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Thyroid-Disrupting Effects of Cadmium and Mercury in Zebrafish Embryos/Larvae

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Abstract: Cadmium (Cd^{2+}) and mercury (Hg^{2+}) are two kinds of non-essential heavy metals. Cd^{2+} and Hg^{2+} can cause thyroid disruption, but very few researchers have investigated the thyroid-disrupting effects of these metals on fish, specifically during their early developmental transition stage from embryos to larvae. In this study, wild-type zebrafish embryos were exposed to varying concentrations (contents) of Cd^{2+} (0, 10, 100, and 1000 μg/L) and Hg^{2+} (0, 0.1, 1, and 10 μg/L) for 120 h. Thereafter, the thyroid hormone contents and transcriptional changes in the genes, including thyroid stimulating hormone-β (*tshβ*), thyroglobulin (*tg*), sodium-iodide symporter (*nis*), thyroid peroxidase (*tpo*), transthyretin (*ttr*), thyroid hormone receptor-α and -β (*thrα*, *thrβ*), types I and II iodothyronine deiodinase (*dio1*, *dio2*), and uridine diphosphate glucuronosyltransferase 1 family a, b (*ugt1ab*) associated with the hypothalamic-pituitary-thyroid (HPT) axis were measured. Results showed that zebrafish embryos/larvae malformation rates were significantly higher in the Cd^{2+} and Hg^{2+} groups. A significant increase in the thyroxine (T4) concentration and a decrease in the triiodothyronine (T3) concentration were observed in the Cd^{2+}-exposed zebrafish embryos/larvae. On the other hand, the T4 and T3 concentrations were observed to be significantly increased after Hg^{2+} exposure. Additionally, changes were noted in the expression patterns of the HPT axis-linked genes after Cd^{2+} and Hg^{2+} exposure. Based on the results of the principal component analysis (PCA), it was concluded that Cd^{2+} exposure significantly affected the thyroid endocrine system at a concentration of 100 μg/L, whereas Hg^{2+} exposure led to a thyroid disruption at a low concentration of 0.1 μg/L. Thus, this study demonstrated that exposure to Cd^{2+} and Hg^{2+} metal ions induced developmental toxicity and led to thyroid disruption in zebrafish embryos/larvae.

Keywords: cadmium and mercury; hypothalamus-pituitary-thyroid axis; thyroid disruption; zebrafish

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1. Introduction

Heavy metal compounds are defined as elements having atomic numbers greater than 20 and atomic densities greater than 5 g/cm [1,2]. They are produced by natural and human activities [3,4]. Heavy metals are not biodegradable or chemically degradable, but they can be bioaccumulated through the food chain in organisms [5,6]. These heavy metal ions enter the aquatic ecosystem through different channels such as geological weathering, atmospheric precipitation, and the discharge of industrial or agricultural waste products [3]. They can be classified as essential and non-essential heavy metals [1,7]. Cadmium (Cd^{2+}) and mercury (Hg^{2+}), as two kinds of nonessential heavy metals, have been considered the priority heavy metals for control in China due to their high toxicity, ubiquity, and constancy [8].

Cadmium has been considered an endocrine disruptor [9,10], since it can be stored in the thyroid gland and can damage the thyroid follicular cells [11–14]. Epidemiological studies have reported that the concentrations of Cd^{2+} in urine were negatively correlated with the 3,5,3′-L-triiodothyronine (T3) and L-thyroxine (T4) levels in the plasma of humans; in addition, a positive link between urinary Cd^{2+} concentrations and plasma thyroid-stimulating hormone (TSH) levels was observed in outdoor workers [15]. Experimental studies have also shown that exposure to Cd^{2+} significantly reduced the thyroid hormone (THs) and TSH levels in rats [12,16–18]. An earlier study conducted on fish observed that the total plasma T4 level in the juvenile rainbow trout was significantly increased after Cd^{2+} exposure for 2–4 h, whereas it was significantly decreased after 7 days [19]. On the other hand, no effect on plasma THs levels was noted when juvenile rainbow trout were exposed to Cd^{2+} for 30 days [20]. In a recent study, it was observed that when Chinese rare minnows were exposed to Cd^{2+} for 4 days, there was a significant decrease in the T4 level, whereas the T3 concentration was unaffected [21].

Another environmentally relevant heavy metal contaminant is mercury. In the aquatic environment, the Hg^{2+} concentrations in polluted waters may range in concentrations of microgram per liter [22]. For example, high Hg^{2+} concentrations (0.2–0.5 μg/L) were observed in the Nura River in Central Kazakhstan [23]. Hg^{2+} is another endocrine disruptor, and hence, its exposure can induce an elevation in the incidence of thyroid follicular cell carcinomas in rats [24]. Epidemiological studies showed that Hg^{2+} was negatively correlated with serum T4 and T3 levels in adults [25]. In fish, Hg^{2+} exposure caused morphological changes in the pituitary and thyroid glands in catfish [26]. Similarly, a significant increase was observed in the plasma cortisol, T4, and T3 levels when the juvenile rainbow trout were exposed to Hg^{2+} [27]. A recent study showed that Hg^{2+} treatment (0, 0.1, and 0.3 mg/L) significantly increased T4 levels but had no effect on the T3 contents in the Chinese rare minnow [28].

THs, synthesized in the thyroid gland, play crucial roles in multiple biological processes in vertebrates, such as growth, differentiation, metabolism, nervous system development, and reproduction [25,29–31]. Thyroid functions are predominantly regulated by the HPT axis. The HPT axis primarily controls the THs' synthesis, transport, and metabolism [32,33].

Although several studies reported that exposure to Cd^{2+} and Hg^{2+} affected the THs levels in animals, the data involving thyroid disruption in fish treated with heavy metals such as Cd^{2+} and Hg^{2+} are insufficient, particularly in the early developmental transition stage from embryos to larvae. In this report, zebrafish embryos were used to evaluate the effects of Cd^{2+} and Hg^{2+} treatment on the thyroid endocrine system. These effects were observed by measuring the levels of THs and gene expression involved in the HPT axis. The aim of the present study was to investigate the mechanisms underlying the responses of the HPT axis to Cd^{2+} and Hg^{2+} exposure in zebrafish embryos/larvae.

2. Experimental Procedures

2.1. Embryo Culture and Exposure

The zebrafish embryos (AB strain) were procured from the China Zebrafish Resource Center (Wuhan, China). Experimental procedures on fish were approved by the Animal Experimental Ethical Inspection of Laboratory Animal Centre, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (No. 20180504001). The Holt buffer (composition of: 3.5 g/L NaCl, 0.05 g/L KCl, 0.025 g/L $NaHCO_3$, and 0.1 g/L $CaCl_2$, pH = 7.0) was used to culture the embryos. The zebrafish embryos were cultured in an incubator and subjected to experimental treatment under conditions that were set as follows: Constant temperatures (28 ± 0.5 °C) with a 14:10 (light:dark) photoperiod. Furthermore, Cd^{2+} and Hg^{2+} stock solutions were prepared after dissolving cadmium chloride ($CdCl_2 \cdot 2.5\ H_2O$, CAS:7790-78-5, purity ≥ 98%, Shanghai Biochemical Technology Co., Ltd. Shanghai, China.) and mercury chloride ($HgCl_2$, CAS:7487-94-7, purity ≥ 99%, Sigma-Aldrich, Saint Louis, MO, USA) in ultrapure water. The embryos were exposed to

varying exposure concentrations of Cd^{2+} (0, 10, 100, and 1000 μg/L), and Hg^{2+} (0, 0.1, 1, and 10 μg/L). 14700 healthy embryos (2 h post-fertilization, 2 hpf) were randomly assigned to forty-two 500-mL glass beakers (350 embryos per beaker) containing 350 mL exposure solution. Each concentration included six replicates. These embryos were exposed to various concentrations of cadmium and mercury for 120 h (5 days), and the exposure solution was replaced by a new solution every 24 h to maintain the Cd^{2+} and Hg^{2+} concentrations. The water samples were collected before the renewal of the exposure medium for measuring the actual Cd^{2+} and Hg^{2+} concentrations. The Cd^{2+} concentration was determined by atomic absorption spectrometry according to the method of the National Standard of China (GB7475-87). The Hg^{2+} concentrations was measured by the method of National Standard of China (GB7468-87). The actual Cd^{2+} and Hg^{2+} concentrations exceeded 80% of the corresponding specific concentration in all water samples. During the period of exposure, the dead embryos were counted and discarded every day, and the developmental status of the live embryos/larvae was observed under a microscope. The morphological changes caused by Cd^{2+} and Hg^{2+} included yolk sac edema, tail malformation, and pericardial edema. The hatching, survival, and malformation rates were recorded one time after exposure for 120 h. The larvae (exposed for 120 h) were anesthetized with 0.03% tricaine methanesulfonate (MS-222, Sigma-Aldrich, Saint Louis, MO, USA) for 3 min at room temperature, randomly sampled for subsequent assays of THs and gene expression levels, and immediately stored at −80 °C.

2.2. RNA Extraction and Quantitative RT-PCR

Twenty larvae were selected randomly from every beaker and pooled into a single sample for gene expression analysis. Every group contained six replicates. The total RNA was extracted with the aid of the Trizol reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's protocols. Agarose gel (1%) electrophoresis was used to estimate the quality of the total extracted RNA. The RNA contents were measured using the Nanodrop lite spectrophotometer (Thermo Fisher, Waltham, MA, USA). FastKing gDNA Dispelling RT SuperMix (Tiangen Biochemical Technology, Beijing, China) was used to synthesize first-strand cDNA following the manufacturer's protocols. Quantitative RT-PCR was carried out using the ABI 7500 System (Applied Biosystems 7500, Carlsbad, CA, USA) with the UltraSYBR mixture (Low ROX) (CWBIO Beijing, China) following the kit instructions. Thermal cycling was done at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. *18sRNA* was selected as a reference gene when calculating the gene expression levels. The $2^{-\Delta\Delta Ct}$ technique was used for analyzing the differences (variations) in the gene expression levels [34]. The quantitative RT-PCR primers were obtained from a previous study [35] and are shown in Table 1.

Table 1. Primer sequences used for RT-PCR in this study.

Gene	Sequences of Primers 5′-3′	Gene Bank	Efficiency (%)
tshβ-F	CCAGACAGACATCCTCATACAC	AY135147	94.92
tshβ-R	GCACGGCAACCTTCATTAAA		
tg-F	CTCTATCCTTTCGGCTGGTATG	XM_001335283	95.33
tg-R	GAAGGAGAGCGGAGACTAAATG		
nis-F	GGTGGCATGAAGGCTGTTGC	NM_0011089391	94.80
nis-R	GATACGGGATCCATTGTTGG		
tpo-F	GCGCTTGGAACACAGTATCA	EU267076	91.69
tpo-R	CTTCAGCACCAAACCCAAAT		
ttr-F	CTCCTGGTGTGTATCGGGTG	BC081488	94.29
ttr-R	AGGATGTCAGTCATGTGCCTT		
thrα-F	GGCTCGGAGTGGTTTCTGA	NM_131396	99.51
thrα-R	CTTGCGGTGGTTGATGTAGTG		
thrβ-F	CACATGCTGTGTTGCAGCTT	NM_131340	93.29
thrβ-R	TCATAAGAGCCAGAGCCCCT		

Table 1. *Cont.*

Gene	Sequences of Primers 5′-3′	Gene Bank	Efficiency (%)
dio1-F	CTGGACCGACAGAAGACGAG	BC076008	100.90
dio1-R	TGCGACATTGCTGAAGTCCT		
dio2-F	CTCGGACACTTGGCTTGACT	NM_212789	106.50
dio2-R	TTGGATCAGGACGGAGAGGT		
ugt1ab-F	CCACCAAGTCTTTCCGTGTT	NM_213422	91.01
ugt1ab-R	GCAGTCCTTCACAGGCTTTC		

2.3. Thyroid Hormone Assays

The whole-body T4 and T3 contents in the larvae were estimated using a previously reported method [36]. Briefly, approximately 200 larvae from each replicate were homogenized with ice-cold phosphate-buffered saline (PBS) with a glass grinder, and the samples were sonicated on ice. Every group included six replicates. The supernatants were obtained after the homogenate samples were centrifuged at 5000 rpm at 4 °C for 15 min. The total protein contents in the supernatants were quantified by the Bradford assay [37]. The THs (T4 and T3) concentrations in supernatants were estimated using the enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp. Wuhan, China; T4: CEA452Ge; T3: CEA453Ge) following kit instructions. The T4 and T3 detection limits were calculated to be 1.29 ng/mL and 51.7 pg/mL, respectively.

2.4. Statistical Analysis

The data related to THs contents and gene expression levels were presented as the mean ± standard deviation (SD). The data derived in the study was analyzed with SPSS 20.0 (IBM, Chicago, IL, USA). The Kolmogorov-Smirnov test was employed for validating the data's normality. The Levene test was utilized for analyzing the homogeneity of variances. After validating the data for normality, the statistical differences observed in the treatment groups were confirmed by means of one-way analysis of variance (ANOVA), followed by Tukey-HSD tests. Principal Component Analysis (PCA) was carried out using Origin 2021 (OriginLab, Northampton, MA, USA). The correlation analysis was confirmed by Spearman's test. Values with a $p < 0.05$ were established as statistically significant.

3. Results

3.1. Developmental Toxicity Caused by Cd^{2+} and Hg^{2+}

The developmental toxicity as a result of exposure to Cd^{2+} and Hg^{2+} is illustrated in Table 2. No significant differences were observed in the hatching and survival rates when zebrafish embryos/larvae were exposed to Cd^{2+}. However, the malformation rates were significantly elevated in the 1000 μg/L Cd^{2+} group. The morphological changes caused by Cd^{2+} included yolk sac edema, tail malformation, and yolk sac edema and pericardial edema (Figure S1B–D). Similar to Cd^{2+}, Hg^{2+} treatment did not affect the survival and hatching rates of the zebrafish embryos/larvae. Moreover, the malformation rate was significantly elevated in the highest Hg^{2+} group (10 μg/L) in comparison to the controls. The morphological changes caused by Hg^{2+} included tail malformation, yolk sac edema, pericardial edema, and spinal curvature (Figure S1E–I).

Table 2. The changes in hatching, malformation, and survival rates in zebrafish embryos/larvae treated with Cd^{2+} and Hg^{2+}.

Cd^{2+} (μg/L)	0	10	100	1000
Hatching (%)	88.75 ± 2.14	87.25 ± 1.00	86.92 ± 1.51	85.00 ± 2.95
Malformation (%)	1.00 ± 0.43	1.75 ± 0.66	3.00 ± 1.09	6.17 ± 1.38 **
Survival (%)	88.08 ± 2.57	85.83 ± 0.95	85.58 ± 0.63	83.92 ± 2.40

Table 2. *Cont.*

Hg^{2+} (μg/L)	0	0.1	1	10
Hatching (%)	90.5 ± 0.75	90.08 ± 0.52	88.58 ± 2.01	87.67 ± 2.32
Malformation (%)	0.92 ± 0.14	1.50 ± 0.66	2.50 ± 0.43	5.00 ± 0.50 **
Survival (%)	89.42 ± 1.77	89.00 ± 0.50	87.67 ± 1.51	85.83 ± 2.08

Notes: The hatching, malformation, and survival rates were recorded after zebrafish embryos/larvae were exposed to Cd^{2+} and Hg^{2+} for 120 h. Each replicate contained 350 embryos/larvae. Each concentration included six replicates. Data are presented as mean ± SD (n = 6). ** $p < 0.01$ denote a significant variation between the treatment and control groups.

3.2. Effects of Cd^{2+} on the Thyroid Endocrine System

Significant upregulation of *tshβ* (1.55 fold), thyroglobulin (*tg*) (2.38 fold), thyroid peroxidase (*tpo*) (1.84 fold), thyroid hormone receptor-α (*thrα*) (2.10 fold), and thyroid hormone receptor-β (*thrβ*) (1.51 fold) were detected in the highest Cd^{2+} group (1000 μg/L) (Figure 1A,B,D,F,G). However, transthyretin (*ttr*) expression was significantly downregulated (0.38 fold) in the 1000 μg/L Cd^{2+} group (Figure 1E). The expression of the type I iodothyronine deiodinase gene (*dio2*) (0.71-, 0.67, and 0.84 fold) and uridine diphosphate glucuronosyltransferase 1 family a, b (*ugt1ab*) genes (0.51-, 0.55-, and 0.66 fold) were downregulated significantly in all the Cd^{2+} treatment groups (10, 100, and 1000 μg/L, respectively) (Figure 1I,J). The results indicated that the sodium-iodide symporter (*nis*) and type I iodothyronine deiodinase (*dio1*) gene expression were not altered after Cd^{2+} exposure (Figure 1C,H).

The T4 contents in 100 and 1000 μg/L Cd^{2+} groups were significantly increased by 2.09 and 1.57 fold, respectively (Figure 1K). In contrast, the T3 contents in the 100 and 1000 μg/L Cd^{2+} groups were decreased significantly by 0.64 and 0.35 fold, respectively (Figure 1L).

3.3. Effects of Hg^{2+} on the Thyroid Endocrine System

The findings in the study showed that *tshβ* (2.60 fold) was upregulated significantly in the 10 μg/L Hg^{2+} group (Figure 2A). *Tg* expression was significantly downregulated by 0.34, 0.32, and 0.70 fold in the 0.1, 1, and 10 μg/L Hg^{2+} groups, respectively (Figure 2B). The *nis*, *tpo*, and *ttr* gene expression levels were significantly upregulated 2.36-, 1.52, and 1.95 fold in the 10 μg/L Hg^{2+} groups, respectively (Figure 2C–E). The expression of the *dio1* (0.53-, 0.59-, and 0.70 fold) and *ugt1ab* (0.30-, 0.41-, and 0.58 fold) genes was downregulated in all Hg^{2+} treatment groups (0.1, 1, and 10 μg/L, respectively) (Figure 2H,J). When the embryos/larvae were exposed to 10 μg/L Hg^{2+}, *dio2* gene expression (1.64fold) was significantly upregulated (Figure 2I), whereas the *thrα* and *thrβ* gene expression levels were not affected (Figure 2F,G).

The whole-body T4 levels in the 0.1, 1, and 10 μg/L Hg^{2+} groups were significantly increased significantly by 1.98, 2.21, and 2.15 fold in comparison to the control samples, respectively (Figure 2K). Meanwhile, the whole-body T3 level was seen to be significantly elevated by 1.43 fold in the 10 μg/L Hg^{2+} treatment group (Figure 2L).

3.4. PCA and Correlation Analysis

Herein, the PCA and correlation analyses were conducted to analyze the relationship between the THs and gene expression levels. The PCA result of Cd^{2+} exposure is shown in Figure 3A. The initial two principal components (PCs) accounted for 73.3% of the total variance. PC1 explained 53.2% of the total variances, while PC2 accounted for 20.1% of the total variances. According to the PCA plot, the separation between the clusters of the control and 100 and 1000 μg/L Cd^{2+} treatment groups was significant. T4 level was positively and significantly correlated with the transcriptional levels of *tshβ*, *tg*, *tpo*, *thα*, *thrβ*, and *dio1*, and negatively correlated with the transcriptional levels of *nis*, and *ttr* (Figure 4A). However, the T3 level exhibited a strong positive correlation with the expression levels of

the *nis* and *ttr* genes and a strong negative correlation with the expression levels of the *tshβ*, *tg*, *tpo*, *thα*, and *thrβ* genes (Figure 4A).

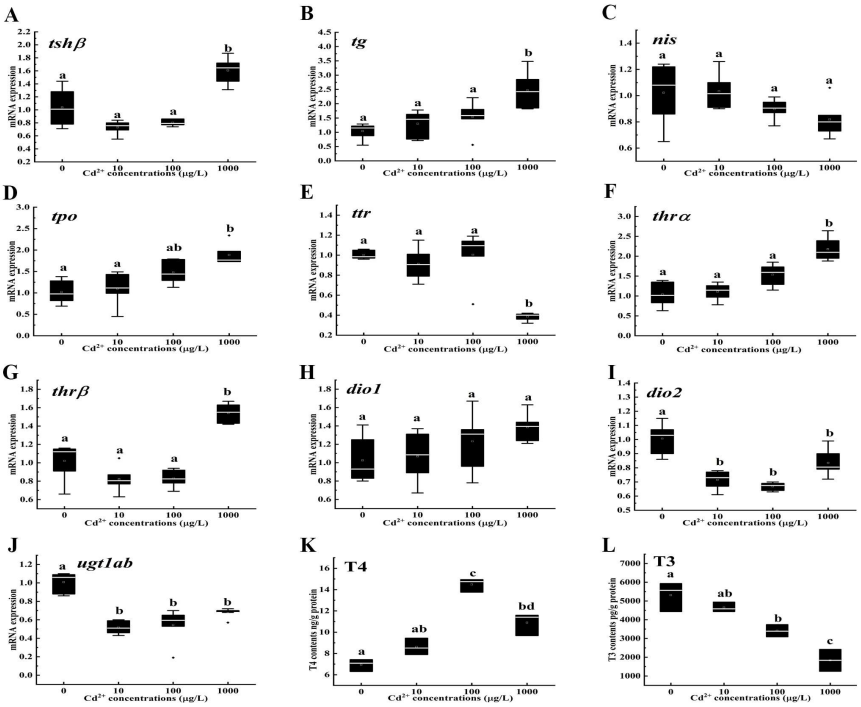

Figure 1. Transcription levels of *tshβ* (**A**), *tg* (**B**), *nis* (**C**), *tpo* (**D**), *ttr* (**E**), *thrα* (**F**), *thrβ* (**G**), *dio1* (**H**), *dio2* (**I**), *ugt1ab* (**J**) and contents of T4 (**K**), T3 (**L**) in zebrafish embryos (2 hpf) that were treated with Cd^{2+} (0, 10, 100, and 1000 μg/L) for 120 h. Data are shown as mean ± SD (n = 6). Different letters denote significant differences between groups ($p < 0.05$).

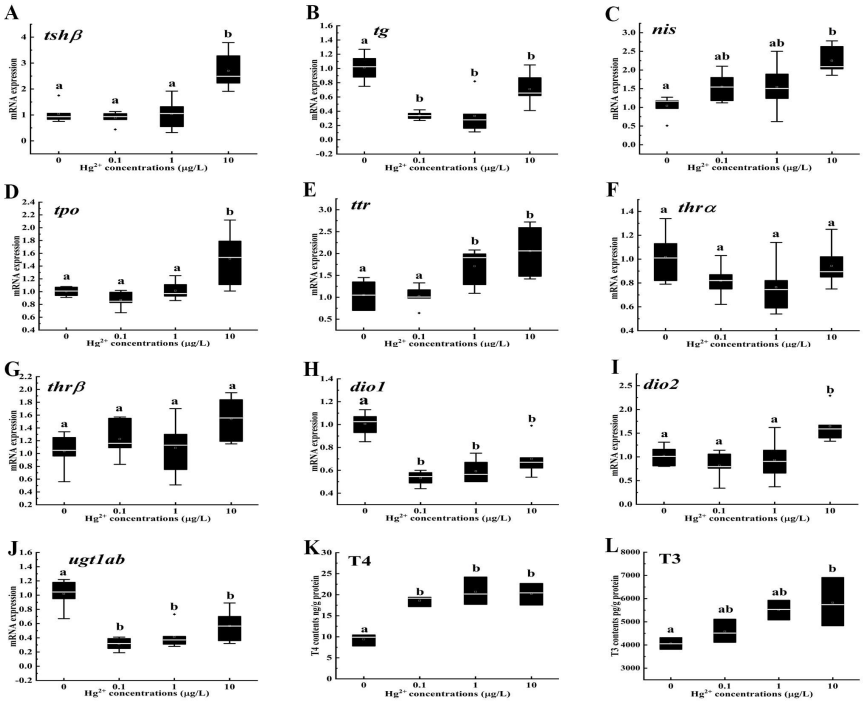

Figure 2. Transcription levels of *tshβ* (**A**), *tg* (**B**), *nis* (**C**), *tpo* (**D**), *ttr* (**E**), *thrα* (**F**), *thrβ* (**G**), *dio1* (**H**), *dio2* (**I**), *ugt1ab* (**J**) and contents of T4 (**K**), T3 (**L**) in zebrafish embryos (2 hpf) that were treated with Hg^{2+} (0, 0.1, 1, and 10 μg/L) for 120 h. Data are shown as mean ± SD (n = 6). Different letters denote significant differences between groups ($p < 0.05$).

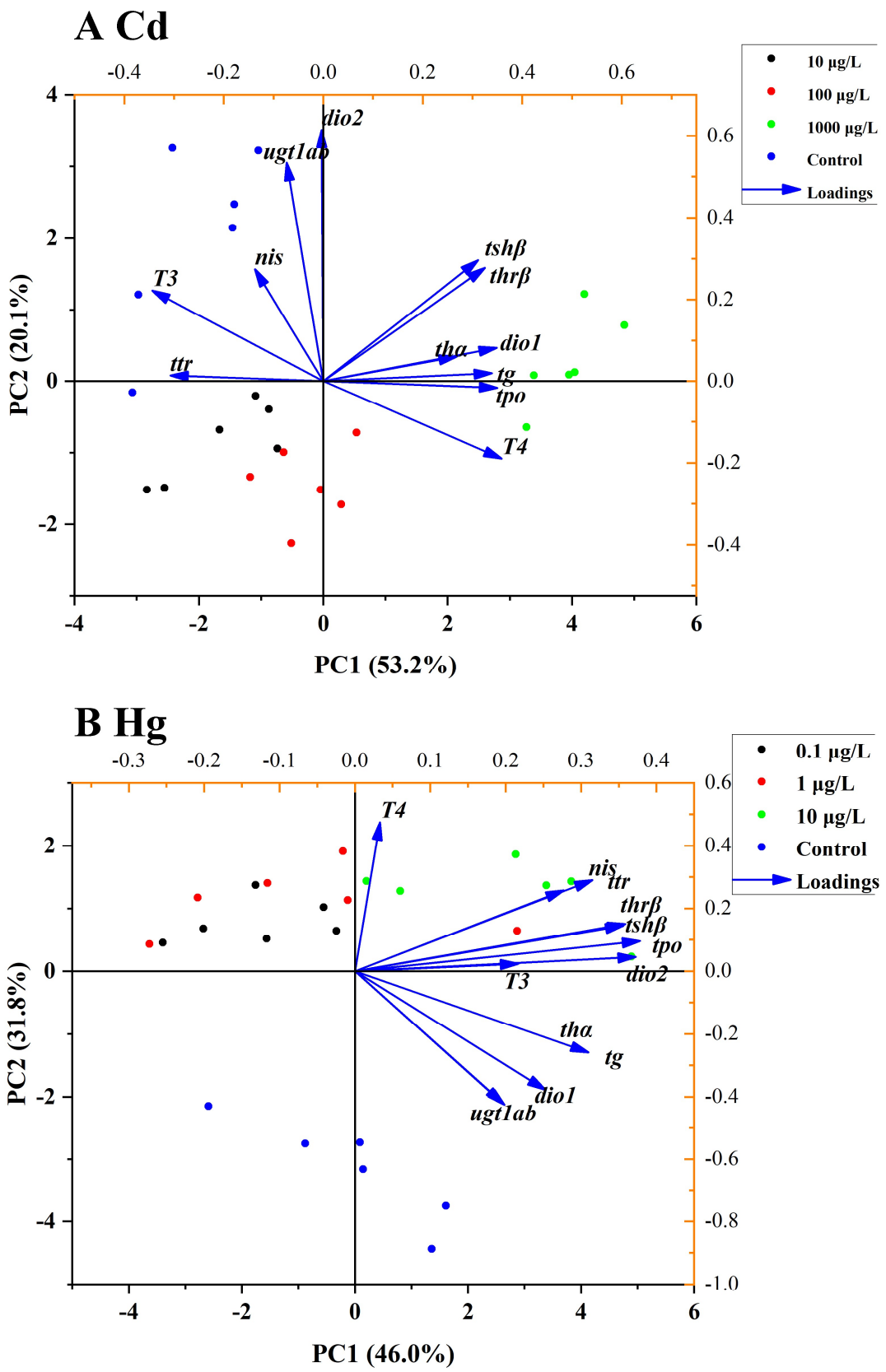

Figure 3. Principal component analysis (PCA) of parameters in zebrafish embryos (2 hpf) that were treated with Cd^{2+} (0, 10, 100, and 1000 μg/L) (**A**) and Hg^{2+} (0, 0.1, 1, and 10 μg/L) (**B**) for 120 h.

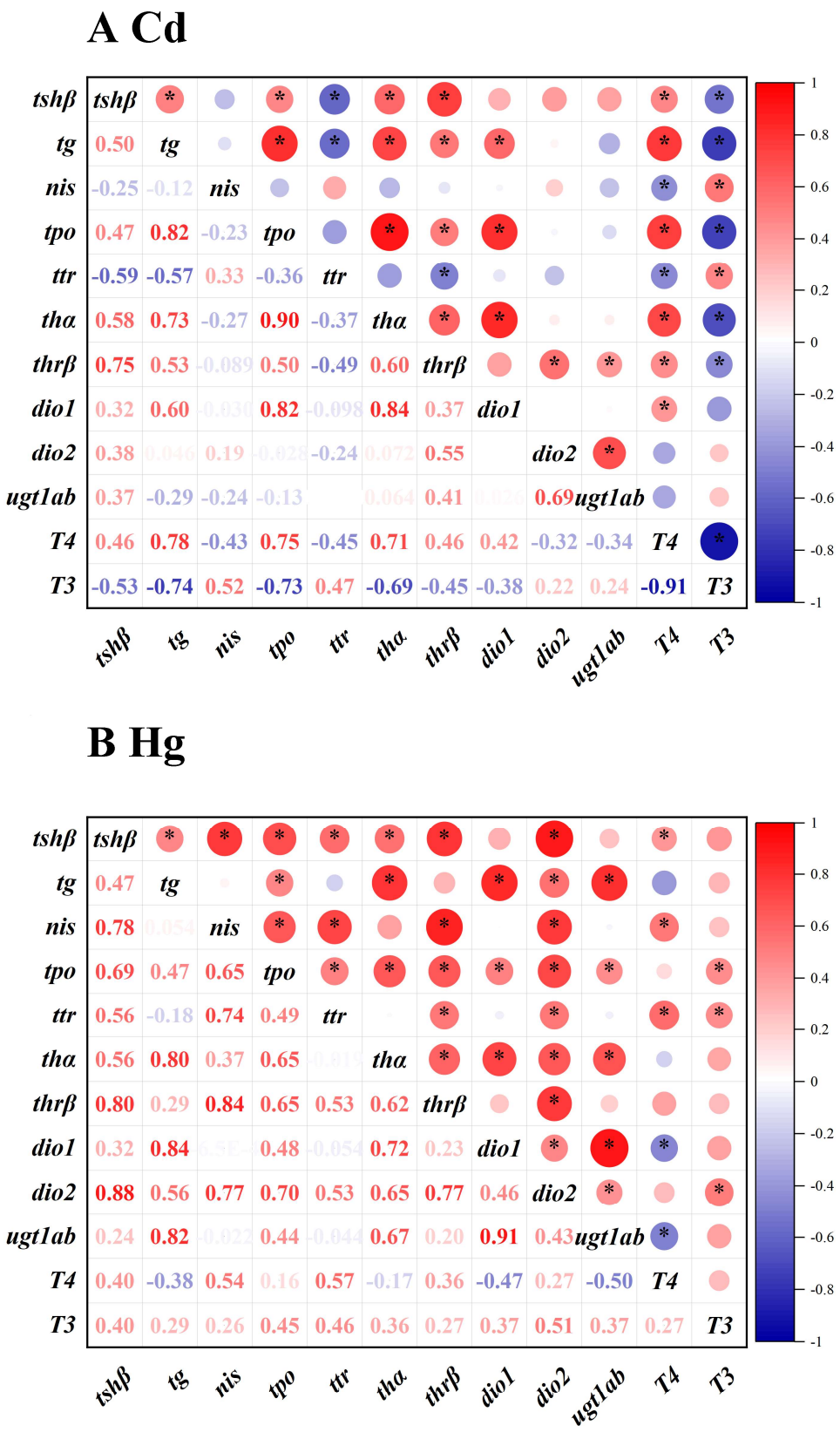

Figure 4. Heatmap of correlation coefficients between the thyroid hormones (THs, including T4 and T3) contents and the gene expression levels in the zebrafish embryos (2 hpf) that were treated with Cd^{2+} (0, 10, 100, and 1000 μg/L) (**A**) and Hg^{2+} (0, 0.1, 1, and 10 μg/L) (**B**) for 120 h. The scale indicates the level of positive (red) or negative (blue) correlation, and * indicates significance (* $p < 0.05$).

The PCA results of Hg^{2+} exposure are shown in Figure 3B. The initial two PCs accounted for 77.8% of the total variance. PC1 accounted for 46.0% of the total variances, while PC2 explained 31.8% of the total variances. Based on the PCA result, the separation between clusters of the control group and all Hg^{2+} treatment (0.1, 1, and 1 μg/L) groups was significant. T4 levels were seen to be significantly and positively correlated with the transcriptional levels of *tshβ*, *nis*, and *ttr* genes and were negatively related to the transcriptional levels of *dio1* and *ugt1ab* genes (Figure 4B). A significant positive correlation was observed between the T3 levels and the *tpo* and *ttr* transcriptional levels (Figure 4B).

4. Discussion

Heavy metals can cause developmental toxicity in fish [38,39]. In the present study, although the hatching and survival rates of the fish were not notably affected by Cd^{2+} and Hg^{2+}, the malformation rates were notably elevated in the zebrafish larvae that were exposed to varying concentrations of Cd^{2+} and Hg^{2+}. Therefore, malformations were found to be a more vulnerable parameter than the hatching and survival rates for assessing developmental toxicity in the zebrafish embryos.

PCA is a useful tool to reduce the dimensionality of large data sets by transforming a large set of variables into a smaller one. Based on the PCA and correlation analyses, THs levels showed a strong correlation with the transcriptional levels of *tshβ*, *tg*, *tpo*, *thα*, *thrβ*, *dio1*, *nis*, and *ttr* in Cd^{2+}-treated embryos/larvae. While the THs levels showed a strong correlation with the transcriptional levels of *tshβ*, *nis*, *ttr*, *dio1*, *ugt1ab*, and *tpo* genes in Hg^{2+}-treated embryos/larvae. Next, we explained the role of these genes in thyroid hormone synthesis.

In vertebrates, the *tshβ* gene encodes for TSH, which stimulates the thyroid gland to generate THs via binding to the corresponding receptor [40]. Thus, the upregulation of *tshβ* could be linked to a higher T4 level. In this study, the *tshβ* transcriptional levels were upregulated significantly, which could explain the enhanced T4 concentrations in the Cd^{2+} and Hg^{2+} treatment groups.

Herein, the transcriptional levels of genes (including *tg*, *nis*, and *tpo*) were determined to be associated with THs biosynthesis. The *nis* gene encodes for the Na-Iodide symporter, which is what actually facilitates the transport (uptake) of iodide into the epithelial cell [41]. The function of the TPO enzyme is to catalyze the iodination of tyrosyl-residues in TG proteins and follow-up coupling of iodotyrosines to produce THs [42,43]. Therefore, the upregulation of *tg*, *nis*, and *tpo* expressions might be related to the increasing T4 levels. In this study, an increase was noted in the T4 concentration, along with the upregulation of *tg* and *tpo* gene expression levels and the upregulation of *nis* and *tpo* gene expression levels in the Cd^{2+} and Hg^{2+} treatment groups, respectively. However, the *tg* gene expression was significantly downregulated, and the T4 levels were increased in the Hg^{2+} exposure groups. This could be attributed to negative feedback regulation in the HPT axis, which downregulates *tg* gene expression to decrease T4 content.

TTR is a carrier protein for THs, and it mediates the transport of THs through the bloodstream to the target tissues [44,45]. Downregulation of the *ttr* gene, which is related to THs transport and metabolic processes in the HPT axis, might be associated with elevated circulating plasma THs levels. Therefore, the significant downregulation of the *ttr* gene might be attributed to the enhanced T4 content in zebrafish larvae after treatment with Cd^{2+}. Consistent with these results, downregulation of *ttr* and enhanced T4 content were reported in zebrafish larvae treated with decabromodiphenyl ethane (DEDPE) [46]. However, earlier reports demonstrated that T4 levels were significantly and positively linked to *ttr* gene expression [36,47]. In line with these studies, the T4 content was significantly increased, along with upregulated *ttr* gene transcription, in the Hg^{2+} treatment groups.

The THR, which belongs to the nuclear receptor superfamily, is a transcription factor that responds to T3. Previous results showed that T4 bound to *trα*, and mediated the expression of THs-regulated genes [48]. Significant upregulation of *thrα* expression was observed after Cd^{2+} exposure in this study, which might be attributed to the higher T4 levels. Previous studies have reported significant upregulation of *thrα* and *thrβ* in Chinese rare

minnow larvae following Hg^{2+} exposure [28]. However, the *thrα* and *thrβ* gene expression levels were not altered in the Hg^{2+} treatment group in this study. These inconsistent consequences might be due to the species-specific effects caused by Hg^{2+} and must be investigated further.

Previous studies reported that Cd^{2+} might be an inhibitor of deiodinase activity and suppress T4 to T3 conversion [49,50]. Similarly, zebrafish embryos/larvae that were exposed to Cd^{2+} exhibited a significant downregulation of the *dio2* gene expression, along with increased T4 and decreased T3 contents in this study. In addition, *dio2* was involved in T4 deiodination to form active T3 [51,52]. Thus, downregulation of the *dio2* gene expression was attributed to the increased T4 level and reduced T3 level in the Cd^{2+} treatment groups. Moreover, *dio1* functions as an outer-ring THs deiodinase and is involved in the metabolism of THs [53]. Therefore, an increase in the T3 content in the Hg^{2+} treatment group was partially attributed to the upregulation of *dio2* and the downregulation of *dio1*. The *ugt1ab* gene was remarkably involved in the THs homeostasis by regulating T4 glucuronidation [54]. An earlier study reported that an increase in the T4 content in zebrafish larvae was due to the downregulation of *ugt1ab* mRNA levels [46]. In this study, the *ugt1ab* gene transcription level was significantly downregulated in the Cd^{2+} and Hg^{2+} treatment groups. Therefore, the increased T4 content could be attributed to the downregulation of *ugt1ab* transcription levels.

Based on the PCA results, it was seen that Cd^{2+} significantly affected the thyroid endocrine system at a concentration of 100 μg/L, whereas Hg^{2+} led to a thyroid disruption at a concentration as low as 0.1 μg/L. Hg^{2+} was a more potent disruptor as compared to Cd^{2+}. The standards for drinking water quality in China (GB5749–2022) listed that the maximal allowable concentrations of Cd^{2+} and Hg^{2+} were 5 and 1 μg/L, respectively. Since Hg^{2+} negatively affects the endocrine thyroid system below this permissible level, the adverse effect of Hg^{2+} on the endocrine system of fish needs to be investigated further.

5. Conclusions

In summary, this study demonstrated that treatment with Cd^{2+} and Hg^{2+} caused developmental toxicity and thyroid disruption in the zebrafish embryos/larvae. Both whole-body THs contents and the expression patterns of genes linked to the HPT axis were altered after exposure to Cd^{2+} and Hg^{2+}. Based on the PCA results, Cd^{2+} significantly affected the thyroid endocrine system at a concentration of 100 μg/L, whereas Hg^{2+} led to a thyroid disruption at a concentration as low as 0.1 μg/L. Further research is necessary to elucidate the mechanisms of thyroid disruption due to heavy metals.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w15010135/s1, Figure S1: Morphology of zebrafish larvae exposure to Cd^{2+} and Hg^{2+}. Normal zebrafish larvae (A); the morphological changes caused by Cd^{2+} (B–D), including yolk sac edema (B), tail malformation (C), and yolk sac edema and pericardial edema (D); the morphological changes caused by Hg^{2+} (E–I), including tail malformation (E), yolk sac edema (I), pericardial edema (H) and spinal curvature (I).

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