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Chronic Toxic Effects of Waterborne Mercury on Silver Carp (*Hypophthalmichthys molitrix*) Larvae

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Abstract: Mercury (Hg) is a kind of heavy metal pollutant widely existing in the aquatic environment, and it is also recognized to have a highly toxic effect on fish. In this study, silver carp (*Hypophthalmichthys molitrix*) larvae were exposed to 0 (control), 1, 5, and 10 μg/L Hg^{2+} for 2 weeks. Antioxidant ability, neurotoxicity, and thyroid hormones (THs) content were evaluated. In comparison with the control, the superoxide dismutase (SOD) activity and the glutathione (GSH) activity were lower in silver carp exposed to 10 μg/L Hg^{2+}. The lowest catalase (CAT) activity was found in the 10 μg/L Hg^{2+}, while malondialdehyde (MDA) content was not significantly different among all groups. Compared with the control, monoamine oxidase (MAO) activity and nitric oxide (NO) content were significantly higher in the 10 μg/L Hg^{2+}, while acetylcholinesterase (AChE) activity significantly decreased. Compared with the control, triiodothyronine (T3) content was significantly higher in the 1 μg/L Hg^{2+} and significantly lower in the 10 μg/L Hg^{2+}; the 1 μg/L and 5 μg/L Hg^{2+} groups had significantly higher thyroxine (T4) content than the other groups. In the 1 μg/L Hg^{2+}, the integrated biomarker response (IBR) index value was the highest. In summary, exposure to Hg could decrease the antioxidant ability, cause changes in neurotoxic parameters, and induce disorders of the thyroid hormone system in silver carp larvae. The results of this study may contribute to the understanding of the adverse effects of chronic mercury poisoning on fish.

Keywords: *Hypophthalmichthys molitrix*; mercury; biochemical responses; thyroid hormones; IBR

Citation: Wang, Y.-J.; Chen, C.-Z.; Li, P.; Liu, L.; Chai, Y.; Li, Z.-H. Chronic Toxic Effects of Waterborne Mercury on Silver Carp (*Hypophthalmichthys molitrix*) Larvae. *Water* **2022**, *14*, 1774. https://doi.org/10.3390/w14111774

Academic Editor: Ken Oakes

Received: 1 April 2022
Accepted: 27 May 2022
Published: 31 May 2022

1. Introduction

Mercury (Hg) is a heavy metal contaminant widely present in aquatic ecosystems, exhibiting teratogenicity, neurological, nephrogenic, and immunotoxicity [1–4]. The impact of various heavy metal pollutants, including Hg, on aquatic environments, has become an increasingly serious problem due to increased human activities [5,6]. Hg has been found in various aquatic environments around the world. For example, the concentration of total Hg reached 16.8 ± 8.4 ng/L in Ya-Er Lake, Xinjiang Province, China [7]. Total Hg was found at 1.8 ± 1.00 ng/L at the bottom of Minamata Bay, Japan [8]. Hg is usually present in the aquatic environment mainly in three forms: elemental mercury (Hg^0), inorganic mercury (IHg), and organic mercury (MeHg), with IHg being the most abundant form in freshwater environments and easily converted to methylmercury through continuous biogeochemical cycling [9]. Methylmercury is more likely to accumulate in sediment-feeding aquatic animals and persists in fish tissue via biomagnification in the food chain [10]. Trace amounts of Hg in aquatic environments accumulate in animals with high nutrient levels through the food chain. It endangers aquatic organisms as well as human health and safety [11]. Due to its high toxicity, it is capable of many adverse effects on reproduction and nervous systems [12]. At present, it has become one of the hot spots of ecotoxicological research.

Previous studies on the toxic effects of Hg in different aquatic animals have been extensively reported [13]. For example, Hg exposure leads to oxidative stress, and genes encoding antioxidant enzymes were found to be significantly upregulated in the spleen, head, and kidney of *Pelteobagrus fulvidraco* [14]. Hg exposure increases the accumulation of Hg in the liver of *Danio rerio* and damages the ultrastructure of the liver [15]. Hg exposure causes neurotoxicity in the marine medaka *Oryzias melastigma* through the induction of oxidative stress and the disruption of cellular metabolism [16]. Hg could cause damage to the immune system of fish, including cell necrosis or necrotizing apoptosis and changes in immune gene expression profiles in marine gilthead sea bream and European sea bass [17,18]. Exposure to Hg at even low concentrations could lead to physiological, biochemical, genetic, metabolic, and morphological abnormalities in fish [19,20]. Silver carp (*Hypophthalmichthys molitrix*) is one of the four major Chinese carp species, as well as one of the most essential freshwater commercial fish, with a global production of 4.79 million tons in 2018 [21,22]. Previous studies on silver carp have focused on growth and development [23], genetic reproduction [24], and ecological regulation [25,26]. However, relatively few studies on the toxicity of heavy metal Hg to silver carp have been reported, especially for nerve damage and related biomarkers such as thyroid hormones (THs).

Therefore, this study aimed to investigate the effect of chronic waterborne Hg exposure on antioxidant ability, neurotoxicity, and THs content in silver carp larvae. Furthermore, this study also provides a comprehensive overview of the physiological stress of chronic waterborne Hg on silver carp larvae via using the integrated biomarker response (IBR) index. This study may increase our understanding of the effects of Hg toxicity on silver carp larvae.

2. Materials and Methods

2.1. Experimental Animals and Test Chemicals

Silver carp larvae were purchased from the Yangtze River Fisheries Research Institute (Jingzhou, China). Silver carp were acclimated in the 120 L plastic box for 7 days, and the experimental water was tap water with full aeration for more than 24 h. Fish were fed hatched brine shrimp (*Artemia nauplii*) twice a day. The experimental conditions were a water temperature of 24.0 ± 1.0 °C, photoperiod 12L:12D, and dissolved oxygen >7.0 mg/L.

Mercuric chloride ($HgCl_2$, purity > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). As a stock solution of 1 g/L $HgCl_2$, deionized water was prepared. Then, the solution was stored in a brown bottle at room temperature, protected from light. Finally, it was diluted to the mass concentration required for the experimental design.

2.2. Experimental Design and Sample Collection

After acclimation, healthy silver carp larvae (average initial weight 0.027 ± 0.004 g; average body length 1.25 ± 0.06 cm) were selected and randomly assigned to 12 tanks (40 × 23 × 25 cm). In the experiment, silver carp larvae were set up in four groups, three parallel in each group. Each tank contained 20 L tap water and 60 silver carp larvae. The selection of test concentrations of Hg^{2+} was based on data from our previous acute toxicology experiments (not yet published). Four concentrations of Hg^{2+} were set at 0, 1, 5, and 10 μg/L in the experiment. All solutions samples were analyzed and the measured concentrations were within 20% of the nominal concentration following OECD guidelines. During the two-week experimental period, half of the water in the tank was replaced with tap water aerated for more than 24 h every day. The temperature was kept at 24.0 ± 1.0 °C, dissolved oxygen >7.0 mg/L, and photoperiod 12L:12D throughout the experimental period. All procedures and animal handling comply with the guidelines approved by the Chinese Society of Laboratory Animal Science. The research has been approved by the Animal Ethics Committee of Yangtze University.

At the end of the exposure experiment, the mucus on the surface of the fish was washed with sterile distilled water. After absorbing the water on the surface of the fish with absorbent tubes, the whole fish were collected in 2 mL Eppendorf tubes and transferred to

the −80 °C refrigerator for the next analysis. Because the silver carp larvae were too small, the whole fish was used for biochemical analysis.

2.3. Biochemical Analysis

The 10% concentration of homogenate was prepared by three whole fish per tank with precooled sterile 0.9% saline solution at a ratio of 10% as m (tissue, g): v (saline solution, ml). The homogenate was centrifuged for 10 min at 4 °C in the centrifuge (SIGMA, Germany). The supernatant was collected and assayed for relevant biochemical parameters. The superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), malondialdehyde (MDA) contents, monoamine oxidase (MAO), nitric oxide (NO), and acetylcholinesterase (AChE) were determined with commercial kits by following the manufacturer's instructions (Jiancheng Bioengineering Research Institute Co., Ltd., Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the content of triiodothyronine (T3) and thyroxine (T4) following the manufacturer's instructions (Uscnlife, Ltd., Wuhan, China). The details of the method were as described in the previous study [27].

2.4. Integrated Biomarker Response

For several biomarkers (antioxidant enzymes, neurotoxicity enzymes, and THs content), IBR index values were calculated following our previous description [28].

2.5. Statistical Analysis

The statistical significance of data was analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range test. Data were presented as the mean ± standard error (SE). A probability score of $p < 0.05$ had been used to determine significance. All statistical analysis was performed with SPSS (version 25). Besides, star plots were drawn using the Origin software (version 9.8).

3. Results

3.1. Antioxidant Ability

Antioxidant enzyme activities of fish exposed to different concentrations of Hg^{2+} are shown in Figure 1. The results showed that the SOD activity of fish was significantly lower than the control after exposure to Hg^{2+} ($p < 0.05$; Figure 1A); Significantly lower GSH activity was found in fish exposed to 10 μg/L Hg^{2+}, relative to the control ($p > 0.05$; Figure 1B). The activity of CAT of fish exposed to 10 μg/L Hg^{2+} was significantly lower than that exposed to 1 μg/L Hg^{2+} ($p < 0.05$; Figure 1C). There was no significant change in MDA content among all groups ($p > 0.05$; Figure 1D). Antioxidant enzyme activities are often used as a biomarker to assess the damage caused by pollutants to aquatic organisms [29]. Under normal conditions, the production of reactive oxygen species (ROS) is in balance with an organism's antioxidant defense system. When the organism is under external pressure, the organism inevitably produces excessive reactive oxygen species, leading to oxidative stress [30]. Hg exposure induces the production of intracellular ROS, which is a distinctive feature of heavy metal toxicity [31,32].

As the first line of antioxidant defense, SOD acts as a superoxide radical scavenger, converting superoxide anion radicals into O_2 and H_2O_2, while CAT decomposes H_2O_2 into H_2O and O_2 to eliminate excess radicals [33–35]. GSH is a non-enzymatic antioxidant that directly eliminates reactive oxygen species under oxidative stress, while MDA is used as a lipid peroxidation metabolite in response to the lipid peroxidation status in the organism [36,37]. Several studies have recently reported that Hg toxicity inhibits the activity of antioxidant enzymes in aquatic animals such as *Pelteobagrus fulvidraco* [14], *Pseudosciaena crocea* [38], and *Apostichopus japonicus* [39]. Similarly, the study results suggest that the SOD, GSH, and CAT activities of silver carp larvae exposed to high concentrations of Hg^{2+} were significantly decreased. Therefore, it is assumed that Hg exposure disrupts the antioxidant defense system of fish, causing oxidative stress. However, more research on the molecular toxicity of Hg exposure for the antioxidant ability of silver carp is required.

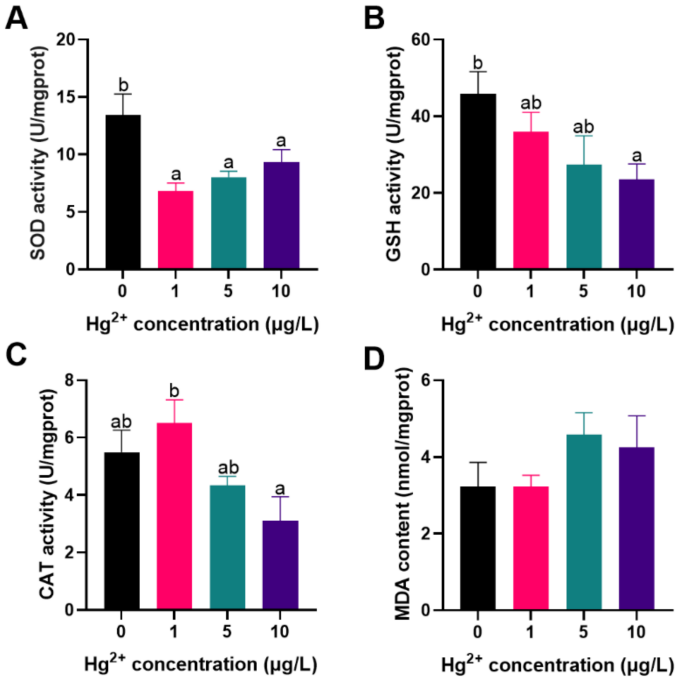

Figure 1. Response to the antioxidant ability of silver carp larvae exposed to Hg^{2+}. (**A**) SOD activity; (**B**) GSH activity; (**C**) CAT activity; (**D**) MDA content. Vertical bars represent the mean ± SE (n = 3). Different letters indicate significant differences between groups ($p < 0.05$).

3.2. Neurotoxic Related Parameters

The effects of Hg^{2+} on the neurotoxic-related enzymes of silver carp larvae are shown in Figure 2. Compared to the control, MAO activity and NO content were significantly increased exposed to 10 μg/L Hg^{2+} ($p < 0.05$; Figure 2A,C). In contrast, the Hg^{2+} exposure groups showed a significant decrease in AChE activity in fish compared to the control ($p < 0.05$; Figure 2B). Chronic Hg exposure was associated with oxidative damage to the central nervous system in fish. Hg in the aquatic environment causes neurotoxicity in aquatic animals through several mechanisms, including oxidative stress, metabolic abnormalities, and cytoskeleton dysfunction [40,41]. MAO regulates many aspects of the central nervous system, including neurotransmission and neuronal electrical activity [42,43]. Different inhibitory effects on MAO activity after different concentrations of Hg stress have been previously reported [44,45]. In this study, 10 μg/L Hg^{2+} increased the MAO activity, which contradicted the findings of previous research. This may be partly due to the small concentration of current Hg or the insensitivity of MAO activity induced by Hg-toxicity, which instead produced excitotoxic effects on fish. NO is a neuroinformatic molecule with a wide range of physiological roles [46]. The current results found that 10 μg/L Hg^{2+} has NO content, which could have toxic effects on fish. AChE is used to degrade acetylcholine, which blocks neurotransmitter excitatory effects on the postsynaptic membrane and maintains normal nerve signal transmission in the organism [47]. The activity of AChE was significantly reduced in the 10 μg/L Hg^{2+} in this study. The possible reason for this speculation was that decreased AChE activity was associated with neuronal damage. Several recent studies have reported the effect of exposure to other heavy metals contamination on the inhibition of AChE in fish, with results similar to those of the present study [48,49].

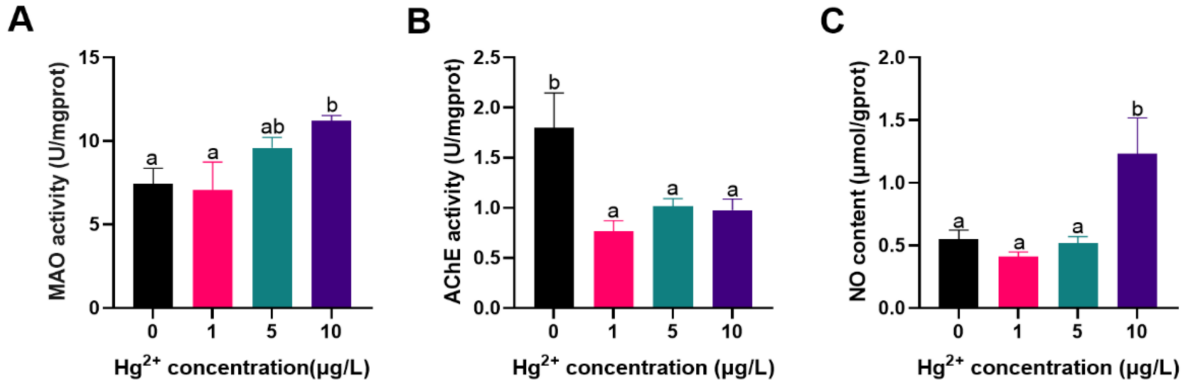

Figure 2. Response to the neurotoxic related parameters of silver carp larvae exposed to Hg^{2+} (**A**) MAO activity; (**B**) AChE content; (**C**) NO content. Vertical bars represent the mean ± SE (n = 3). Different letters indicate significant differences between groups ($p < 0.05$).

3.3. Thyroid Hormones (THs) Content

THs content was quantified as shown in Figure 3. Compared to the control, T3 content was significantly increased in the 1 μg/L Hg^{2+} and significantly decreased in the 10 μg/L Hg^{2+} ($p < 0.05$; Figure 3A); T4 content of fish in the 1 μg/L and 5 μg/L Hg^{2+} groups were significantly higher than that in other groups ($p < 0.05$; Figure 3B). THs are important regulators in the early growth and development of animals [50]. Environmental contaminants that interfere with THs synthesis have been the subject of interest in aquatic toxicology studies [50–52]. T3 and T4 are the main forms of thyroid production and are essential for fish growth and development, osmotic pressure regulation, and nutrient metabolism [53,54].

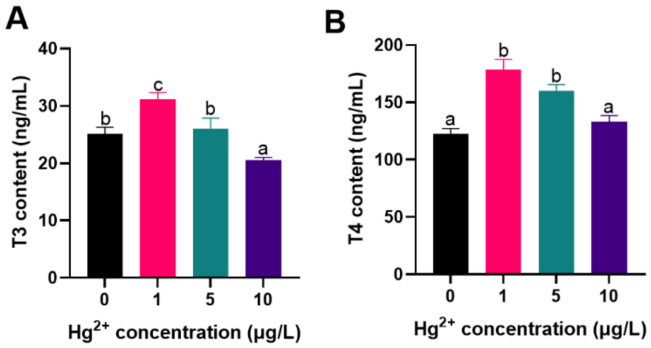

Figure 3. Response to the thyroid hormones levels of silver carp larvae exposed to Hg^{2+}. (**A**) T3 content; (**B**) T4 content. Vertical bars represent the mean ± SE (n = 3). Different letters indicate significant differences between groups ($p < 0.05$).

In this study, T3 content was significantly decreased in fish exposed to high concentrations of Hg^{2+} and T4 content was significantly increased after exposure to low concentrations of Hg^{2+}, suggesting that the toxicity of Hg induces thyroid dysfunction. In a previous study, low concentrations of Hg exposure had increased the content of whole-body THs in zebrafish larvae [55]. This variation reflects the fact that Hg-induced thyroid dysfunction varies depending on fish species, developmental stage, and treatment dosage.

3.4. Integrated Biomarker Response

The IBR index values were calculated by integrating the measured nine biomarker responses, and the results for each experimental group were shown in Figure 4. The IBR index values showed that the highest pressure was caused in the 1 μg/L Hg^{2+} (13.2661) and the lowest pressure was caused in the 5 μg/L Hg^{2+} (8.1793). The IBR is a novel index that integrates biomarker responses. It is an effective tool for assessing pollutant sensitivity us-

ing multiple biomarker responses [56,57]. The IBR index values standardize biomarkers to represent environmental stresses, thus providing a concise and comprehensive assessment of the health of organisms under various stress situations [58]. The IBR index showed the highest value in the 5 μg/L Hg^{2+}, indicating that the 5 μg/L Hg^{2+} is at the highest stress. In general, high concentrations of Hg^{2+} stress should have higher overall stress of toxicity. The IBR results of this study did not match the expected results. It is speculated that the reason for this may be the higher overall stress on fish due to excitotoxicity produced by a low concentration of Hg^{2+}.

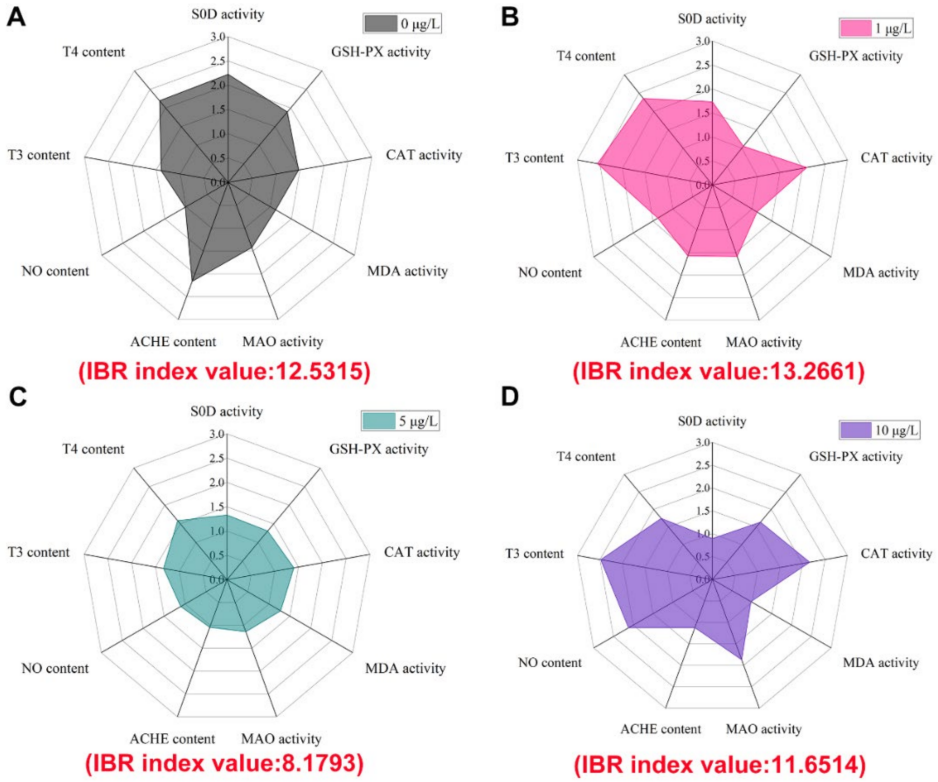

Figure 4. Star plot and IBR index values of normalized biomarker data in different groups. (**A**) 0 μg/L; (**B**) 1 μg/L Hg^{2+}; (**C**) 5 μg/L Hg^{2+}; (**D**) 10 μg/L Hg^{2+}.

Although IBR index values can reflect the biotoxicity effects of mercury exposure and contribute to assessing the effects of toxic contaminants on aquatic animals, relatively few reports have used the IBR index to evaluate the toxicity of heavy metals. Furthermore, the type and number of biomarkers selected are also crucial for the calculation of IBR index values [59,60]. Therefore, the IBR index as a quantitative tool to assess the effects of heavy metal toxicity stress on fish still needs more research.

4. Conclusions

Overall, this study indicates that chronic waterborne Hg exposure could decrease the antioxidant capacity of silver carp larvae and cause changes in neurotoxic parameters. At the same time, waterborne Hg exposure could lead to abnormal levels of THs in silver carp larvae and may induce thyroid dysfunction. Furthermore, the IBR index value indicated that a low concentration of Hg^{2+} (1 μg/L) exposure generated stronger stress than Hg^{2+} exposure under the highest concentration (10 μg/L). This study provides current biochemical data on the toxicity of Hg, which will be essential for understanding the risk of Hg in aquatic environments.

Author Contributions: Y.-J.W.: Culture and Sampling and Indices Measurement; C.-Z.C.: Writing-Original draft preparation; P.L.: Writing- Reviewing and Editing; L.L.: Data analysis; Y.C.: Conceptualization; Z.-H.L.: Conceptualization and Overall guidance. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Engineering Research Center of Ecology and Agricultural Use of Wetland, Ministry of Education (Yangtze University) (KFT202006); National Key Research and Development Program of China (2018YFD0900902, 2018YFD0900905).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Yangtze University.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data of this paper are from our experiment and based on the previous published papers cited in our paper.

Conflicts of Interest: The authors declare no conflict of interest.

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