Aquatic Toxicology 138–139 (2013) 35–42

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Thyroid endocrine disruption in zebrafish larvae following exposure to hexaconazole and tebuconazole

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ARTICLE INFO

Article history:
Received 6 February 2013
Received in revised form 3 April 2013
Accepted 4 April 2013

Keywords:
Tebuconazole
Hexaconazole
Thyroid endocrine disruption
Gene transcription
Zebrafish larvae

ABSTRACT

The widely used triazole fungicides have the potential to disrupt endocrine system, but little is known of such effects or underlying mechanisms of hexaconazole (HEX) and tebuconazole (TEB) in fish. In the present study, zebrafish (*Danio rerio*) embryos were exposed to various concentrations of HEX (0.625, 1.25 and 2.5 mg/L) and TEB (1, 2 and 4 mg/L) from fertilization to 120 h post-fertilization (hpf). The whole body content of thyroid hormone and transcription of genes in the hypothalamic-pituitary-thyroid (HPT) axis were analyzed. The results showed that thyroxine (T4) levels were significantly decreased, while triiodothyronine (T3) concentrations were significantly increased after exposure to HEX and TEB, indicating thyroid endocrine disruption. Exposure to HEX significantly induced the transcription of all the measured genes (i.e., corticotrophin-releasing hormone (CRH), thyroid-stimulating hormone (TSHβ), sodium/iodide symporter (NIS), transthyretin (TTR), uridine diphosphate glucuronosyltransferase (UGT1ab), thyronine deiodinase (Dio1 and Dio2), thyroid hormone receptors (TRα and TRβ)) in the HPT axis, but did not affect the transcription of thyroglobulin (TG). However, TEB exposure resulted in the upregulation of all the measured genes, excepting that TG, Dio1and TRα had not changed significantly. The overall results indicated that exposure to HEX and TEB could alter thyroid hormone levels as well as gene transcription in the HPT axis in zebrafish larvae.

1. Introduction

Endocrine disrupting chemicals (EDCs) are compounds that interfere with endocrine (or hormone system) in both wildlife and humans. EDCs can modulate the endocrine system through multiple mechanisms of action and possibly cause cancerous tumors, birth defects, and other developmental disorders. Animal studies have indicated that many of those adverse effects were often permanent and some EDCs could act as epigenetic modulators which led to potential transgenerational effects (Anway et al., 2005; Waring and Harris, 2011). The adverse effects of EDCs which have been observed in many vertebrate species cause growing concern among researchers and policy makers. 127 pesticides were identified as having endocrine disrupting properties, including the 91 listed by the Pesticide Action Network (Tebourbi et al., 2011). Consequently, endocrine disrupting pesticides (EDPs) are the largest group of EDCs in numbers compared to other chemical groups.

Triazoles are a class of fungicides largely used in agriculture as crop protection products. Their antifungal activity is due to their ability to inhibit the P450 enzyme (CYP51), which blocks the conversion of lanosterol to ergosterol causing disruption of fungal wall (Di Renzo et al., 2007). But the inhibition potency of these triazole fungicides is not limited to fungi, they may also inhibit other P450-mediated activities resulting in various adverse effects (Robinson et al., 2012). Several triazoles have been reported to alter the concentrations or genes transcription involved in steroid homoeostasis and thirteen triazoles have been identified as endocrine disruptors (Goetz and Dix, 2009; Hester and Nesnow, 2008; Liu et al., 2011; Mnif et al., 2011). Hexaconazole (HEX) and tebuconazole (TEB) are both triazole fungicides, which are applied on a number of crops in China such as grapes, rice, fruits, and vegetables because of their broad-spectrum antifungal activity. The two fungicides had been classified by the US EPA as Group C-Possible Human Carcinogen (U.S. EPA, 2006). TEB is persistent in soils and presents moderate mobility (EFSA, 2008). It is classified as toxic to aquatic organisms and may cause

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0166-445X
http://dx.doi.org/10.1016/j.aquatox.2013.04.001

long-term adverse effects in the aquatic environment (Bayer CropScience Limited, 2005). The presence of TEB in stream water has increased in recent years (Montuelle et al., 2010) and its concentrations detected in surface waters was up to 175–200 μg/L (Elsaesser and Schulz, 2008). Even more, it was found out in human being with the maximal concentrations of 19.2 μg/L and 2.22 ng/kg in urine and hair samples from farm workers, respectively (Fustinoni et al., 2012; Schummer et al., 2012). HEX is highly active and efficiently acts to inhibit sterol synthesis in many fungi, particularly ascomycetes and basidiomycetes (Worthington, 1991). HEX is highly persistent with the field soil degradation DT_{50} of 225 d, and no degradation of HEX is found in river water incubated at 20 °C for 3 weeks (Tsukatani et al., 2008). Accordingly, there was a potential for exposure to TEB and HEX, thus causing endocrine disrupting effects on human beings and wildlife, especially on aquatic species. But there were few studies regarding their endocrine toxicity, especially disruption on the thyroid hormonal homeostasis of fish.

Thyroid hormones (THs) play a crucial role in the regulation of development, growth, immune, metabolism, energy provision, reproduction and behavior in vertebrates (Jugan et al., 2010). Fish thyroid homeostasis is subject to the regulation of hypothalamic-pituitary-thyroid (HPT) axis. Many different groups of chemicals may interfere with thyroid hormonal homeostasis, such as triadimefon (Liu et al., 2011), perfluorooctane sulfonate (PFOS) (Shi et al., 2009), microcystin-leucine-arginine (MCLR) (Yan et al., 2012). Previous studies suggested that the influence of a chemical on HPT axis, including the alterations of gene transcriptions, hormone levels and enzyme activities, could be applied to evaluate the effect of thyroid endocrine disruption (Chen et al., 2012; Picard-Aitken et al., 2007; Yu et al., 2010). In addition, using gene transcription patterns as endpoints could observe toxic effects at toxicant concentrations that did not cause morphological effects (Hermsen et al., 2012; Yang et al., 2007a) and provide further insight into the mechanisms of action of toxicants.

Zebrafish (*Danio rerio*) are widely used as a predominant test model for the assessment of EDCs due to its small size, ease of culture, high reproductive capacity, rapid organogenesis, morphological and physiological similarities to mammals, etc. (Segner, 2009). Some previous studies suggested that zebrafish embryos/larvae were an ideal model fish for investigating endocrine disruption by chemicals in the laboratory (Chen et al., 2012; Kanungo et al., 2012; Liu et al., 2011; Tu et al., 2013). Therefore, zebrafish embryos were employed as a model in our study. In order to investigate the effect of TEB and HEX on thyroid development of zebrafish embryos, and to discuss the possible mechanisms underlying toxic response, gene transcription in the HPT axis were quantitatively examined, meanwhile, the levels of THs (T3 and T4) were also measured in the present study. The information obtained will add new knowledge about the endocrine toxicity mechanisms of triazoles.

2. Materials and methods

2.1. Chemicals

TEB (technical grade AI: 98%) was purchased from Shanghai Yuanji Chemical Co., Ltd. (Shanghai, China). HEX (technical grade AI: 96.3%) was purchased from Zhejiang Jiahua Group Co., Ltd. (Zhejiang, China). Dimethyl sulfoxide (DMSO) and MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

2.2. Zebrafish maintenance and embryo exposure

Adult zebrafish (*D. rerio*) of the wild-type (AB strain) were raised in a flow-through system with dechlorinated tap water (pH 7.0–7.4) at a constant temperature (28 ± 1 °C). The light regime was 14-h light, 10-h dark. Fish were fed with freshly hatched brine shrimp (*Artemia nauplii*) twice a day in a quantity that was consumed within 5 min. Five days before spawning, females were separately housed to optimize egg production. Males and females were paired in spawning boxes on the afternoon the day before spawning in a ratio of 2:2. Spawning was triggered once the light was turned on the following morning and was usually completed within 30 min.

Eggs from different spawning boxes were collected within 1 h of spawning, were pooled and washed in standardized water (Standardization, 1996) (117.6 mg of $CaCl_2{\cdot}2H_2O$, 49.3 mg of $MgSO_4{\cdot}7H_2O$, 25.9 mg $NaHCO_3$ and 2.3 mg KCl in 1 L of deionized water) to remove any coagulated eggs and debris. At 2 h post-fertilization (hpf), embryos were examined under a dissecting microscope, and those embryos that had developed normally and reached the blastula stage were selected for subsequent experiments. Fertilization rate of the batch of eggs used was at least 90%. Standardized water was aerated during at least half an hour before addition of test chemicals.

Stock solutions of the test chemicals were prepared in DMSO in concentrations 1000 times the highest final exposure concentrations and stored at −20 ± 1 °C before use. Stock solutions were serially diluted in DMSO and 500 μL of each of these were mixed with 500 mL of standardized water in beakers to give a total DMSO concentration of 0.1% in each test solution. Normal embryos (approximately 400) were randomly distributed into glass beakers containing 500 mL of HEX and TEB solution at various nominal concentrations (HEX: 0, 0.625, 1.25, 2.5 mg/L; TEB: 0, 1, 2, 4 mg/L) until 120 hpf. The selected exposure concentrations were previously ascertained by performing a range-finding study; this study revealed that after exposure to the lowest concentration of HEX and TEB, the malformation rates showed a trend toward an increase but the tendency was not statistically significant. Both the control and exposure groups with 6 replicates in each exposure concentration received 0.01% (v/v) DMSO. During the experimental period, embryos were kept in an incubator at 28 ± 0.5 °C with a photoperiod of 14 h light:10 h dark. Chorions, dead eggs and larvae were removed and 50% of the exposure solution was renewed daily. After exposure, the larvae were placed in MS-222 (40 mg/L), and then divided into two groups: one group were placed in *RNAlater* (Ambion, Austin, TX) to stabilize RNA and stored at −20 °C for RNA isolation, and the other group were frozen in liquid nitrogen, and stored at −80 °C for hormone analysis.

2.3. Thyroid hormone extraction and measurement

T3 and T4 levels were measured by using enzyme-linked immunosorbent assay (ELISA) as described by Yu et al. (2011). The test kits were purchased from R&D Company (USA). 300 larvae were used for thyroid hormone extraction according to manufacturer's description of ELISA kit (R&D, USA). The extraction efficiencies were determined by addition of 100 μl ^{125}I radioiodinated T4 and T3 (Beijing North Institute of Biotechnology, Beijing, China) to larvae ($n = 4$). The mean recoveries for larval samples were 67.3% T4 (61.6–74.3%) and 65.7% T3 (62.2–73.7%). The ELISA for T3 and T4 was validated for use with zebrafish samples by demonstrating parallelism between a series of diluted and spiked samples in relation to the standard curve attached to the ELISA kit. The detection limits for T3 and T4 were 0.6 ng/mL and 3 ng/mL, respectively.

2.4. RNA isolation and quantitative real-time polymerase chain reaction

To begin RNA isolation, *RNAlater* was removed and total RNA was extracted from 30 homogenized zebrafish larvae by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), all procedures followed the manufacturer's instruction and the published protocols. The RNA samples were dissolved in ribonuclease-free water, the concentration of RNA was measured on the NanoDrop spectrophotometer (ND-2000; NanoDrop Technologies Inc., Wilmington, DE), and then stored at −80 °C until the process of reverse-transcriptase polymerase chain reaction. Approximately 1 μg RNA was retrotranscribed by PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Biochemicals, Dalian, China) following the manufacturer's instructions. The kit could remove the genomic DNA contamination, and the product from the reverse transcription was diluted 10 times and 2 μL of each diluted sample, corresponding to 10 ng of the reverse transcribed RNA, was used for the real-time PCR. Quantitative real-time PCR was carried out using the SYBR Green PCR kit (Takara Biochemicals, Dalian, China) and analyzed on an ABI StepOnePlus™ Real-Time PCR System (PerkinElmer Applied Biosystems, Foster City, CA, USA). The primer sequences of the selected genes were performed as described previously by Yu et al. (2010) and Jin et al. (2008), listed in Table 1. The thermal profile was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A melting temperature-determining dissociation step was performed at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s at the end of the amplification phase.

All the samples were analyzed in triplicate and the mean value of these triplicate measurements were used for the calculations of the mRNA transcriptions. The housekeeping gene β-actin was used as an internal standard. The transcription level of each target gene was normalized to its β-actin mRNA content. The fold change of the tested genes was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Dissociation curve analysis was performed for each gene to check the amplification of untargeted fragments only one peak was observed for each amplification, indicative for the amplification of the target gene only. Gene transcription data are presented as change relative to control animals within the same treatment.

2.5. Statistics

Data were statistically analyzed using the SPSS (version 16.0; USA). All quantitative data were expressed as the mean ± S.E. of the mean (S.E.M.). One-way ANOVA was applied to calculate statistical significance followed by Dunnett's test as a post hoc test to independently compare each exposure group to the control group. The LSD test was used as a post hoc test for multiple comparisons between groups. A probability of $P < 0.05$ was considered statistically significant.

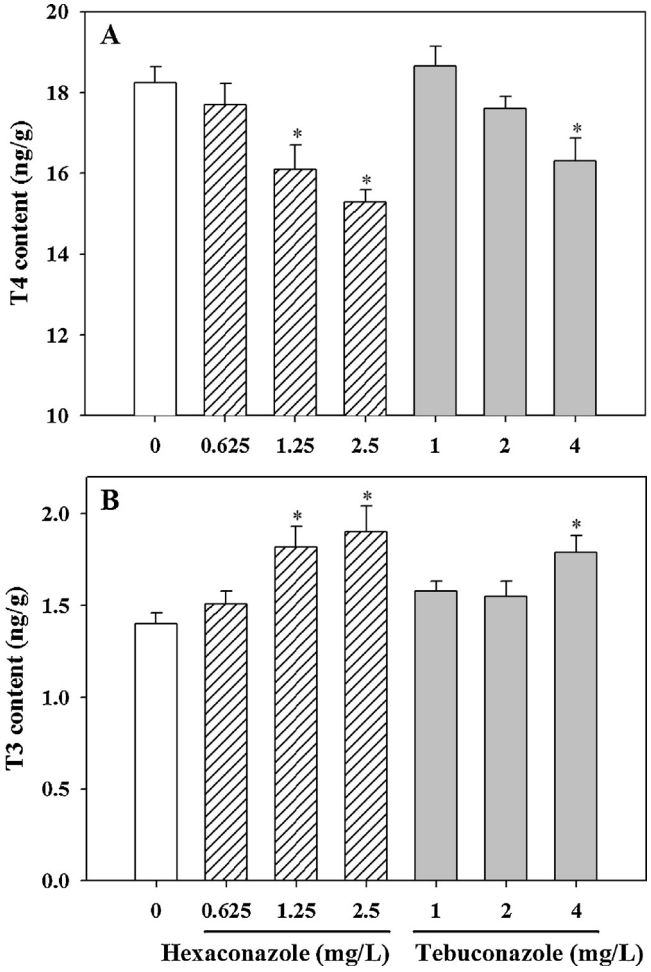

Fig. 1. Whole body content of T4 (A) and T3 (B) in zebrafish larvae exposed to various concentrations of Tebuconazole and Hexaconazole until 120 h. Data expressed as mean ± S.E. ($n = 4$), $^*P < 0.05$, relative to control.

3. Results

3.1. Developmental toxicity

There were no significant effects on hatching, malformation and survival after exposure to HEX (0.625, 1.25 and 2.5 mg/L) and TEB (1, 2 and 4 mg/L) relative to the control until 120 hpf.

3.2. Whole-body TH content

The body total THs were measured in the larvae at 120 hpf, results were listed in Fig. 1. The whole-body T4 and T3 contents

Table 1
Primers used for the quantification of the mRNA transcription by real-time PCR.

Gene name	Sense primers (5′–3′)	Antisense primer (5′–3′)	Gen bank accession no.
β-Actin	atggatgaggaaatcgctgcc	ctccctgatgtctgggtcgtc	AF057040
CRH	ttcgggaagtaaccacaagc	ctgcactctattcgccttcc	NM_001007379
TSHβ	gcagatcctcacttcacctacc	gcacaggtttggagcatctca	AY135147
NIS	ggtggcatgaaggctgtaat	gatacggcatccattgttgg	NM_001089391
TG	ccagccgaaaggatagagttg	atgctgccgtggaatagga	XM_001335283
Dio1	gttcaaacagcttgtcaaggact	agcaagcctctcctccaagtt	BC076008
Dio2	gcataggcagtcgctcattt	tgtggtctctcatccaacca	NM_212789
UGT1ab	ccaccaagtctttccgtgtt	gcagtccttcacaggctttc	NM_213422
TTR	cgggtggagtttgacacttt	gctcagaaggagagccagta	BC081488
TRα	ctatgaacagcacatccgacaagag	cacaccacacacggctcatc	NM_131396
TRβ	tgggagatgatacgggttgt	ataggtgccgatccaatgtc	NM_131340

in the control group were detected to be 18.25 ± 0.38 ng/g and 1.43 ± 0.06 ng/g, respectively. In HEX treatment groups, the total T4 levels were significantly decreased by 11.8% and 16.2% exposed to 1.25 and 2.5 mg/L, respectively, compared with those in the control ($P < 0.05$) (Fig. 1A). In contrast, the total T3 levels were significantly increased by 30% and 35.7% exposed to 1.25 and 2.5 mg/L, respectively ($P < 0.05$) (Fig. 1B). In TEB treatment groups, significant difference was only observed in the 4 mg/L exposure group, in which the T4 level was significantly decreased (10.7%) (Fig. 1A) and the T3 level was significantly increased (27.8%) ($P < 0.05$) (Fig. 1B).

3.3. Gene transcription profile

The transcription of the CRH gene was significantly up-regulated by over 3-fold relative to the control in the 1.25 and 2.5 mg/L HEX exposure groups (Fig. 2A), while the 1 and 2 mg/L TEB exposure up-regulated CRH gene over 1.6-fold relative to the control (Fig. 2A). Thyroid stimulating hormone (TSHβ) gene transcription was significantly up-regulated by 2.34-, 2.06- and 10.55-fold after exposure to 0.625, 1.25 and 2.5 mg/L HEX, respectively, while 2.47- and 2.18-fold after exposure to 2 and 4 mg/L TEB, respectively (Fig. 2B).

Upon treatment with 1.25 and 2.5 mg/L HEX, the transthyretin (TTR) gene transcriptional was significantly up-regulated by 6.93- and 9.83-fold, respectively, while by 4.17- and 3.11-fold in the 2 and 4 mg/L TEB exposure groups (Fig. 2C). The mRNA levels of uridine diphosphate glucuronosyltransferase (UGT1ab) were significantly increased 1.6-, 2.71- and 4.21-fold after exposure to 0.625, 1.25 and 2.5 mg/L of HEX, respectively, while 2.88- and 6.5-fold after exposure to 2 and 4 mg/L of TEB, respectively (Fig. 3A). The sodium/iodide symporter (NIS) gene transcription was significantly up-regulated 3-fold after exposure to 2.5 mg/L HEX, while 1.67- and 5.36-fold after exposure to 2 and 4 mg/L TEB (Fig. 3B). Transcription of the thyroglobulin (TG) gene showed no change in all the HEX and TEB exposure groups (Fig. 3C).

In zebrafish, two isoforms of deiodinases (Dio1 and Dio2) have been identified. HEX exposure significantly up-regulated Dio1 gene transcription (1.8- and 3.73-fold) in the 1.25 and 2.5 mg/L exposure groups (Fig. 4A). However, the levels of Dio1 gene transcription were not altered in any TEB treated groups (Fig. 4A). With regard to the gene transcription of Dio2, exposure to 1.25 and 2.5 mg/L HEX led to induced mRNA transcription by 5.65- and 13.83-fold, respectively, exposure to 2 and 4 mg/L TEB led to induced mRNA transcription by 4.36- and 3.28-fold, respectively (Fig. 4B).

There were no significant changes in the mRNA transcription of TRα when exposed to TEB (Fig. 5A). While the HEX exposure significantly up-regulated TRα gene transcription (1.64-, 1.96- and 4.73-fold) in the 0.625, 1.25 and 2.5 mg/L exposure groups (Fig. 5A). Transcription of TRβ was increased by 2.94- and 5.32-fold in the 1.25 and 2.5 mg/L HEX groups, respectively, while by 1.97- and 2.61-fold in the 2 and 4 mg/L TEB groups, respectively (Fig. 5B).

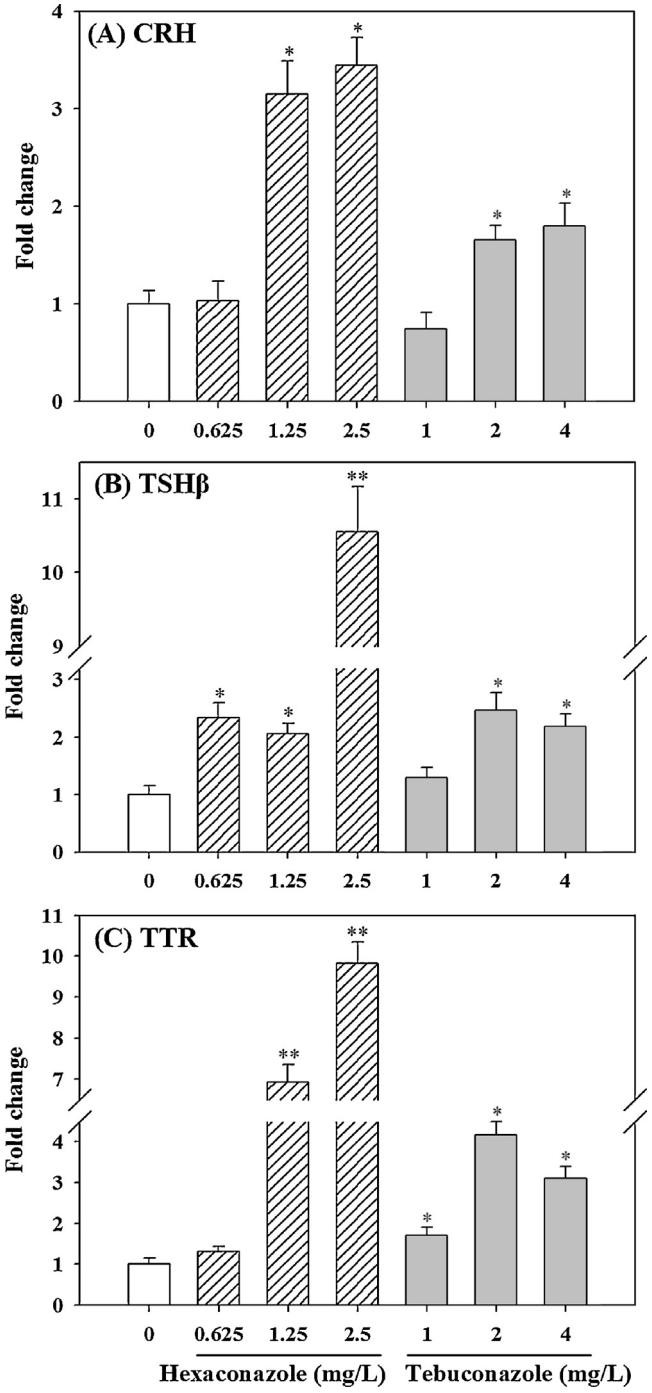

Fig. 2. Relative transcription levels of (A) CRH, (B) TSHβ and (C) TTR in zebrafish larvae exposed to various concentrations of tebuconazole and hexaconazole until 120 h. The results were evaluated as the relative ratio of the transcription level of each mRNA to that of β-actin. Data expressed as mean ± S.E. ($n = 4$), $^{*}P < 0.05$; $^{**}P < 0.01$, relative to control.

4. Discussion

In the present study, a significant decrease in T4 levels and increase in T3 levels were detected in zebrafish larvae after exposure to HEX and TEB, suggesting HEX and TEB can induce thyroid disruption. This result was consistent with previous findings, in which TEB exposure was found to decrease the plasma T4 levels in rats (Kjærstad and Miljøstyrelsen, 2007). On the contrary, the T4 level was increased and the T3 level was decreased in the zebrafish larvae after exposed to triadimefon, which was also a triazole fungicide (Liu et al., 2011). This indicated that triazole fungicides might have the abilities to act as endocrine disruptors via a range of different mechanisms.

CRH and TSH secretions function as common regulators of the HPT axis in fish are triggered by changes in the concentrations of circulating THs (De Groef et al., 2006), evaluation of CRH and TSHβ genes transcription can be used to determine whether environmental chemicals cause thyroid dysfunction (Yu et al., 2011). The present data showed that CRH and TSHβ gene transcriptions were both significantly upregulated after HEX and TEB treatment. Previous studies indicated that T4 modulated the transcription of CRH

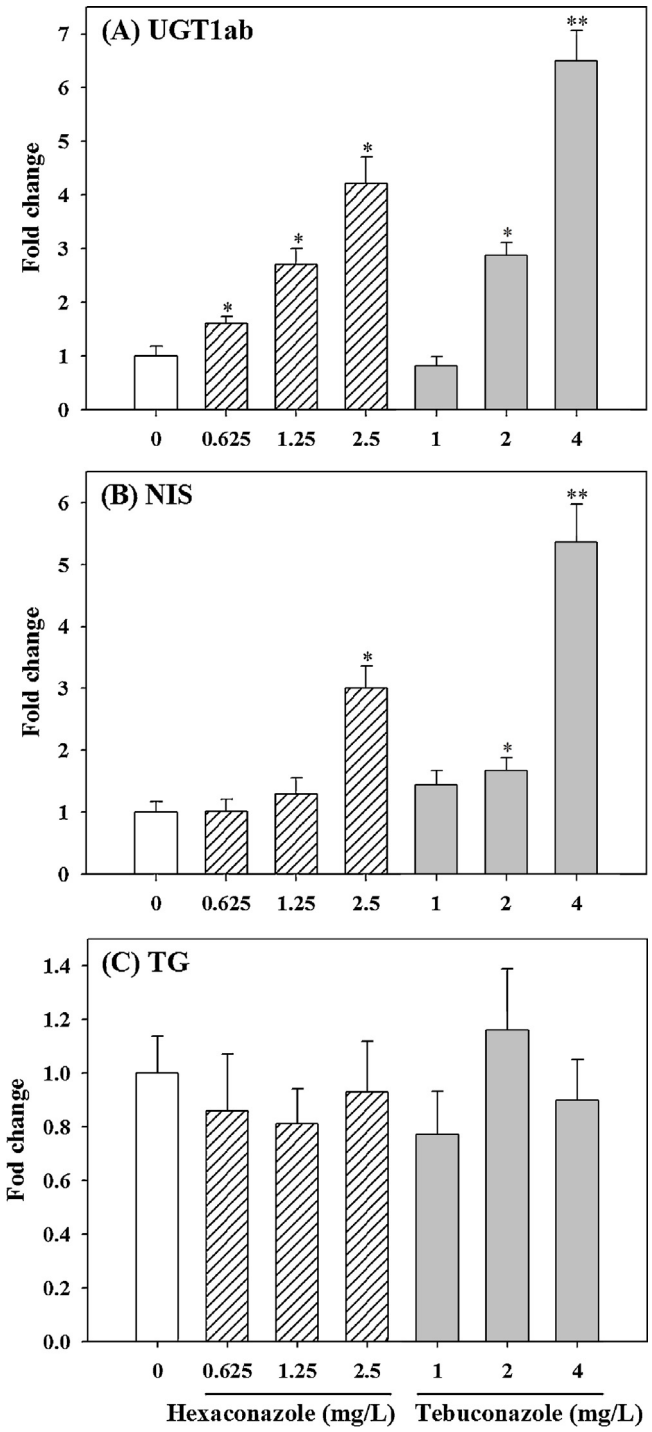

Fig. 3. Relative transcription levels of (A) UGT1ab, (B) NIS and (C) TG in zebrafish larvae exposed to various concentrations of tebuconazole and hexaconazole until 120 h. The results were evaluated as the relative ratio of the transcription level of each mRNA to that of β-actin. Data expressed as mean ± S.E. ($n = 4$), $^{*}P < 0.05$; $^{**}P < 0.01$, relative to control.

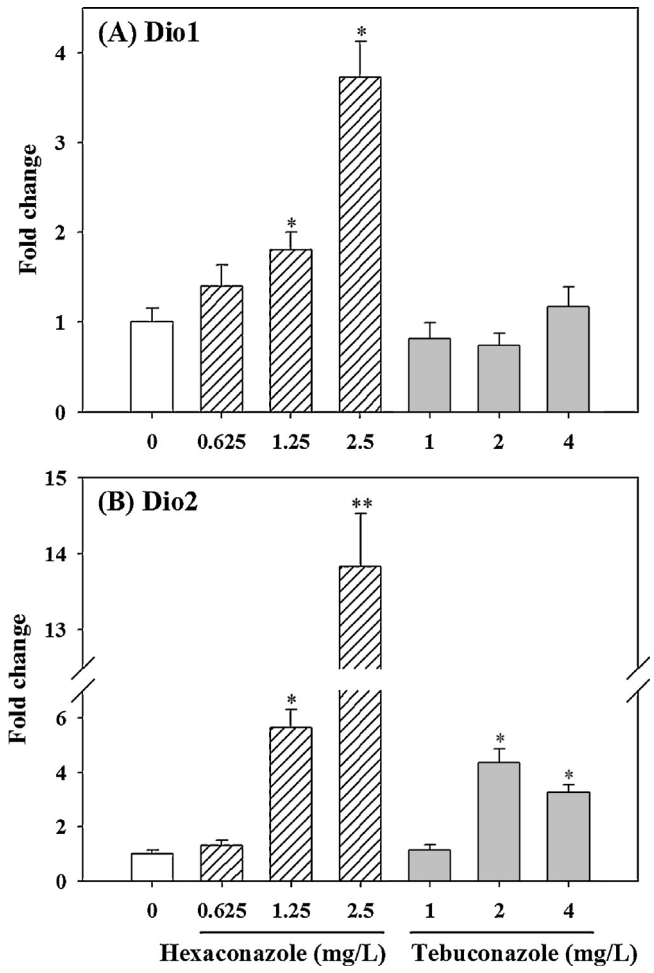

Fig. 4. Relative transcription levels of (A) Dio1 and (B) Dio2 in zebrafish larvae exposed to various concentrations of tebuconazole and hexaconazole until 120 h. The results were evaluated as the relative ratio of the transcription level of each mRNA to that of β-actin. Data expressed as mean ± S.E. ($n = 4$), $^{*}P < 0.05$; $^{**}P < 0.01$, relative to control.

and TSH in fish via a negative feedback mechanism, reductions of T4 and accompanying upregulation of CRH and TSHβ gene transcription have been reported in zebrafish larvae when exposed to BDE-209 (Chen et al., 2012), PBDE mixture DE-71 (Yu et al., 2010) and MCLR (Yan et al., 2012). On the other hand, long-term exposure to DE-71 increased the levels of plasma T4 in zebrafish, which was associated with decreased transcription of CRH and TSHβ in the brain (Yu et al., 2011). Together with these findings, our results suggested that the upregulation of CRH and TSHβ could be attributed to the hypothalamus and pituitary negative feedback mechanism for the regulation of the decreased T4 levels.

TTR binds THs and transports them to various target tissues (Morgado et al., 2007b). Several environmental contaminants that share structural similarity to the THs may interfere with TH homeostasis by binding TTR in vertebrates, thus disrupting the TH axis (Morgado et al., 2007a). Therefore, TTR gene was used as an indicator for thyroid system disruption in several studies (He et al., 2012; Li et al., 2009; Yu et al., 2011). THs may directly or indirectly regulate plasma TTR (Morgado et al., 2007b), previous studies observed that the decreases in TTR were coincident with situations in which THs were reduced (He et al., 2012; Yu et al., 2010). However, the TTR gene transcription increased in lampreys when serum TH concentrations were at their lowest (Manzon et al., 2007). Hence, the plasma TH concentrations were unlikely to be responsible for differences in the TTR transcription (Li et al., 2009). In our studies, TTR gene transcription in zebrafish larvae were up-regulated after HEX and TEB treatment, which might impact the binding and transport of THs and thus posed a potential risk to thyroid functions, further investigations are needed to discover the mechanism in the increased TTR gene transcription.

THs can be glucuronidated or sulfated by different uridine diphosphate glucuronosyltransferase (UGT) or sulfotransferase (SULT) enzymes (Jugan et al., 2010). Yu et al. (2010) found that

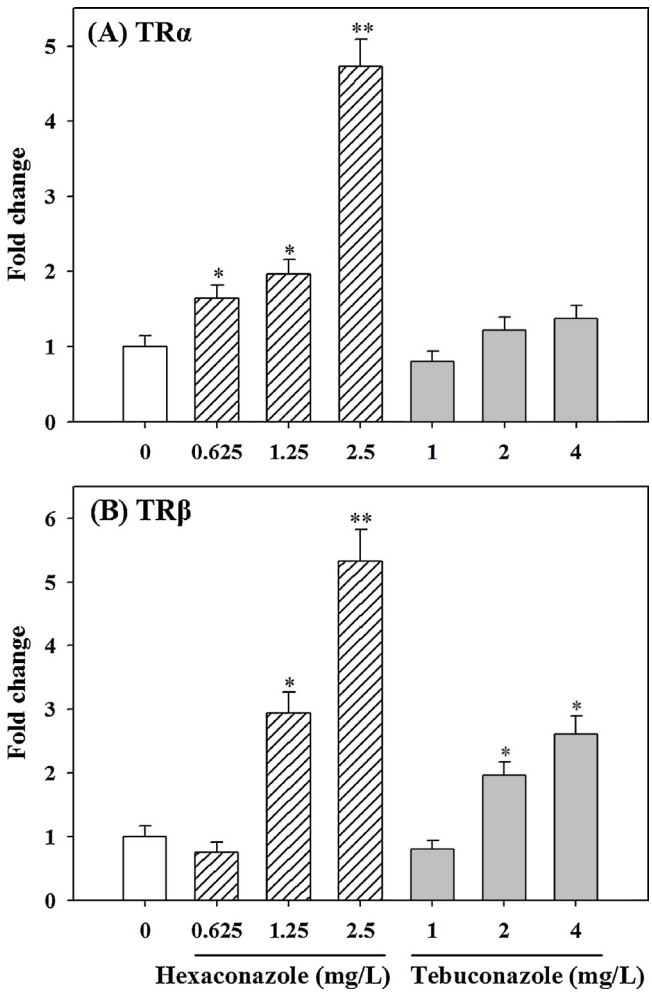

Fig. 5. Relative transcription levels of (A) TRα and (B) TRβ in zebrafish larvae exposed to various concentrations of tebuconazole and hexaconazole until 120 h. The results were evaluated as the relative ratio of the transcription level of each mRNA to that of β-actin. Data expressed as mean ± S.E. ($n = 4$), $^{*}P < 0.05$; $^{**}P < 0.01$, relative to control.

zebrafish larvae exposed to DE-71 for 14 days exhibited reduced levels of circulating T4 and increased UGT1ab gene transcription. The decreased T4 and over expressed UGTs were also reported in rats treated with triazoles fungicides: myclobutanil, propiconazole and triadimefon (Hester et al., 2006; Wolf et al., 2006). Consistent with these previous reports, a significant upregulation of UGT1ab mRNA expression was also observed in our experiment. This might increase T4 elimination and resulted in decreased T4 level, UGTs might play a role in decreasing circulating T4 levels in our study.

The NIS is a transmembrane glycoprotein that transports sodium and iodide across the basolateral plasma into follicular cells of the thyroid gland, which is the first step in the synthesis of thyroid hormone (Dohán and Carrasco, 2003). TG is the protein precursor of thyroid hormone and can be used by the thyroid gland to produce the thyroid hormones T4 and T3. Therefore, changes of the NIS and TG may alter the THs production, and NIS and TG mRNA can be useful markers to monitor the thyroid activity during development (Manchado et al., 2008; Shi et al., 2009). A recent study found that MCLR could reduce the circulating T4 and T3 levels in the zebrafish larvae and up-regulate the NIS and TG gene transcriptions (Yan et al., 2012). The present data showed that both HEX and TEB could up-regulate the transcription of NIS gene while TG mRNA transcription did not change significantly. This indicated the HEX and TEB could induce thyroid endocrine disruption in developing zebrafish larvae, the upregulation of NIS gene transcription could possibly be explained as a compensatory mechanism to the T4 reduction.

There are three types of deiodinases found in fish: type 1 (Dio1), type 2 (Dio2), and type 3 (Dio3), Dio1 and Dio2 are capable of converting T4 into T3 while Dio3 is a purely inactivating enzyme (Orozco and Valverde, 2005). Dio1 plays a minimal role in plasma TH homeostasis, knockdown of Dio1 alone seemed have no influence on zebrafish developmental progression (Walpita et al., 2010), but it had a considerable influence on iodine recovery and TH degradation (Van der Geyten et al., 2005). In contrast, Dio2 exclusively catalyzes outer-ring deiodination and plays a pivotal role in producing active T3 (Yu et al., 2010). Previous studies have indicated that the gene transcriptions of deiodinases were sensitive molecular biomarkers for thyroid disruption in fish that had been exposed to environmental contaminants (Li et al., 2009; Picard-Aitken et al., 2007; Shi et al., 2009). In this study, both HEX and TEB treatment could induce the Dio2 transcription, this may be associated with a reduction in the levels of circulating T4. For Dio1, transcription of the Dio1 gene showed no change in all the TEB exposure groups, but were significantly up-regulated in 1.25 and 2.5 mg/L HEX exposure groups. These are in line with previous reports showing that hypothyroidism increases Dio1 and Dio2 activities and expression of their mRNA while hyperthyroidism suppresses them in fish (Orozco and Valverde, 2005). Although Dio1 does not seem to be very important in euthyroid conditions, it may be crucial under depleted thyroid status as is the case when T3 production by Dio2 is inhibited (Walpita et al., 2010). Induced Dio1 in zebrafish larvae upon HEX treatment in our study could possibly be explained as increased TH degradation which further demonstrated HEX impaired the THs signaling thereby to influence the larvae development and growth.

The biological activity of TH is predominantly mediated by two thyroid hormone receptors (TRs), TRα and TRβ (Yang et al., 2007b). In this experiment, we found TRβ mRNA transcription was significantly increased while TRα did not significantly change in zebrafish larvae exposed to TEB. On the other hand, both TRα and TRβ mRNA transcription were up-regulated by HEX. The different effects on TRα and TRβ gene transcriptional patterns were also observed in the zebrafish larvae when exposed to PFOS: TRα and TRβ were up- and down-regulated, respectively (Shi et al., 2009). Hence, our results together with previous studies further demonstrated that TRα and TRβ might serve different functions. Chen et al. (2012) found that the upregulation of TRs might due to the increased T3 levels and would influence the transcription of other genes involved in thyroid function. We could speculate that HEX and TEB increased T3 levels, so that the TRs were significantly up-regulated, this may be feedback systems attempt to respond to the disturbance of HPT axis homeostasis. Abnormal TRα and TRβ might lead to the failure of THs to bind and activate the appropriate post-receptor response cascades (Liu et al., 2011). Thus, we infer that altered transcription levels of TRs in this study might play important roles in the induction of thyroid disruption by HEX and TEB.

Nowadays, more than 1400 commercial triazole products are registered in China (http://www.chinapesticide.gov.cn/service/aspx/B3.aspx). This might do great harm to environment and health due to their widely application. It was reported that the sex ratio of zebrafish had a significant increase in terms of males when treated with 0.5 mg/L of triadimefon (Liu, 2011). Furthermore, the combined effects induced by different triazole fungicides that share several mechanisms might be additive. Given the moderate mobility, persistence (persistent in soils for TEB (EFSA, 2008), and field soil degradation DT_{50} of 225 d for HEX (Tsukatani et al., 2008)), environmental exposure level (up to 175–200 μg/L for TEB (Elsaesser and Schulz, 2008), which was very close to the effective concentrations in the present study) of the two fungicides, it was considered

that TEB and HEX might have a high risk to aquatic species. It was suggested that the use of these two fungicides should be controlled in China, especially the south areas and the high groundwater level areas.

In summary, we found that exposure of HEX and TEB to zebrafish larvae increased the T3 levels while decreased the T4 concentrations. Furthermore, a series of genes transcription involved in the HPT axis were changed. The results demonstrated HEX and TEB exposure might result in thyroid endocrine toxicity, thus affected the normal development of zebrafish larvae. To obtain a more complete toxicological profile of HEX and TEB, further studies are needed to examine the effects on adult fish and a long-term exposure. Taking into account the extensive use of triazole fungicides, the thyroid endocrine toxicity of triazole fungicides should be paid for more attention.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (No. 31101458), and the Natural Science Foundation of Zhejiang Province (No. Z12C140006).

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