the food chain, and eventually accumulated in upper consumers (Jewett et al. 2003; Guardiola et al. 2016; Graves et al. 2017). Such accumulation can result in high concentrations of MeHg in top predators, such as carnivorous fish, eventually threatening various physiological and biochemical processes in the organisms.

Fish accumulate MeHg in biological tissues either by ingestion or by direct exposure through their body, but accumulation is rarely uniform in all tissues. Uptake pathways of MeHg differ substantially among tissues, such as the liver, gills, kidneys, brain, and muscle (Wang and Wong 2003; Mela et al. 2007; Maulvault et al. 2016; Peng et al. 2016). Previous studies indicated that the pattern of MeHg distribution in fish was closely related to tissue-specific bioavailability, uptake rate, and mechanisms of MeHg (Leaner and Mason 2004; Peng et al. 2016). It is commonly believed that organic Hg, especially MeHg, is more neurotoxic than inorganic Hg and accumulates easily in the central nervous system (CNS) of fish (Pletz et al. 2016; Cariccio et al. 2018). Previous studies have demonstrated the biochemical and physiological effects of mercury on fish CNS and sensory structures as well as behavioral shifts (Pereira et al. 2019), although many questions related to the toxicokinetics and the mechanisms underlying these biochemical and physiological effects have not yet been fully answered. However, MeHg accumulation in fish, above a certain level, can disturb various physiological processes in various tissues (Arini et al. 2016), especially by oxidative damage to fish body tissues, which has been demonstrated to be a general pathway of MeHg toxicity (Larose et al. 2008; Cappello et al. 2016b; Strungaru et al. 2018) associated with increased reactive oxygen species (ROS) production. Generally, MeHg accelerates the generation of ROS (such as superoxide anion O_2^- , hydroxyl radicals $\cdot OH$, hydrogen peroxide H_2O_2 , alkoxy radical $RO\cdot$, and lipid hydroperoxide LOOH), which may ultimately lead to cell and tissue damage in fish (do Nascimento et al. 2008). To counteract oxidative stress, fish have developed a sophisticated array of antioxidant defense systems that are species specific and dependent on developmental stage (Larose et al. 2008; Cambier et al. 2012; Mozhdeganloo et al. 2015; Maulvault et al. 2017). Fish react by enhancing the levels of antioxidants (e.g., catalase (CAT); superoxide dismutase (SOD); glutathione (GSH); glutathione S-transferase (GST)) to participate in MeHg detoxification and ROS elimination (Larose et al. 2008). If the antioxidant defense capacity is overwhelmed by ROS production oxidative stress occurs, potentially causing intracellular damage and lipid peroxidation (LPO) in various tissues (Brandão et al. 2015; Mozhdeganloo et al. 2015; Cappello et al. 2016a).

Flounder (*Paralichthys olivaceus*) is an essential species for fisheries and is commonly found in the waters of

northern Chinese coasts (Huang 2010). In the past few decades, human-centric activities, such as agriculture, industry, and urbanization, have grown very fast in coastal areas. The release of metals, such as Hg, into coastal waters is a potential threat to local populations and fishery habitats (Huang et al. 2012a). For example, the total Hg and MeHg concentrations in Jinzhou Bay of the Bohai Sea, a spawning and nursery ground for flounder, were reported to be 39–430 $ng L^{-1}$ and 0.05–0.28 $ng L^{-1}$, respectively (Wang et al. 2009). Moreover, MeHg can be biotransformed from Hg compounds, eventually increasing the levels of MeHg in aquatic organisms. Overdoses of MeHg could adversely affect the antioxidant defenses of fish, finally reducing the recruitment of flounder populations. The antioxidant defenses in flounder may react differently to tissue oxidative stress induced by MeHg, and the mechanisms underlying the antioxidant defenses need to be fully explained.

We hypothesize in the current study that MeHg accumulation in flounder juveniles is tissue specific and dose-dependent, and the antioxidant defenses in tissues function differently to cope with oxidative stress caused by MeHg exposure. This hypothesis was assessed by rearing flounder juveniles and exposing them continuously to varying MeHg concentrations for 30 d. After the exposure test, the metal accumulation, contents, or activities of antioxidants (CAT, SOD, GST, and GSH) and the tissue LPO levels (liver, gills, and muscle) in the flounder at early life stages (ELs) were evaluated. The flounder we used for the exposure test at ELs is sensitive to the toxicity of waterborne MeHg even at low levels, as demonstrated in previous studies. The gills, liver, and muscle tissues of fish have been widely used for toxicity tests of metals and persistent organic pollutants, which also have been identified as sensitive to investigated toxicants. Therefore, on the basis of reference to previous literature, we chose these three tissues in flounder juveniles after the MeHg exposure test to examine how MeHg influenced the contents or activities of antioxidants to cope with LPO levels, eventually revealing the tissue-specific antioxidant defenses of flounder juveniles against the oxidative stress caused by MeHg. These observations will illuminate the mechanisms underlying MeHg toxicity to flounder juveniles and provide information for evaluating the impacts of MeHg on the recruitment of wild flounder populations.

Materials and Methods

Fish for Experiments

Flounder juveniles (7.42 ± 0.39 cm in total length; 6.65 ± 0.49 g in body weight) were supplied by the Yellow Sea Fishery Station, Haiyang, Shandong Province, in July 2017. Before the toxicity test, 500 juveniles were

acclimatized in an indoor pond (2 m³) for 1 week in filtered flowing seawater. The primary chemical parameters (mean \pm standard deviation [SD]) of the seawater were pH 7.7 ± 0.2 , salinity 29.9 ± 0.7 , and dissolved oxygen 6.5 ± 0.2 mg L⁻¹. During acclimation, the temperature of the water was 26.2 ± 1.0 °C, and a photoperiod of 13L:11D was provided. Fish were fed with commercial pellets (Santong Central Feedstuff Co. Ltd., Shangqiu, China) three times per day. Feces and uneaten feed in the pond were removed regularly.

Test Protocol

The test for toxicity was performed in polyethylene tanks (300 L capacity) kept indoors for a 13L:11D photoperiod. The analytical reagent methylmercury chloride or CH₃ClHg (CAS No: 115-09-3; purity $\geq 99.5\%$; Sigma-Aldrich Chemical Co., USA) was used for tests. Flounder juveniles of similar size were treated with the blank control (0 $\mu\text{g L}^{-1}$) and MeHg (0.1, 1.0, 10.0, and 20.0 $\mu\text{g L}^{-1}$; per treatment in triplicate). Each tank was filled with 200 L of test solution, and 30 fish were stocked. The MeHg concentrations were sublethal to the fish, as determined by preliminary tests. More specifically, MeHg was determined to cause detrimental impacts on embryonic-larval flounder at 13–15 $\mu\text{g L}^{-1}$ concentrations of waterborne MeHg exposure, resulting in high mortality, morphological abnormalities, reduced growth and yolk absorption (Ren et al. 2019a). Moreover, MeHg at ≥ 10.0 $\mu\text{g L}^{-1}$ caused active antioxidant defenses and immune responses in larval flounder after 35 d of exposure (Ren et al. 2019b). Additionally, we referred to a few similar studies of waterborne MeHg exposure (nominal concentrations: 0.25–10.00 $\mu\text{g L}^{-1}$; duration time: ≤ 30 d) to juvenile fish to determine the test protocol for the present test (Zhou et al. 2001; Guardiola et al. 2016; Wu et al. 2018). Therefore, on the basis of these previous results, we selected 0–20.0 $\mu\text{g L}^{-1}$ and 30 d as the experimental concentrations and duration, respectively. During the exposure test, seawater and feeding management were identical to those used during fish acclimation. Each day, the test solutions in each tank were renewed thoroughly. Throughout the test, aeration was performed gently for all tanks and was stopped after 30 d of exposure of the fish to MeHg.

To accurately estimate the nominal MeHg levels in the test solutions, every 5 days beginning from Day 1 of the test, a solution sample was withdrawn (50 mL) for testing from one tank for each treatment, treated with 0.4% HCl, and kept at -20 °C in glass bottles (borosilicate) until analysis for MeHg.

At the end of the test, ten fish were arbitrarily sampled per tank to determine MeHg accumulation. After sacrifice (Metacaine, MS-222), the tissues (liver, gills, and muscle) were dissected. The tissue samples were then washed

thoroughly with chilled physiological saline (0.9% NaCl) from Cisen Pharmaceutical Co., Ltd. (Jining, China) and preserved in liquid nitrogen in centrifuge tubes rinsed with acid until MeHg determination. Another ten fish from each tank were identically sampled, dissected, pretreated, and preserved in liquid nitrogen for the measurements of biochemical markers.

The testing protocol was designed according to the recommendations of the Laboratory Animal Guideline for Ethical Review of Animal Welfare (Standardization Administration of China 2018) with consent from the Chinese Academy of Sciences, the Institute of Oceanology, Committee on the Ethics of Animal Experiments.

Chemical Analysis

In the solutions for analysis, the MeHg concentrations were determined by distilling samples of each unfiltered solution and performing aqueous ethylation followed by detection through a purge and trap-GC-AFS (Model III, Brooks Rand Labs, USA) as per EPA Method 1630 (United States Environmental Protection Agency 1998; Support Information in Supplementary Material). The ratio of the absolute difference of the measured and nominal concentrations relative to the nominal concentration, expressed as a percentage, defined the error in MeHg levels and was used to assess the accurate reflection of the nominal MeHg levels in the test solutions throughout the entire test.

The sampled liver, gill, and muscle tissues from ten juveniles per tank were collected as a single sample to estimate the accumulation of MeHg in dry weight ($\mu\text{g g}^{-1}$ DW). The samples were thawed, lyophilized and made into fine powder. The powdered samples were chemically analyzed by trap-GC-AFS detection (Model III, Brooks Rand Labs, USA) according to EPA method 1630 (United States Environmental Protection Agency 1998), as described earlier.

Biochemical Analysis

The sampled liver, gill, and muscle tissues from ten other juveniles per tank were collected as a single sample to estimate the biochemical markers (CAT, SOD, GSH, and GST) conferring antioxidant activity and malondialdehyde (MDA). After thawing, samples were surface-dried using absorptive paper, and the weight was measured (closest to 0.01 g of body mass). The test samples and physiological saline (0.9% NaCl; a 1:9 ratio by weight) were transferred to an ice-water bath and homogenized. After that, the homogenate was centrifuged for 15 min (3500 \times g, 4 °C) in an Eppendorf 5840R (Eppendorf AG, Hamburg, Germany). The supernatant was analyzed immediately to estimate the contents or activities of the chosen biochemical markers. The measurements for all specified markers were performed

using corresponding kits available from Jiancheng Bioengineering Institute (Nanjing, China). The absorbance was estimated on an Epoch2 microplate reader from Biotek Epoch Instruments, Inc. (Winooski, USA).

The CAT, SOD, and GST activities were assayed according to the protocols of Marklund and Marklund (1974), Beers and Sizer (1952), and Habig et al. (1974), respectively. The definition of one unit of SOD activity was the enzyme amount exhibiting 50% inhibition of the autooxidation rate of 0.1 mM of pyrogallol in 1 mL of reaction solution per mg protein per minute. The definition of one unit of CAT was the enzyme amount that catalyzed the degradation of 1 μM H_2O_2 per mg protein per minute. One unit of GST activity was defined as the enzyme amount that catalyzed the conjugation of 1 μM of CDNB with GSH per mg protein per minute. The absorbance was estimated at 450 nm for SOD, 405 nm for CAT, and 412 nm (with 1-chloro-2,4-dinitrobenzene as the fluorescent reagent) for GST. The activities of the three enzymes were expressed in units of U mg^{-1} prot.

The GSH content was determined as per Beutler (1963) by analyzing the sample with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) as the fluorescence reagent at 405 nm absorbance. The GSH content was presented in units of $\mu\text{mol g}^{-1}$ prot. The level of LPO was reflected by the measurement of MDA content, which was assayed by the method of Ohkawa et al. (1979). The definition of one unit of MDA content was the amount produced after thiobarbituric acid reaction per mg protein and expressed as nmol mg^{-1} protein. The absorbance was determined at 532 nm. Following Bradford (1976), the total protein was determined using bovine serum protein as the standard, and the absorbance was estimated at 562 nm.

Data Analysis

Data were presented as the mean \pm SD and assessed for the assumptions of homogeneity and normality of variance using the Kolmogorov–Smirnov test and Levene test, respectively. The data on biochemical marker contents or activities in tissues satisfied both assumptions and were analyzed by two-way ANOVA (analysis of variance) to examine the

function of the MeHg concentration on each biochemical marker among tissues. The MeHg accumulation data were logarithmically transformed for two-way ANOVA to examine the effect of the MeHg concentration on MeHg accumulation among tissues. In both two-way ANOVAs, for post hoc multiple comparisons, Tukey's test was applied between the means of MeHg treatments and the control or between the means of tissues for each variable. At $p < 0.05$, the differences were deemed significant. All statistical data were analyzed by using IBM SPSS 22 (SPSS Inc., Chicago, IL) for Windows.

A method for combining all measured biomarker responses into one general “stress index” termed “integrated biomarker response” (IBR) was used to compare the potential toxic effects of these treatments on fish tissues (Beliaeff and Burgeot 2002; Serafim et al. 2012; Feng et al. 2015). The biochemical data for each tissue and treatment were standardized, and Si values were scored for the selected markers (CAT, SOD, GSH, and GST) and MDA as per previously published procedures. The star plots in Excel (Office 2018) were generated using the Si scores. The area of the star plot indicates the IBR of each treatment, that is, the combined responses to the two stressors in terms of antioxidant defense. The detailed calculations were performed according to Beliaeff and Burgeot (2002).

Results

Level of Metal in Test Solutions and Accumulation in Tissues

Overall, the estimated concentrations of MeHg in the test samples were all close to the nominal values over the course of the toxicity test, with errors ranging from 7.5 to 12.4% (Table 1). MeHg accumulation showed clear tissue specificity and dose dependence in the flounder liver, gills, and muscle (two-way ANOVA, $p < 0.05$; Appendix Table 2).

In the liver, metal accumulations in all MeHg treatments were significantly higher than in the control (for all

Table 1 Methylmercury accumulations (mean \pm SD) in three tissues (liver, gills, and muscle) of flounder juveniles after MeHg exposure for 30 days

MeHg concentration ($\mu\text{g L}^{-1}$)		MeHg accumulation in tissues ($\mu\text{g g}^{-1}$)		
Nominal	Measured	Liver	Gills	Muscle
Control	$< 10^{-5}$	$0.01 \pm 0.001^{a***}$	$0.03 \pm 0.003^{a***}$	$0.01 \pm 0.002^{a*}$
0.1	0.09 ± 0.01 (10.0%)	$0.05 \pm 0.01^{b***}$	$0.09 \pm 0.004^{b***}$	$0.03 \pm 0.001^{b*}$
1.0	0.88 ± 0.02 (12.0%)	$0.96 \pm 0.09^{c***}$	$0.19 \pm 0.01^{c**}$	$0.14 \pm 0.001^{c*}$
10.0	8.76 ± 0.33 (12.4%)	$7.55 \pm 0.13^{d***}$	$6.41 \pm 0.15^{d***}$	$3.40 \pm 0.04^{d*}$
20.0	21.49 ± 1.05 (7.5%)	$13.38 \pm 0.06^{e***}$	$6.86 \pm 0.01^{d**}$	$3.22 \pm 0.03^{d*}$

Different asterisks or superscripts indicate significant differences between tissues at a MeHg concentration or between MeHg concentrations in a specific tissue (two-way ANOVA, Tukey's test, $p < 0.05$), respectively. Numbers in the parentheses are the errors of the measured MeHg concentrations in the test solutions

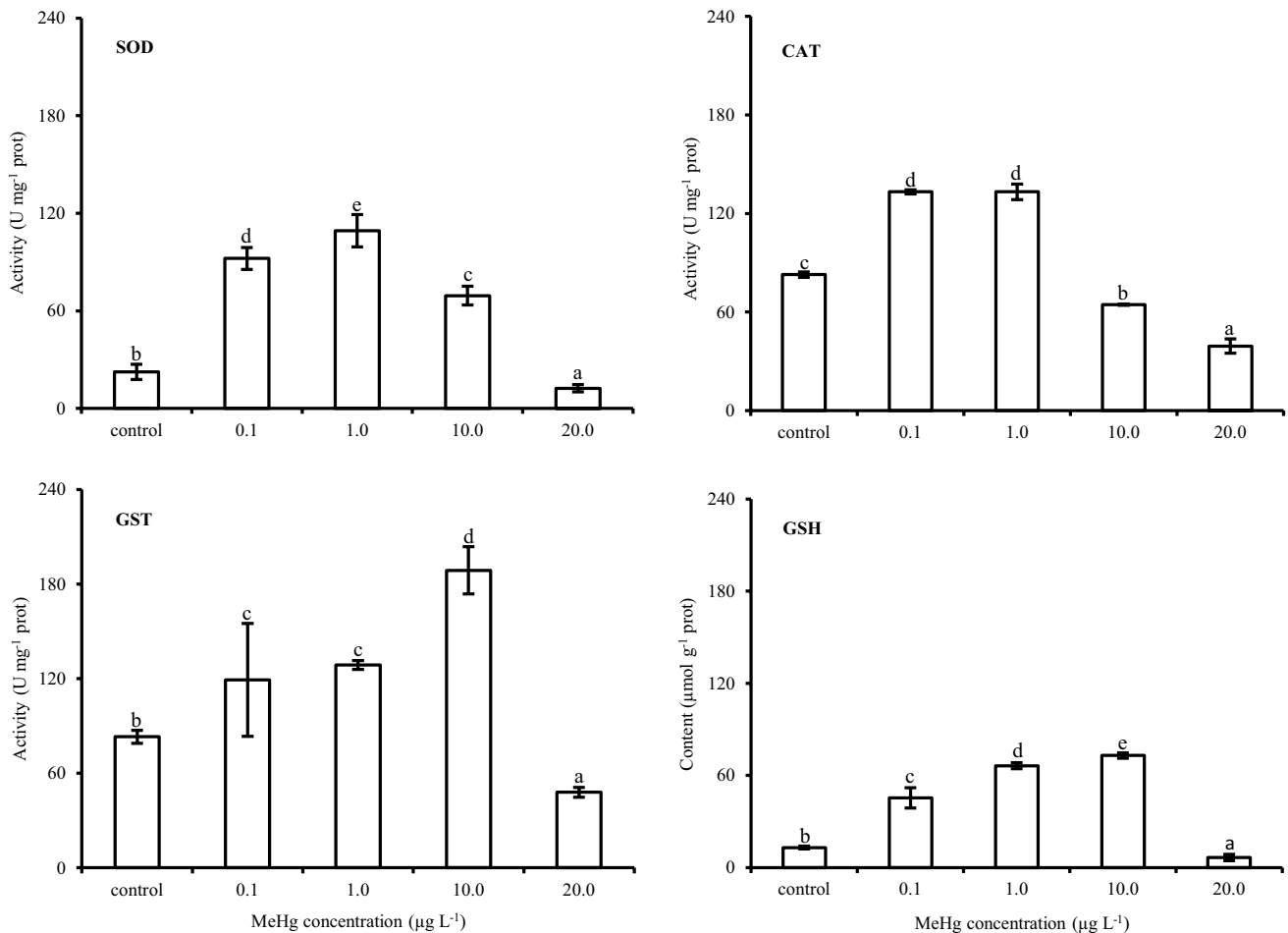


Fig. 1 Activities of SOD, CAT, and GST, as well as GSH content in the liver of flounder juveniles exposed to MeHg. Data are expressed as the mean \pm SD ($n=3$ per treatment). Treatments without the same

superscript were significantly different at $p < 0.05$ (one-way ANOVA, Tukey's test)

comparisons, $p < 0.05$ in Tukey's test; Table 1), reaching 5, 96, 755, and 1338 times the control levels at 0.1, 1.0, 10.0, and 20.0 $\mu\text{g L}^{-1}$, respectively. In the gills, the metal accumulations at 0.1, 1.0, 10.0, and 20.0 $\mu\text{g L}^{-1}$ were 3, 6, 214, and 229 times that in the control ($p < 0.05$ for all comparisons; Table 1), respectively. Metal accumulations in the muscle at 0.1, 1.0, 10.0, and 20.0 $\mu\text{g L}^{-1}$ were 3, 14, 340, and 322 times that in the control ($p < 0.05$ for all comparisons; Table 1), respectively.

The liver generally accumulated the highest level of MeHg, followed by the gills and muscle (for all comparisons, $p < 0.05$ in Tukey's test; Appendix Table 2). At 1.0, 10.0, and 20.0 $\mu\text{g L}^{-1}$, the metal accumulations in the liver were 5.1–6.9, 1.2–2.2, and 2.0–4.2 times those in the gills and muscle, respectively. Metal accumulations in the gills were 1.4–2.1 times those in the muscle at the same metal concentrations.

Antioxidant Responses to Metal Exposure in Tissues

The antioxidant responses, including the activities of CAT, SOD, and GST and the GSH contents in the tissues of flounder juveniles, were markedly affected by the MeHg concentration and tissue (two-way ANOVA, $p < 0.05$; Appendix Table 3).

In the liver, the activities of SOD and GST as well as GSH content increased markedly at 0.1–10.0 $\mu\text{g L}^{-1}$ and declined significantly at 20.0 $\mu\text{g L}^{-1}$ compared with those in the controls (Tukey's test, $p < 0.05$ for all comparisons; Fig. 1). The CAT activity increased significantly at 0.1–1.0 $\mu\text{g L}^{-1}$ and decreased significantly at 10.0–20.0 $\mu\text{g L}^{-1}$ compared with that in the control ($p < 0.05$).

In the gills, unlike CAT activity, the activities of SOD and GST and the GSH content showed the same trends as in the liver. The activities of SOD and GST and the GSH content were significantly increased at 0.1–10.0 $\mu\text{g L}^{-1}$ but

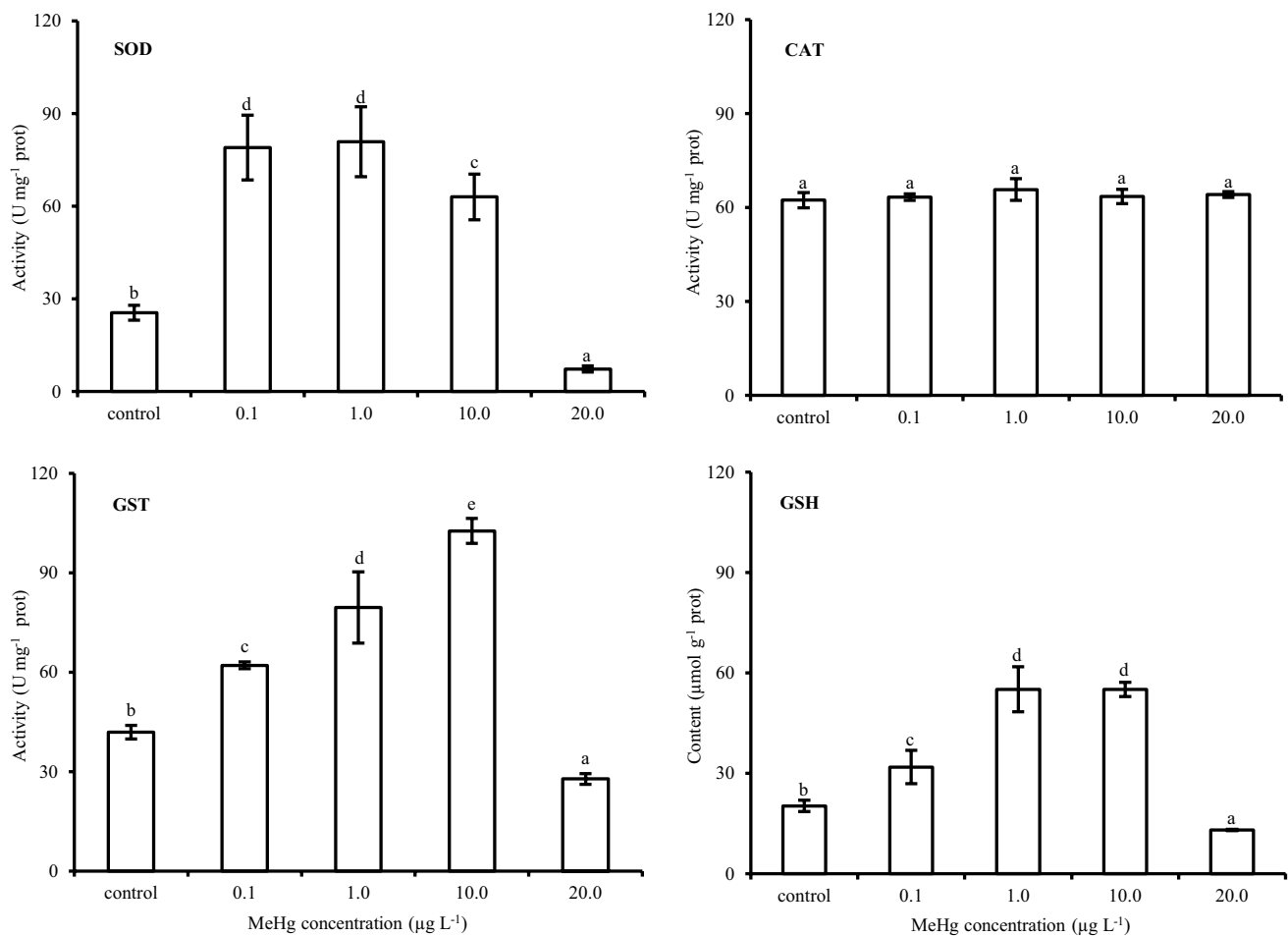


Fig. 2 Activities of SOD, CAT, and GST, as well as GSH content in the gills of flounder juveniles exposed to MeHg. Treatments without the same superscript were significantly different at $p < 0.05$ (one-way ANOVA, Tukey's test)

significantly decreased at $20.0 \mu\text{g L}^{-1}$ compared with those in the controls (Tukey's test, $p < 0.05$ for all comparisons; Fig. 2). However, the CAT activity did not differ significantly between the MeHg-treated samples and the control ($p > 0.05$).

In the muscle, the activities of SOD and GST and the GSH content were significantly increased at all MeHg concentrations compared with those in the controls (Tukey's test, $p < 0.05$ for all comparisons; Fig. 3). However, the activity of CAT did not vary markedly between the MeHg treatments and the control ($p > 0.05$).

LPO and IBR in Tissues

The levels of LPO showed a significant tissue-specific and dose-dependent pattern in the flounder (two-way ANOVA, $p < 0.05$; Appendix Table 4). In all three tissues, the levels

of LPO increased significantly with enhanced MeHg concentration ($p < 0.05$ for each tissue; Fig. 4). The LPO levels in the liver and gills were significantly higher in all MeHg treatments than in the controls (Tukey's test, $p < 0.05$). In the muscle, the LPO increased significantly at 10.0 – $20.0 \mu\text{g L}^{-1}$ relative to that in the control ($p < 0.05$), although it did not vary significantly between 0.1 – $1.0 \mu\text{g L}^{-1}$ and the control ($p > 0.05$).

At all MeHg treatments, the liver showed the highest level of LPO, followed by the gills and muscle ($p < 0.05$ for all comparisons; Appendix Table 4). The LPO levels in the liver were 2.9–5.7, 2.2–5.0, and 2.2–3.7 times those in the gills and muscle at 1.0, 10.0, and $20.0 \mu\text{g L}^{-1}$. The LPO levels in the gills were 1.7–2.3 times those in the muscle across the MeHg treatments. The LPO levels did not vary significantly among tissues in the control ($p > 0.05$).

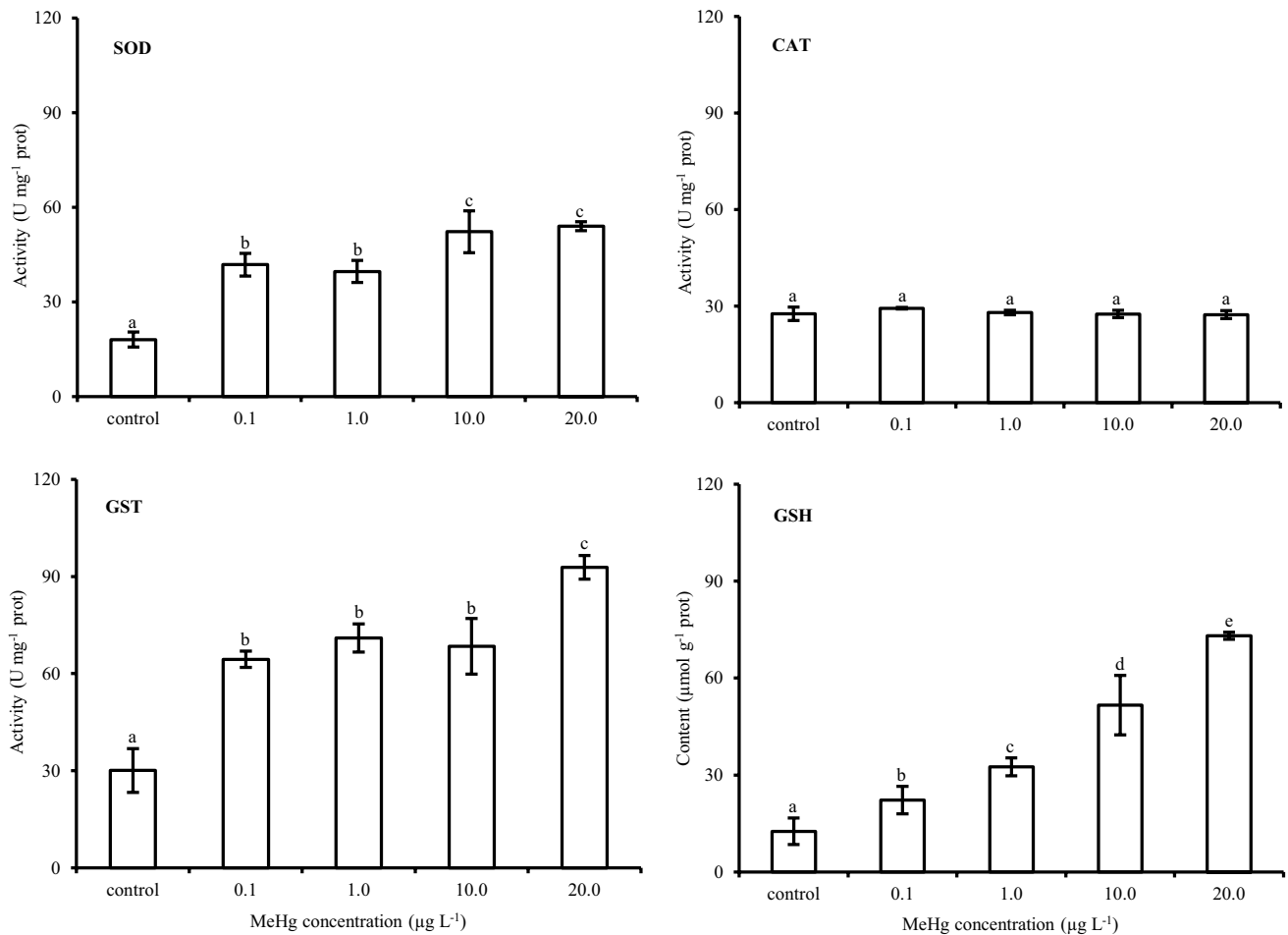


Fig. 3 Activities of SOD, CAT, and GST, as well as GSH content in the muscle of flounder juveniles exposed to MeHg. Treatments without the same superscript were significantly different at $p < 0.05$ (one-way ANOVA, Tukey's test)

The star plots that reflected the antioxidant defense responses to MeHg exposure in the three tissues are shown in Fig. 5. The IBR averaged 11.08 ± 7.36 (0.33–16.81), 3.16 ± 2.34 (0.16 ± 5.59), and 1.49 ± 1.32 (0.43 ± 3.36) in the liver, gills, and muscle of the flounder in the MeHg treatments, respectively (Fig. 6). The IBR ranking across treatments (tissue (MeHg concentration, $\mu\text{g L}^{-1}$)) was ordered as follows: liver (1.0) > liver (10.0) > liver (0.1) > gills (10.0) > gills (1.0) > muscle (20.0) > gills (0.1) > liver (control) > muscle (10.0) > muscle (1.0) > gills (control) > muscle (0.1) > liver (20.0) > gills (20.0) > muscle (control).

Discussion

Tissue-Specific Accumulation of MeHg in Fish

In this study, MeHg accumulation was enhanced in the three tissues of flounder juveniles in a significant dose-dependent manner. This finding is consistent with that observed in other species of fish, such as Atlantic salmon (*Salmo salar*; dietary exposure 0–10 mg kg^{-1} ; Berntsen et al. 2003), sweetlips (*Plectorhinchus gibbosus*; waterborne exposure 0.02–1.83 $\mu\text{g L}^{-1}$; Wang and Wong 2003), and white sturgeon (*Acipenser transmontanus*; at 0–1000 $\mu\text{g kg}^{-1}$ oral exposure; Huang et al. 2012b), under MeHg exposure. Methylmercury was not distributed consistently in the examined tissues of flounder

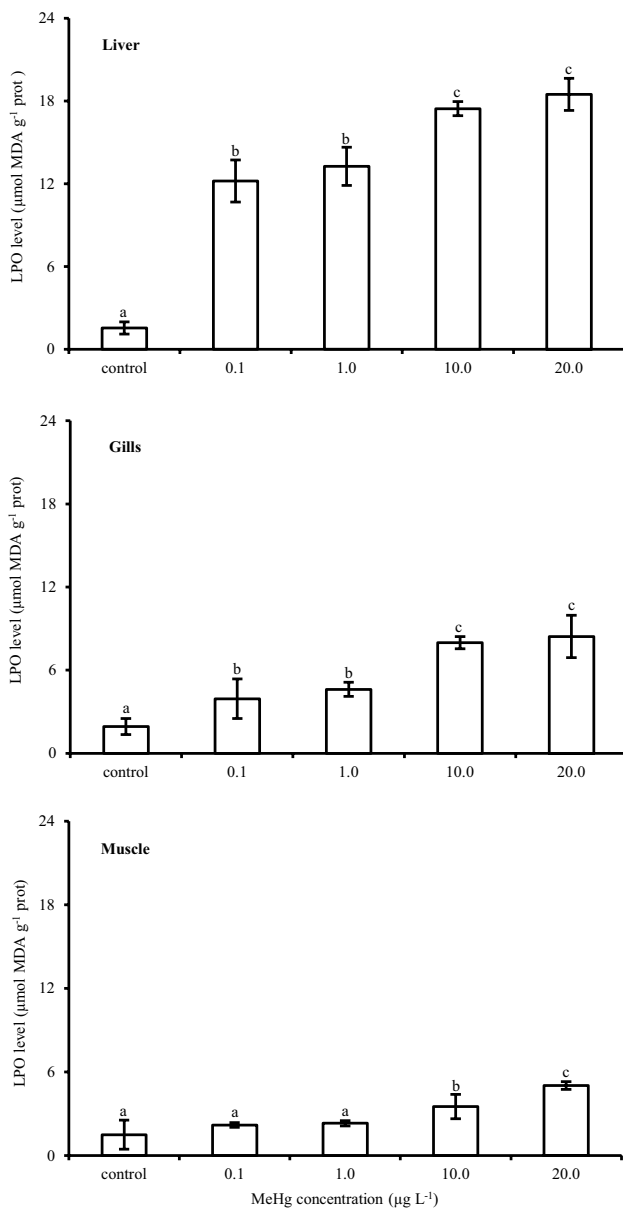


Fig. 4 The levels of LPO in the liver, gills, and muscle of flounder juveniles exposed to MeHg. Treatments without the same superscript were significantly different at $p < 0.05$ (one-way ANOVA, Tukey's test)

juveniles; rather, accumulation decreased in the order liver > gills > muscle, similar to the findings in other fish species, such as sheephead minnows (*Cyprinodon variegatus*; liver > gills > rest tissues; Leaner and Mason 2004), gilthead seabream (*Sparus aurata*; liver > muscle; Guardiola et al. 2016), and rabbitfish (*Siganus canaliculatus*; liver > intestine > gills > muscle; Peng et al. 2016). Generally, the liver of fish showed higher accumulation of

MeHg than other tissues did. This confirmed earlier findings of the relatively fast contamination of internal tissues by MeHg (Berntssen et al. 2003). Once MeHg is absorbed into the gills, skin, or intestinal tract of fish, it is likely to diffuse across the intestinal barrier into internal tissues (e.g., liver and kidney) due to its lipophilicity (Berntssen et al. 2003). Furthermore, high accumulation of MeHg is generally believed to be closely related to the functional and structural characteristics of the liver in fish. The liver, which is known to be highly metabolically active, has a vital role in metal detoxification and elimination (Huang et al. 2012a). Due to its association with thiol complexes, especially thiol groups (–SH), MeHg has high mobility in fish tissues. MeHg may accumulate in tissues, including the liver, which synthesizes proteins and other enzymes containing Cys residuals and –SH groups at high levels. Through a series of biological processes of detoxification and elimination, MeHg can be disassimilated and excreted (Huang et al. 2012b). In this study, MeHg accumulation in the gills of flounder juveniles ($0.09\text{--}6.86\ \mu\text{g g}^{-1}$ at all MeHg exposure concentrations) was comparable to that in the gills of white sturgeon ($5.99\text{--}17.15\ \mu\text{g g}^{-1}$) after short-term dietary MeHg exposure (Huang et al. 2012b). Because gills are one of the first organs to contact waterborne MeHg directly, a large amount of MeHg also accumulates in this tissue. Methylmercury in an aquatic environment can enter the gills directly through physiological activities, such as respiration, osmotic pressure, and acid–base balance regulation. Therefore, the gills are likely to accumulate MeHg to a relatively high level (Huang et al. 2012a). There are reports of MeHg accumulation at high levels in the gills of other fish species, such as sweetlips (gills > viscera > other tissues; Wang and Wong 2003) and rabbitfish (gills > liver > intestine > muscle; Peng et al. 2016), during waterborne MeHg exposure. In contrast, MeHg accumulations in the muscle of flounder juveniles in this study were only 14.6–60.0% and 33.3–73.7% of the levels in the liver and gills, respectively. The largest fraction of the fish weight is represented by the muscles; thus, the MeHg concentration here was generally lower than in other tissues (e.g., liver and gills) because of growth dilution. Some studies have shown that this tissue-specific discrepancy also may be related to the duration of exposure. For example, tissue MeHg accumulation in white sturgeon followed the order of gills > liver > muscle after 48 h of exposure (Huang et al. 2012b), whereas after 8 weeks of exposure, the order of tissue MeHg accumulation was muscle > gills > liver (Lee et al. 2011). Moreover, Guardiola et al. (2016) observed that although Hg accumulation in the muscle was always lower than that in

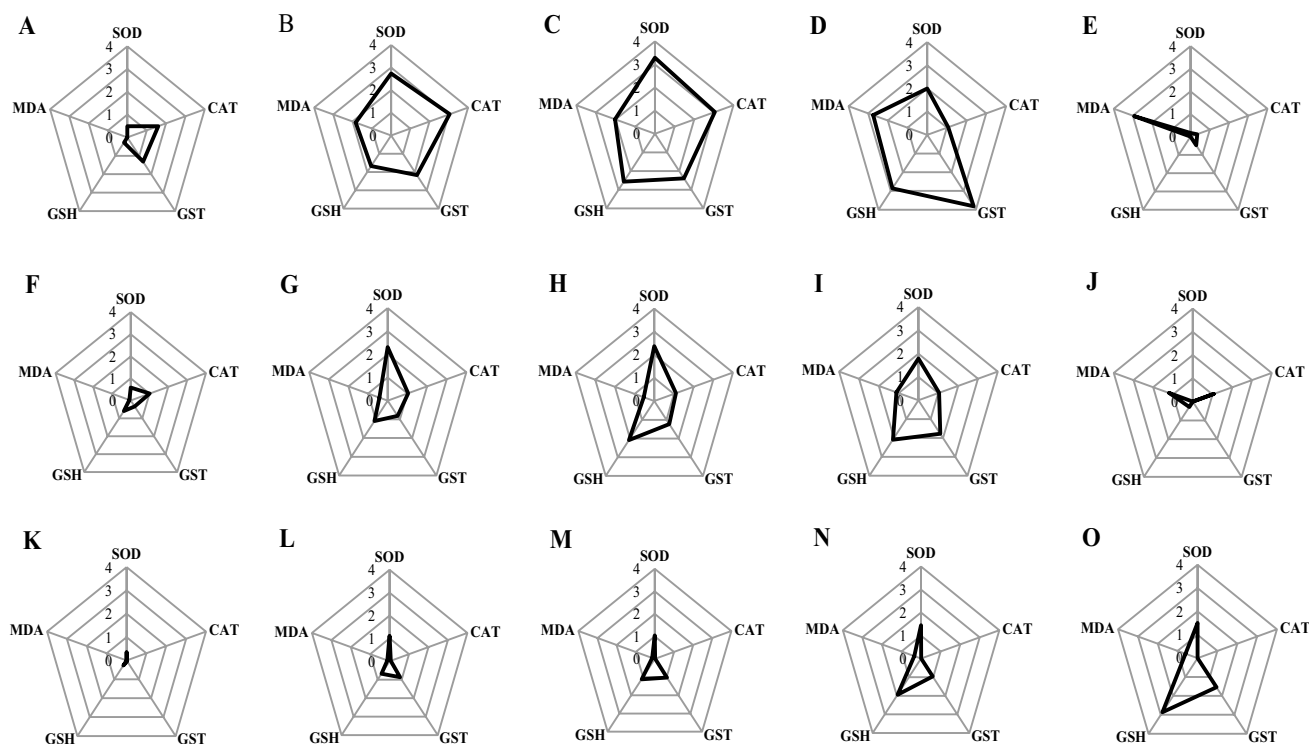


Fig. 5 Star plots of antioxidant biochemical markers among tissues of flounder juveniles exposed to MeHg (**a**, liver at control; **b**, liver at $0.1 \mu\text{g L}^{-1}$; **c**, liver at $1.0 \mu\text{g L}^{-1}$; **d**, liver at $10.0 \mu\text{g L}^{-1}$; **e**, liver at $20.0 \mu\text{g L}^{-1}$; **f**, gills at control; **g**, gills at $0.1 \mu\text{g L}^{-1}$; **h**, gills at

$1.0 \mu\text{g L}^{-1}$; **i**, gills at $10.0 \mu\text{g L}^{-1}$; **j**, gills at $20.0 \mu\text{g L}^{-1}$; **k**, muscle at control; **l**, muscle at $0.1 \mu\text{g L}^{-1}$; **m**, muscle at $1.0 \mu\text{g L}^{-1}$; **n**, muscle at $10.0 \mu\text{g L}^{-1}$; **o**, muscle at $20.0 \mu\text{g L}^{-1}$)

the liver of gilthead seabream under MeHg exposure, Hg accumulation in the muscle increased significantly as the duration extended from 2 d to 10 d or 30 d, whereas that in the liver decreased. These results suggested that muscle tissue acted as a final reservoir that gradually accumulated MeHg from other tissues. Likewise, flounder juveniles exposed to other metals exhibited similar outcomes (e.g., cadmium and inorganic Hg; Cao et al. 2012; Huang

et al. 2012a). The accumulation of MeHg with high tissue specificity in flounder juveniles indicated that following waterborne exposure, MeHg was first absorbed through the physiological activities of the gills and then concentrated in metabolically active tissues, such as the liver, and finally distributed widely in muscle tissue. In addition to species-specific factors, factors such as the exposure type (dietary or waterborne), the metal form (organic or inorganic forms), and the duration and dose of exposure greatly influence MeHg accumulation.

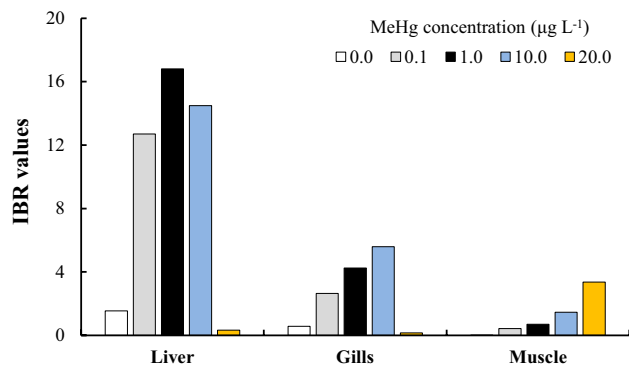


Fig. 6 Integrated biomarker response (IBR) of antioxidant defense among liver, gills, and muscle tissues in flounder juveniles under MeHg exposure across the treatments

Tissue-Specific Antioxidant Defenses of Fish Under MeHg Exposure

Methylmercury is known as an oxidative stressor (Antunes dos Santos et al. 2018) that can induce ROS formation, initiating an important mechanism of fish tissue injury through oxidative stress. To counteract oxidative stress, fish have developed a sophisticated antioxidant defense system involving antioxidant enzymes (such as SOD, CAT, and GST) and nonenzymes (e.g., GSH), as the modulation of antioxidant levels is an important adaptive response of organisms to adverse conditions (Mela et al. 2014; Mozhdeganloo et al. 2015; Guardiola et al. 2016; Maulvault et al. 2017). In a

normal scenario, antioxidant defense systems can eliminate intracellular ROS, preserving the cell redox state. Nevertheless, damage to membranes, DNA, and proteins in the cells of exposed fish occurs when ROS levels exceed the capacity of the antioxidant system to scavenge or when the antioxidant defenses fail (do Nascimento et al. 2008; Mozhdeganloo et al. 2015; Guardiola et al. 2016).

The CAT-SOD system normally constitutes the first line of defense against ROS induced by MeHg because of the ability of SOD to catalyze O_2^- and H^+ into H_2O_2 , which is the less toxic form, whereas CAT catalyzes the conversion of H_2O_2 into O_2 and H_2O (Maulvault et al. 2017). In previous studies, CAT and SOD activities in different tissues of experimental fish usually responded differently to MeHg exposure. For example, MeHg exposure did not affect SOD activity but decreased CAT activity in the liver of tiger tetra (*Hoplias malabaricus*; at 1.05–10.5 $\mu\text{g g}^{-1}$ injected concentrations for 5 d; Mela et al. 2014). In seabass (*Dicentrarchus labrax*), however, the SOD and CAT activities were increased significantly in the liver and brain, whereas those activities in the muscle were not significantly affected when fish were exposed to MeHg (at 8 mg kg^{-1} DW dietary concentration for 28 d; Maulvault et al. 2017). We also need to take into account the conditions of fish exposure, including the exposure duration and dose as well as experimental factors, which could generally influence the observations of SOD and CAT activities in various tissues. Guardiola et al. (2016) observed increased activities of CAT and SOD in the liver of gilthead seabream under short-term MeHg exposure (2 d) but not under extended exposures (10 or 30 d). In this study, the CAT and SOD activities in the livers of flounder juveniles were significantly increased at lower concentrations and decreased at higher concentrations. Tissue type also played a significant role in antioxidant responses to MeHg stress because of the increased sensitivity of liver enzymes compared with those in the gills and muscle. This may be because in fish, the liver is the major organ for the detoxification and excretion of toxic substances and has a critical role in redox metabolism (Berntssen et al. 2003; Huang et al. 2012a). Additionally, MeHg accumulation in the liver may induce these enzymes to eliminate the generated ROS. However, high MeHg exposure decreased the activities of enzymes in the liver of flounder, similar to the observations in tiger tetra (Neto et al. 2008). Maulvault et al. (2017) reported that a reduction in enzyme activity reflects a reduced capacity of the liver to eliminate ROS in response to MeHg. In addition, MeHg can directly bind to antioxidant enzymes, resulting in diminished protective action against the formation of ROS. We noticed an increase in SOD activity but a decline in CAT activity in flounder

juveniles exposed to 10.0 $\mu\text{g L}^{-1}$ MeHg. In general, a concurrent response in CAT and SOD activities was observed upon pollutant exposure, as revealed in previous studies (Guardiola et al. 2016; Maulvault et al. 2017). The excessive H_2O_2 produced by SOD might exceed the scavenging ability of CAT and eventually inhibit CAT activity. Overall, the same alteration of SOD activity as in the liver also was confirmed in the gills: SOD activity increased to defend against continuous ROS and oxidative stress at lower levels of MeHg exposure but decreased at higher exposure. However, no marked difference in CAT activity was observed in the gills of flounder after exposure to MeHg for 30 d. Monteiro et al. (2013) reported similar findings in the neotropical fish *Hoplias malabaricus* and attributed the results to the fact that the H_2O_2 level can be reduced by diffusion in the surrounding water, and the remainder can be eliminated by basal gill CAT activity. The lack of increase in CAT activity in the gills also may be associated with the competitive effects exhibited by glutathione peroxidase (GPx), which can regulate the level of H_2O_2 catabolism (Huang et al. 2012a). Methylmercury exposure increased SOD activity, but there was no effect of CAT activity in the muscles of flounder. MeHg accumulation in muscle was lower than in the liver and gills and might not have reached the threshold of inducing oxidative stress. Therefore, the first line of antioxidant defenses was sufficient to counteract the ROS-induced damage in muscle.

As a major nonprotein thiol, GSH can act as a substrate for conjugation with xenobiotics as well as an antioxidant to protect cellular components from oxidative stress induced by ROS (Larose et al. 2008). As with fish of other species, such as mullet snakehead (*Channa punctatus*; Rana et al. 1995) and matrinxã (*Brycon amazonicus*; Monteiro et al. 2010), exposed to inorganic Hg, we observed increased GSH levels in the liver and gills (0.1–10.0 $\mu\text{g L}^{-1}$) as well as muscle (0.1–20.0 $\mu\text{g L}^{-1}$) of flounder exposed to MeHg. Due to the high affinity of GSH to MeHg, the increase in GSH contents in these tissues might be an adaptive mechanism for binding to MeHg, thereby mitigating its toxicity. In addition, the increased GSH contents were explained as an increase in amino acid substrate uptake and increased activities of biosynthetic enzymes, leading to the protection of fish from oxidative stress (Monteiro et al. 2010). A significant decrease in GSH contents was observed in the liver and gills of the flounder treated with the highest MeHg levels. Similarly, GSH depletion was observed in the liver of rainbow trout (*Oncorhynchus mykiss*; Mozhdeganloo et al. 2015) and in the gills of golden grey mullet (*Liza aurata*; Cappello et al. 2016a). Methylmercury-induced decreases in GSH contents may occur because MeHg binds directly to the -SH groups

of GSH, thus reducing the available intracellular pool of GSH (Elia et al. 2003; Cappello et al. 2016a, b). Moreover, extreme oxidative stress may decrease GSH contents due to the oxidation of GSH (Monteiro et al. 2010).

GST is a GSH-dependent antioxidant enzyme that conjugates a wide variety of electrophilic compounds with GSH to form less toxic substances (van der Oost et al. 2003). The change trend of GST activities in the gills and liver ($0.1\text{--}10.0\ \mu\text{g L}^{-1}$) and muscle ($0.1\text{--}20.0\ \mu\text{g L}^{-1}$) was consistent with that of GSH in this study, indicating an adaptive and protective response to MeHg accumulation in flounder juveniles. Similar results have been reported on the increased GST activity in the gills of golden grey mullet (from a wild Hg-polluted area; Cappello et al. 2016a) and in the liver, brain, and muscle of seabass (at $8\ \text{mg kg}^{-1}$ DW dietary concentration for 28 d; Maulvault et al. 2017) when exposed to MeHg. It is known that the GST enzyme can conjugate with GSH to resist the toxicity of MeHg and has the ability to scavenge LOOH, similar to other antioxidants, such as GPx, in fish tissues (Cappello et al. 2016a). Conversely, GST activities in the liver and gills decreased at the highest MeHg concentration, similar to those observed in the liver of tiger tetra (at $1.05\text{--}10.5\ \mu\text{g g}^{-1}$ injected concentrations for 5 d; Mela et al. 2014). Because GST uses GSH as a co-factor, the decline in levels of GSH in the liver and gills also might inhibit GST activity. Methylmercury might be removed from the system by direct conjugation with GSH by the GST enzyme followed by excretion, resulting in decreased GSH contents, as well as GST activity, especially in the liver and gills of flounder with high MeHg accumulation. Compared with that in the liver and gills, the MeHg accumulation in muscle was relatively low, and the enhancement in GSH contents and GST activities can effectively inhibit ROS production and prevent oxidative damage.

LPO is a significant contributor to cellular function loss under oxidative stress and is a self-propagating, complex, and highly destructive process that enhances cell membrane rigidity. Therefore, LPO often is used as a valuable oxidative stress marker (Berntssen et al. 2003; Monteiro et al. 2013; Mozhdeganloo et al. 2015). After 30 d of MeHg exposure, the LPO level in the liver, gills, and muscle of flounder juveniles showed dose-dependent and tissue-specific trends. Although most of the antioxidants (i.e., CAT, SOD, GST, and GSH) detected in all the investigated tissues of the flounder exposed to MeHg were significantly increased, the antioxidant defenses were not sufficient to protect against LPO. Significant increases in LPO levels occurred in all three tissues. The levels were highest in the liver and lowest in the muscle, which might be associated with tissue-specific antioxidant defenses and MeHg accumulation. Similarly,

significant increases in the levels of LPO induced by MeHg were observed in the livers of tiger tetra (Neto et al. 2008) and rainbow trout (Mozhdeganloo et al. 2015). However, Berntssen et al. (2003) did not observe oxidative damage in the liver, although the accumulation of MeHg in the liver was higher than that in other tissues. They attributed the findings to the redox-defense system's adaptive response (such as SOD and GPx) in the liver, which responded actively to MeHg-induced oxidative stress. Similar to the results in the liver, the LPO level in the gills of flounder increased significantly in a dose-dependent manner. This observation is consistent with the common belief that exposure to Hg compounds causes LPO in fish gills (Monteiro et al. 2010, 2013; Huang et al. 2012a). Gills are the first target of waterborne MeHg and are one of the main absorption sites because of their permeability and large surface area. In this study, the increase in LPO levels in the gills of flounder juveniles indicated that the gills were vulnerable to oxidative damage. In contrast, a significant decline in the levels of LPO in the gills of golden grey mullet from MeHg-polluted water was reported by Cappello et al. (2016a), who suggested that this might be related to enhanced membrane stabilization through related metabolic activities. Compared with the liver and gills, muscle is the terminal tissue of the MeHg transfer chain. The muscle of flounder was affected least by oxidative stress under MeHg exposure. The activities of GST and SOD and the GSH content were significantly increased in the muscle at all MeHg concentrations, indicating a possible adaptive response of fish with an effective antioxidant system, which could effectively scavenge ROS and prevent oxidative damage in muscle. The increases in GST activity in flounder juveniles also might be related to the severe oxidative stress reflected by the increased LPO levels in the liver, gills, and muscle with increasing MeHg concentration.

As previously suggested (Beliaeff and Burgeot 2002; Kim et al. 2010; Serafim et al. 2012; Feng et al. 2015), the IBR often reflects the toxicity stress or "health status" of organisms by combining biomarker signals under analysis, especially their antioxidant responses to stressors. In this study, the large IBR values generally corresponded to high MeHg exposure treatments in the liver, gills, and muscle, implying increased stress with increased MeHg concentrations. These observations might be closely related to the high accumulation of MeHg in flounder tissues, which induced ROS production, oxidative stress, and tissue damage, eventually causing severe injuries to the antioxidant system. To avoid oxidative damage, antioxidants, such as SOD, CAT, and GST as well as GSH, responded actively to toxicity stress, although they acted differently under

different exposure conditions. In the liver, the high values of IBR at 0.1–1.0 $\mu\text{g L}^{-1}$ were associated with SOD and CAT, whereas the values at 10.0 $\mu\text{g L}^{-1}$ were associated with GST, as shown by the star plots in Fig. 5. Additionally, the large values of IBR in the gills were closely related to GST and GST at 1.0–10.0 $\mu\text{g L}^{-1}$. These findings might be due to the tissue-specific antioxidant defenses against MeHg toxicity. As previously mentioned, SOD and CAT act as the first line of defense for the antioxidant system, playing major roles in eliminating ROS in a timely manner to avoid oxidative damage. These metabolic activities occur more frequently in liver, which is the main detoxification site (Berntssen et al. 2003; Huang et al. 2012a; Maulvault et al. 2017). In this study, part of the MeHg entered the fish body through the gills and accumulated easily in this tissue, causing the active responses of the GSH-GST systems to combine with MeHg and eventually transport it out of the body (van der Oost et al. 2003; Larose et al. 2008; Cappello et al. 2016a, b). However, at the highest exposure (20.0 $\mu\text{g L}^{-1}$), the IBR values in the liver and gills were all reduced compared with the levels at other exposures, which did not seem to correspond to the meaning of the IBR index that high IBR values revealed high toxicity stress. This finding was closely related to the significantly decreased activities or contents of antioxidants due to high MeHg accumulation in these two tissues in flounder. In contrast, unlike CAT, the levels of SOD, GST, and GSH all contributed to the dose-dependent IBR values in the muscle, probably as a consequence of the continuously increased oxidative stress with increasing MeHg accumulation. In this case, various members of the antioxidant system acted synchronously on MeHg toxicity. Above all, the ranking of IBR revealed that the antioxidant biochemical markers of the flounder juveniles reacted differently to survive oxidative stresses at different MeHg exposure concentrations, which was closely related to the tissue-specific antioxidant defenses and MeHg accumulation.

Conclusions

After 30 d of waterborne exposure, MeHg accumulation in flounder juveniles was dose dependent and tissue specific in the order liver > gills > muscle. The flounder exhibited tissue-specific antioxidant defenses in response to MeHg exposure, which may indicate different rates of production of free radicals and varying antioxidant potential in the three investigated tissues, as well as different MeHg

accumulation in tissues. These data show that the LPO levels in the three tissues increase in a dose-dependent manner, indicating that antioxidant induction is not sufficient to scavenge the ROS generated by MeHg. Compared with other tissues, the liver is the most susceptible to oxidative damage and the most sensitive to MeHg intoxication in flounder juveniles. The experimental data obtained from flounder juveniles in this study can serve as a useful reference for comparing biomarker responses with those in fish living in MeHg-contaminated waters in the wild. The increase in LPO levels and alterations in antioxidant defense systems can be used as biomarkers in assessing MeHg toxicity in aquatic ecosystems. Further studies must be performed to evaluate the mechanisms of MeHg accumulation and release in different tissues of fish for a better understanding of the physiological effects of MeHg in natural populations.

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Authors' Contributions The study conception and design were mainly performed by Liang Cao, Shuozeng Dou, and Zhonghua Ren. Material preparation, data collection, and analysis were mainly performed by Jinhu Liu, Dayan Zhou, and Wenting Cui. The first draft of the manuscript was written by Zhonghua Ren. The revision and editing of revised manuscript were mainly performed by Zhonghua Ren, Zhenbo Lv, Jinhu Liu, and Liang Cao. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Appendix

See Tables 2, 3 and 4.

Table 2 Results of two-way ANOVA analysis of MeHg accumulation affected by MeHg concentration and tissue of flounder juveniles

Factor	SS	df	MS	F	p value
Concentration	47.78	4	11.94	11,437	<0.001
Tissue	0.63	2	0.32	303	<0.001
Interaction	1.61	8	0.20	193	<0.001

Table 3 Results of two-way ANOVA analysis of the activities or contents of each biochemical marker affected by MeHg concentration and tissue of flounder juveniles

Biochemical marker	Factor	SS	df	MS	F	p value
SOD	Concentration	24,365	4	6091	158	<0.001
	Tissue	3010	2	1505	39	<0.001
	Interaction	12,925	8	1616	42	<0.001
CAT	Concentration	3846	4	961	56	<0.001
	Tissue	17,192	2	8596	502	<0.001
	Interaction	24,237	8	3030	177	<0.001
GST	Concentration	28,361	4	7090	57	<0.001
	Tissue	26,833	2	13,416	108	<0.001
	Interaction	22,831	8	2854	23	<0.001
GSH	Concentration	17,777	4	4444	249	<0.001
	Tissue	1938	2	969	54	<0.001
	Interaction	1641	8	205	11	<0.001

Table 4 Results of two-way ANOVA analysis of MDA contents affected by MeHg concentration and tissue of flounder juveniles

Biochemical marker	Factor	SS	df	MS	F	p value
MDA	Concentration	448	4	112	180	<0.001
	Tissue	758	2	379	608	<0.001
	Interaction	211	8	26	42	<0.001

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