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Antioxidative responses and bioaccumulation in Japanese flounder larvae and juveniles under chronic mercury exposure

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ABSTRACT

This study investigated the sub-lethal effects of waterborne mercury on growth, bioaccumulation and antioxidative responses of larvae and juveniles of Japanese flounder (*Paralichthys olivaceus*). Fish were exposed to 0–10 μg $Hg^{2+}L^{-1}$ solutions from embryonic to the juvenile stages for 80 days. Antioxidative responses to mercury exposure were studied in metamorphosing larvae (18 days post hatching, dph), settling larvae (33 dph) and juveniles (78 dph). Results showed that increasing mercury concentration led to increased mercury bioaccumulation and reduced flounder growth. Of the antioxidants investigated, superoxide dismutase (SOD) and catalase (CAT) activities at the three developmental stages were sensitive to mercury exposure and increased with increasing mercury concentration. Glutathione (GSH) content was elevated in metamorphosing larvae, but decreased in juveniles as mercury concentration increased. Glutathione-*S*-transferase (GST) activity did not significantly vary with mercury concentration in either larvae or juveniles. Mercury exposure did not affect malondialdehyde (MDA) content of larvae, but significantly increased MDA content of juveniles. Results suggest that flounder larvae and juveniles have the potential to manipulate the levels of antioxidants such as SOD, CAT and GSH, which protect flounder from oxidative stress induced by mercury exposure. These antioxidants could serve as biomarkers of mercury contamination in the aquatic environment.

1. Introduction

Mercury is one of the most toxic metals in the aquatic environment (Vieira et al., 2009). Mercury can cause various damages to the central nervous system and endocrine system of fish that can induce permanently detrimental effects on their development through all life stages (Alvarez et al., 2006). Mercury has also been proven to disrupt vital functions such as reproduction, osmoregulation, orientation, foraging, predator recognition and communication in fish (Zillioux et al., 1993; Weis and Weis, 1995). Early life stages (ELS) of fish are particularly sensitive to mercury exposure and developmental problems have been reported in fish embryos, larvae and juveniles exposed to mercury, even at μg L^{-1} exposure levels (Dave and Xiu, 1991). These problems include inhibition of enzyme activities, yolk membrane rupture, reduction in hatching success and survival, promotion or delay in hatching time, embryonic and larval malformations, abnormal swimming behavior and low feeding ability (Weis and Weis, 1995; Latif et al., 2001; Zhou et al., 2001; Devlin, 2006). Meanwhile, the aquatic environment is often at risk of mercury pollution from natural and anthropogenic sources (Verlecar et al., 2008). Increased loads of mercury in the aquatic environment usually lead to elevating mercury bioaccumulation in fish tissue, thereby resulting in various health problems in fish consumers (Guilherme et al., 2008a).

Like other toxic metals, mercury usually triggers redox reactions generating reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), lipid hydroperoxide (LOOH), alkoxyl radical ($RO^{\bullet}$) and singlet oxygen ($^1O_2$) in fish (Livingstone, 2001; Larose et al., 2008; Verlecar et al., 2007). ROS cause oxidative damage to biological molecules such as lipids, proteins and DNA, which consequently alters the structure and function of molecular membranes and ultimately leads to cell and tissue damages (Livingstone, 2001; Ritola et al., 2002; Verlecar et al., 2008). Under normal physiological conditions, fish could detoxify and remove ROS in cells by antioxidative defense systems and thus maintain a balance between ROS generation and neutralization. The antioxidative defense systems are comprised of enzymatic and non-enzymatic components such as superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase (GST), glutathione peroxidase (GPX), glutathione (GSH) and β-carotene (Ritola et al., 2002). If the antioxidants are not effective in detoxifying ROS, the antioxidative defense systems may be overwhelmed. In such cases, oxidative stress will occur in fish tissues and lead to various types of intracellular

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damage such as lipid peroxidation (LPO) (Ritola et al., 2002; Elia et al., 2003; Guilherme et al., 2008b). In recent years, oxidative stress biomarkers have been increasingly investigated to explore the mechanisms by which metals like mercury may act toxicological effects on the biological processes of fish (Elia et al., 2003; Guilherme et al., 2008a,b). These studies find that the antioxidative defense mechanisms fish develop to counteract oxidative stress are species-specific and depend primarily on the type of exposure (e.g. waterborne or dietary), the form of mercury (e.g. elemental, inorganic or organic), the dose and the exposure duration.

Japanese flounder (*Paralichthys olivaceus*) is a demersal fish and is widely distributed along the northern Chinese coastal waters. It is of commercial importance, but the wild population has drastically decreased in recent years due to overfishing and marine pollution. In the Bohai Sea, where the spawning and nursery grounds of flounder are located, heavy metals represent one group of common pollutants. Of them, mercury concentration ranged from 0.002 to 0.15 μg L^{-1} in these waters. However, it reaches as high as 2.59 μg L^{-1} in some extreme sites like the discharging outlets of industrial waste water (Liu et al., 2003). Meanwhile, in a number of recent fishery investigations, abnormally developed or undeveloped dead flounder embryos and larvae were often observed in this area. Biological damages of metals such as mercury to their ELS are thereby considered as potential risks for the decline of fishery resources in this region (unpublished data). Therefore, information about the toxicity of pollutants including mercury to the ELS of flounder is essential to better understand their development, survival and growth in nature. To our knowledge, however, there is little literature about the oxidative stress of flounder to pollutants, and even less is known about larvae and juveniles exposed to mercury.

The main purpose of the present study was to investigate the antioxidants of flounder larvae and juveniles responding to chronic mercury exposure at sub-lethal concentrations. To do so, we conducted an investigation of mercury bioaccumulation in flounder tissue and the effects of mercury exposure on their growth. We also examined the effects of long-term sub-lethal mercury exposure on their antioxidative responses. The antioxidants investigated in the present study included SOD, CAT, GST and GSH. The oxidative damage was evaluated by determining LPO and was measured by malondialdehyde (MDA) content.

2. Materials and methods

2.1. Fish rearing and experimental design

Fertilized eggs of Japanese flounder (*P. olivaceus*) were obtained from Shunyuan Fish Hatchery Station, Rizhao, China. Eggs were kept in incubators at an estimated density of 800 eggs L^{-1} at 18 ± 1 °C for 2 h. During this time, the normally developing embryos floated in the surface water, while the unfertilized or dead embryos settled on the bottom of the incubators. Only floating viable embryos were used for toxicity tests.

Approximately 1000 viable fertilized eggs were transferred to each of the 30-L polyethylene experimental tanks. Tanks were placed randomly in an indoor pond with a constant temperature of 18 ± 1 °C (maintained by thermostat-controlled water baths). A light regime of 14 h of light and 10 h of dark was provided. After 2 h of acclimation, eggs were exposed to waterborne mercury treatments. Four nominal concentrations and one control were set at 0 (control), 0.2, 1, 5, 10 μg Hg^{2+} L^{-1}. We conducted three replicates at each concentration setting. Analytical-reagent mercury chloride ($HgCl_2$, purity>99.5%; CAS No: 7487-94-7; Sigma-Aldrich Chemical Co., USA) was used as a test chemical. A stock solution of 100 mg Hg^{2+} L^{-1} was prepared by dissolving mercury chloride in deionized water every day. This solution was equilibrated for 24 h and then diluted to produce the designated Hg^{2+} concentrations in filtered seawater (hardness, 6124.6 ± 272.3 mg L^{-1} as $CaCO_3$; pH, 8.1 ± 0.1; dissolved oxygen concentration, 7.5 ± 0.2 mg L^{-1}; salinity, 33 ± 1‰) for solution renewal on the following day.

Larvae hatched within 58 h post fertilization (hpf). Following the routine feeding management scheme for Japanese flounder, larvae were offered docosahexaenoic acid (DHA) enriched rotifers (*Brachionus plicatilis*) as food at a density of 5–8 individuals mL^{-1} once a day. Feeding began at 2 days post hatching (dph) after they opened their mouth and initiated feeding. Feeding continued until 14 dph. From 15 to 32 dph, larvae were fed both rotifers and DHA enriched brine shrimp (*Artemia* sp.) nauplii. During this period, the pelagic larvae completed metamorphosis and started to live benthically. On 33 dph, we allowed 100 newly settled juveniles in each experimental tank. Juveniles were fed a commercial diet until the end of the test on 78 dph.

Beginning at the initial feeding, two-thirds of the test solution in the experimental tanks was renewed every day with solution of the same mercury concentration. This process continued until 14 dph. After 14 dph, the test solution was renewed thoroughly every day until the termination of the test. Gentle aeration was provided throughout the test. Dead individuals and uneaten food were removed every day throughout the test.

Fish were sampled from each experimental tank at 18 dph (200 metamorphosing larvae, 20 days after exposure), 33 dph (20 settling larvae, 35 days after exposure) and 78 dph (3 juveniles, 80 days after exposure) for the present study. Five additional juveniles were sampled at 78 dph from each experimental tank at the end of the test for mercury bioaccumulation quantification and growth measurements. Sampled fish were stored in acid-rinsed vials in liquid nitrogen until biochemical analyses. After all samplings were conducted, the rest of the settling larvae were sacrificed and discarded on 33 dph because they were mercury contaminated. The rest of the juveniles at the end of the test were stored in liquid nitrogen for other study.

2.2. Solution sampling and chemical analyses

Solution samples for chemical analyses were collected every day from one of the three replicates at each concentration. Samples were acidified with 1% (v/v) HNO_3 solution and were measured using automated atomic fluorescence spectrometry (AFS) (Titan AFS-610A; Beijing, China). The error of mercury concentration, expressed as the percentage of the absolute difference of the measured and nominal concentrations by the nominal concentration, was used to assess the effectiveness of mercury concentration in the test solutions.

2.3. Biological analyses

In order to determine mercury bioaccumulation and fish growth, the fish sampled at the end of the test were analyzed individually. Fish were thawed, washed with deionized water and dried with absorptive papers. Next, they were weighed to the nearest 0.01 g in body mass (W_B) and measured to the nearest 0.1 mm in total length (L_T). These measurements allowed us to determine growth. The individuals (weighing 0.14–0.72 g) were each transferred into sealed, acid-washed polytetrafluoroethylene containers with concentrated HNO_3 (1 mL for 0.1 g sample) at 50 °C for digestion overnight. The solutions were then diluted with ultrapure water and mercury bioaccumulation (total mercury) in each individual was quantified using the AFS method (Liang et al., 2003).

For biochemical analyses, the 200 metamorphosing larvae (18 dph) and 20 settling larvae (33 dph) sampled from each tank were pooled as one sample because the larvae were too small to be analyzed individually. Three juveniles (78 dph) from each experimental tank were analyzed individually. Fish were thoroughly washed with ice-cold physiological salt water (0.9% NaCl), and then