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# Antioxidant responses, hepatic intermediary metabolism, histology and ultrastructure in *Synechogobius hasta* exposed to waterborne cadmium

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ABSTRACT

The present study was conducted to determine antioxidant responses, hepatic intermediary metabolism, histology and ultrastructure in *Synechogobius hasta* after a 15-days of waterborne cadmium (Cd) exposure at the concentrations of 0 (control), 0.10, 0.17 and 0.29 mg Cd/l (corresponding to 0, 12.6%, 21.5% and 36.7% of 96 h $LC_{50}$), respectively. Growth performance and survival declined, but hepatosomatic index (HSI) increased with increasing waterborne Cd levels ($P<0.05$). Waterborne Cd exposure also significantly increased lipid contents in whole body and liver ($P<0.05$), and Cd accumulation in whole body, muscle and vertebrae. Waterborne Cd exposure changed hepatic enzymatic activities involved in intermediary metabolism, induced antioxidant responses and increased lipid peroxidation level in the gill, liver and spleen. Waterborne Cd exposure also caused considerable histological alterations of gill, liver and spleen, led ultrastructures damage of liver and spleen of fish, and induced fatty liver for this fish species.



## 1. Introduction

Cadmium (Cd) is a widespread aquatic environmental pollutant, associated with a broad spectrum of human activities and products such as plastics, ceramics, glass and vehicle tires (Thompson and Bannigan, 2008) and recognized as one of the most deleterious heavy metal pollutants without any biological function (Hallenbeck, 1984). Many studies have been conducted to determine the biological response of aquatic animals to Cd intoxication. For example, exposure to Cd could result in the delay in ontogenetic development, low hatchability, high morphological abnormality (Witeska et al., 1995; Williams and Holdway, 2000; Jezierska et al., 2009), reduce survival and growth (Hansen et al., 2002), disturb osmotic and ion regulation, alter the activities of several enzymes and cell injuries (Heath, 1995). Although the biochemical and toxicological properties of Cd have been widely studied, the effect of sub-lethal concentrations of Cd on fish are not entirely understood (Isani et al., 2009), and knowledge about the effects of Cd on relevant antioxidant parameters in extra-hepatic tissue and hepatic nutrient metabolism is lacking. Cd has been found to stimulate lipid peroxidation in fish causing serious tissue damage (Bagchi et al., 1996), but these studies mainly focused on liver and little is known on the change of antioxidant responses and lipid peroxidation in gill and spleen. Furthermore, to supply the energy demand for detoxification and repair processes in fish exposed to Cd, the nutrients must be mobilized in the liver. Inside the cells, each metabolic pathway is continuously regulated in order to maintain homeostasis and, in general, few key enzymes control the metabolic flux, such as pyruvate kinase (PK), lipoprotein lipase (LPL) and hepatic lipase (HL), lactate dehydrogenase (LDH), succinate dehydrogenase (SDH) and malic dehydrogenase (MDH). To our knowledge, little is known about the effect of waterborne cadmium exposure on these enzymes involved in nutrient metabolism after exposing fish to Cd.

*Synechogobius hasta* are widely distributed over the southern coast of Liaoning Province, China. The fish species has been identified as a species destined for diversification of Chinese marine aquaculture (Luo et al., 2008). In recent years, commercial farming of this fish has become of increasing interest in northern China because of its euryhalinity, rapid growth, good taste and high market value (Luo et al., 2008). At present, several studies have been conducted to determine the characteristics of nutrient physiology for the fish species in our laboratory (Luo et al., 2008, 2009). We also found that under natural coasts where the fish lived, *S. hasta* developed fatty liver syndrome, whose reasons were unknown. Recently, we demonstrated that waterborne

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copper exposure could induce fatty liver syndrome for this fish species (Liu et al., 2010). As a continuous study involved in the reason of fatty liver occurrence for the fish species, the present experiment was aimed to investigate the effects of waterborne Cd exposure on growth, hepatic nutrient metabolism, histology and ultrastructures, and antioxidant responses in *S. hasta,* and explored whether waterborne Cd exposure could induce the occurrence of fatty liver syndrome for the fish species.

## 2. Materials and methods

The research was conducted in Panjin Guanghe Fisheries Co. Ltd. Two experiments with waterborne Cd exposure were conducted. The first experiment was involved in the acute Cd toxicity for *S. hasta*, determining the 96 h median lethal concentration ($LC_{50}$). The second experiment evaluated chronic effects of waterborne Cd exposure on growth, nutrient metabolism, histology and ultrastructure, and antioxidant responses of liver, gill and spleen in *S. hasta*. For the two experiments, Cd was added as $CdCl_2$ (99.9% in purity, SCRC, Shanghai, China) in distilled water for stock concentrations. Individual test solutions in the first and second experiment were obtained by adding the appropriate volume of the primary stock to the dilution water. The Cd concentration in the test tanks was measured using inductivity coupled plasma mass spectrometry (ICP–MS, IRIS Advantage (HR), Thermo Jarrel Ash Corporation, Boston, MA, USA). We assured that the experiments performed on animals, animal care, and all protocols followed the ethical guidelines of Huazhong Agricultural University for the care and use of experimental animals for the protection of human subjects and animal welfare.

### 2.1. Experiment 1: acute toxicity for the determination of 96 h $LC_{50}$

*S. hasta* were taken from the local ponds and transferred to indoor fiberglass tanks (300 l in water volume) for 10-day acclimatization. The test consisted of six concentration groups (zero control, 0.10, 0.20, 0.40, 0.80 and 1.60 mg Cd/l, respectively), three replicates per group, with 10 fish in each replicate. During the 96 h experiment, each tank was filled with 200 l sand-filtered seawater and they were aerated continuously. Each test solution was renewed 100% daily. Water temperature was maintained at 26 °C, dissolved oxygen was $6.28 \pm 0.2$ mg/l, pH 8.4 and salinity 28.

During the exposures, mortality was monitored at 0, 4, 8 and 12 h and then at each 12 h interval to the end of the test (96 h of exposure), and used to calculate the 96 h $LC_{50}$. The criteria for death were no gill movement and no reaction to gentle prodding. Dead fish were removed and discarded after each observation. The $LC_{50}$ at 96 h were computed using the probit analysis computer program (Finney, 1971) and was 0.79 mg/l.

### 2.2. Experimental 2: sub-lethal experiment

According to the first experiment, $LC_{50}$ of Cd for *S. hasta* after 96 h exposure was 0.79 mg/l. In the second experiment, *S. hasta* were exposed to four Cd treatments at the concentrations of zero control, 0.10, 0.17 and 0.29 mg Cd/l (corresponding to 0, 12.6%, 21.5% and 36.7% of 96 h $LC_{50}$), respectively, three replicates per treatment and 20 fish per replicate. Prior to the experiment, the fish from a local pond were kept in 300 l circular fiberglass tanks for 10-day acclimatization. Actual measured Cd value in natural seawater was 0.70 μg/l. At the beginning of the experiment, uniform-sized fish (mean initial body weight: $13.73 \pm 0.67$ g, Mean ± SD) were stocked in 12 fiberglass tanks with 20 fish each. Every tank was filled with 200 l sand-filtered seawater. The experiment was carried out in static aquarium system, and continuously aerated under relatively constant temperature ($25 \pm 1$ °C), pH (8.4) and salinity (28), normal photoperiod (14 h light/10 h darkness), hardness ($156.8 \pm 3.2$ mg $l^{-1}$ as $CaCO_3$).

During the acclimation and experimental period, fish were fed trash fish twice a day at 10% (based on the wet weight of the trash fish) of fish weight. The Cd content of trash fish was not determined directly. However, since the organisms of all treatments were fed the same food and the same daily ration, any histological alternation in comparison to the control group was taken as indicative of the effect of waterborne Cd used in the experiment. To ensure the water quality and maintain Cd levels, water was replaced 100% every 12 h by sand-filtered seawater, and Cd exposure was carried out by the dilution in distilled water of $CdCl_2$, and Cd concentrations were monitored during the experimental period by the methods of ICP–MS. Dead juveniles and uneaten food were removed every day in the morning (1100 h) and afternoon (1400–1600 h) when the water was renewed. The sub-lethal experiment continued for 15 days.

### 2.3. Sampling and samples analysis

At the end of the 15-days sub-lethal experiment, 24 h after the last feeding, all fish were counted and weighed to determine survival, weight gain (WG) and specific growth rate (SGR). After obtaining the final total weight of fish in each tank, four fish per tank were randomly selected, weighed and measured for body lengths, then frozen at −70 °C for subsequent determination of whole body composition. Remaining fish from each aquarium were randomly selected, dissected in ice to obtain gill, liver, white muscle, vertebrae samples and condition factor (CF), viscerosomatic index (VSI) and hepatosomatic index (HSI) were calculated. They were then stored at −70 °C for subsequent proximate analysis of crude protein, lipid and Cd content of various tissues. Crude protein ($N \times 6.25$) was determined by the Kjeldahl method after an acid digestion using an auto-Kjeldahl system (2300-Auto-analyzer, Sweden). Crude lipid was determined by the ether-extraction. Cd content of whole body, white muscle and vertebrae were analyzed using ICP–MS. The Cd detection limits are 0.01 ng/ml and 0.2 ng/g dry weight. Quality control blanks and standards were run every 20 samples. The accuracy of the method was evaluated by calibration vs. an international standard (Tort-2, lobster hepatopancreas, National Research Council of Canada). Recovery of metals ranged from 94% to 104%. The Cd concentrations in the tissue were expressed as μg/g dry weight. These analyses were conducted in duplicate.

For enzymatic analysis, liver, gill and spleen were removed using sterile forceps, placed in sterile 15 ml glass tissue grinders and stored at −70 °C (not longer than 2 wks) for subsequent analysis. When analyzing, samples were homogenized in 0.1 M Tris–HCl buffer at 4 °C, pH 7.4, to make a 10% (W/V) homogenate. The homogenates were centrifuged at 16000 g for 5 min at 4 °C and then the supernatants were collected for enzyme analysis. The assays were run in triplicate. The following enzyme activities were measured: superoxide dismutase (SOD, EC1.15.1.1) as described by Misra and Fridovich (1972), glutathione peroxidase (GPx, EC 1.11.1.9) by the method of Paglia and Valentine (1967) with modifications according to Lawrence and Burk (1978), catalase (CAT, EC 1.11.1.6) (Aebi, 1984), succinate dehydrogenase (SDH, EC 1.3.99.1) (Slater and Bonner, (1952), pyruvate kinase (PK, EC 1.11.1.7) (Carbonell et al., 1973), lactate dehydrogenase (LDH, EC1.1.1.27) (Jones and Sidell, 1982), malic dehydrogenase (MDH, EC 1.1.1.40) (Ochoa, 1955), hepatic lipase (HL, EC 3.1.1.3) following Ehnholm et al. (1975) and modified by Burgaya et al. (1989). Lipoprotein lipase (LPL, EC 3.1.1.34) activity was measured using labeled triolein-$^3$H as a substrate, according to the modified methods by Ballart et al. (2003). All enzyme activities were expressed based on soluble protein. Soluble protein content of homogenates was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Lipid peroxidation was also determined in samples according to the method described by Livingstone et al. (1990) in terms of malondialdehyde (MDA) equivalents using the thiobarbituric acid (TBA) reaction. These methods have been used with success in our recent study (Liu et al., 2010).

For histological observation, the left liver, the spleen (sliced into 3 mm thick slabs), and the second gill arch of the right side of each fish were collected and fixed in 10% neutral buffered formalin for 24 h. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax. Sagittal sections of 6 μm thickness were stained with haematoxylin and eosin (Woods and Ellis, 1994), and then prepared for light microscopy analysis.

For the observation of the ultrastructures of liver and spleen, the methods from Li et al. (2007) were used here. In brief, specimens of liver and spleen were prefixed in 2.5% glutaraldehyde solution, diced into 1 $mm^3$, followed by three 15 min rinses with 0.1 M phosphate buffer (pH 7.4). Post-fixation was in cold 1% aqueous osmium tetroxide for 1 h. After rinsing with phosphate buffer again, the specimens were dehydrated in a graded ethanol series of 50–100% and then embedded in Epon 812. Ultrathin sections were sliced with glass knives on a LKB–V ultramicrotome (Nova, Sweden), stained with uranyl acetate and lead citrate and examined under a HITACHI H-600 electron microscope.

### 2.4. Statistical analysis

Results are presented as mean ± SD. Prior to statistical analysis, all data were tested for normality of distribution using the Kolmogornov–Smirnov test. The homogeneity of variances among the different treatments was tested using the Barlett's test. Then they were subjected to one-way ANOVA and Duncan's multiple range test. Difference was considered significant at $P < 0.05$. All statistical analyses were performed using the SPSS 10.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA).

## 3. Results

Growth (WG and SGR) and survival significantly declined with increasing waterborne Cd levels ($P < 0.05$) (Table 1). CF and VSI showed no significant differences among the treatments ($P > 0.05$) (Table 2), but HSI increased with the waterborne Cd concentration ($P < 0.05$). For whole body composition, the lowest lipid content and the highest protein content were observed for fish in the control. Hepatic crude protein and moisture contents showed no significant differences among the treatment ($P > 0.05$),