Toxicological signature for thyroid endocrine disruption of dichlorooctylisothiazolinone in zebrafish larvae

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Research Article

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Abstract

Dichlorooctylisothiazolinone (DCOIT), which is one of the isothiazolinone preservatives, is applied to water-based adhesives in food packaging. This study investigated the effects of DCOIT on the embryonic growth and thyroid endocrine system using zebrafish. Organism-level (hatchability, survival, and growth), hormone-level (triiodothyronine (T3) and thyroxine (T4)), gene-level (genes associated with the hypothalamus-pituitary-thyroid axis), and microRNA-level (microRNAs related to thyroid endocrine disruption) endpoints were measured. Significant rise in embryonic coagulation, delayed hatching, and decreased larval length were observed in fish exposed to greater than 0.3 μg/L DCOIT. Lower contents of T3 and T4 were observed after exposure to DCOIT, which was accompanied by the upregulation of genes associated with the thyrotropin releasing hormone and thyroid stimulating hormone and the downregulation of genes associated with the thyroid hormone receptors and deiodination. Strong influence of DCOIT on dre-miR-193b and -499 may be a critical mechanism to inhibit transcription of trαa and trβ, which in turn may affect thyroid hormones and development of the organism. Our findings suggest that hypothyroidism induced by the exposure to DCOIT is potentially associated with genetic and microRNA-level changes, which eventually affects development.

1. Introduction

Dichlorooctylisothiazolinone (DCOIT), which is one of the isothiazolinones, is characterized by a heterocyclic compound with nitrogen and sulfur aromatic ring (Herman et al., 2019). This chemical has been often used as a substitute for organotin compounds, which are banned due to their potential for endocrine disruption (Hiromori et al., 2014). DCOIT is used in water-based adhesives to maintain the quality of packaged food and prevent contamination (Zhong et al., 2019). In addition, it is used as an active substance in the commercial antifouling biocide SeaNine-211™ or Kathlon™ 910SB to suppress undesirable biofouling phenomenon (Chen and Lam, 2017; dos Santos et al., 2020). Several studies have attempted to detect DCOIT in the aquatic environment (Chen et al., 2012; Liu et al., 2017; Wieck et al., 2018), marine sediment (García et al., 2020), polyvinyl alcohol cooling towels (Kawakami et al., 2014), and commercial water-based adhesives (Zhong et al., 2019). DCOIT was detected up to 54.3 ng/L in the influent and 4.20 ng/L in the effluent obtained from ten wastewater treatment plants (Liu et al., 2017). In marine sediments, SeaNine-211 was found at a concentration of 2.1 ng/g (García et al., 2020).

With the frequent use of DCOIT and its detection in aquatic environments, toxicity studies have been conducted to investigate the effects of this substance on humans and ecosystems. One epidemiological study reported that exposure to DCOIT caused allergic contact dermatitis (Umekoji et al., 2016). DCOIT alters cellular metabolism and increases generation of reactive oxygen species at the mitochondrial and cellular levels (Kim et al., 2021). Among the four isothiazolinones, namely methylisothiazolinone (MIT), chloromethylisothiazolinone (CMIT), octylisothiazolinone (OIT), and DCOIT, DCOIT is the most toxic compound in marine bacterium *Vibrio fischeri* and green algae *Scenedesmus vacuolatus* (Arning et al., 2009). Moreover, it inhibits egg production in the copepod *Acartia tonsa* (Wendt et al., 2016). Recent studies have reported that a mixture of CMIT/MIT (Chatterjee et al., 2021), MIT and OIT (Lee et al., 2022)

interfere with the development of zebrafish at an early stage of growth and disrupt the thyroid endocrine function. However, there is no information on developmental retardation and effects on thyroid endocrine system due to exposure to DCOIT.

Thyrotropin releasing hormone (TRH), thyroid stimulating hormone (TSH), and thyroid hormones play a crucial role in maintaining homeostasis in the hypothalamus, pituitary, and thyroid gland, respectively. They control the secretion of thyroid hormones through feedback circuits within the hypothalamus-pituitary-thyroid (HPT) axis (Deal and Volkoff, 2020). Hypothyroidism, in which the thyroid gland produces less triiodothyronine (T3) and thyroxine (T4), leads to delayed growth, lack of activity, and poor performance (Marino et al., 2008). Current evidence suggests that hypothyroidism induced by endocrine disrupting chemicals affects fish development through regulation of hormones, genes, and microRNAs (miRNAs) in the HPT axis (Lee et al., 2022; Liu et al., 2013; Wang et al., 2013). For example, significant decrease in T3 and T4 and shortened body length were observed after exposure to OIT, and this was accompanied by decrease in *tra* and *trβ* genes and increase in dre-miR-193b and − 499 (Lee et al., 2022). Higher lipophilicity (log Pow 4.79; Arning et al., 2009) of DCOIT may lead to higher bioavailability and toxicity, but studies on its effects on thyroid hormone action and adverse health outcomes are limited.

Small non-coding RNA, miRNA, has been utilized as a biomarker of several endocrine disrupting chemicals to assess underlying toxic mechanism (Lee et al., 2018; Lee et al., 2022; Tran and Kim, 2020). MiRNA can repress the translation of target mRNAs, and a decrease in the amount of specific mRNAs is an important consequence of this molecular event (Gulyaeva and Kushlinskiy, 2016). Two miRNAs responsible for genes associated with phenotypic tail defects, namely dre-miR-137 and − 141, were overexpressed in zebrafish embryo exposed to triphenyl phosphate (Tran and Kim, 2020). Four miRNAs involved in aromatization and reproductive effects were identified in male zebrafish exposed to bisphenol S (Lee et al., 2018). Identifying the molecular mechanisms regulating miRNA expression can explain the changes in transcription of protein coding genes.

The purpose of this study was to assess the toxicity of DCOIT on the embryonic stages of zebrafish and its effect on the thyroid endocrine system. Zebrafish embryo/larvae is an ideal model organism to understand the actions of hormonal-, molecular-, and miRNA- level mechanisms underlying developmental processes (Reinwald et al., 2021). Basic developmental endpoints were observed to investigate the malformation, growth delay, and acute lethality. Furthermore, thyroid disruption at a hormonal-level (T3 and T4), genetic-level (genes associated with HPT axis), and miRNA-level (miRNAs potentially regulate the transcription of *tra*, *trβ*, and *deio2* genes) was studied.

2. Materials And Methods

2.1. Chemicals and DCOIT exposure in fish

DCOIT (CAS No. 64359-81-5, purity > 95%) was purchased from Merck (Merck KGaA, Dramstadt, Germany). Dimethyl sulfoxide (DMSO) was used as a solvent (0.01% v/v) in the exposure medium.

Zebrafish pairs (AB strain) were cultured in-house in a flow-through system (ZebTEC, Buguggiate, Italy) at 26 ± 1°C under 14 h light:10 h dark photoperiod. Among the collected fertilized eggs, only healthy embryos were used for the experiments. Following the fish embryo acute toxicity test guideline 236 (Organization Economic Cooperation and Development, 2013) with minor modification, zebrafish embryos were randomly placed in 24-well plates and exposed to various concentrations (0, 0.003, 0.03, 0.3, 3, and 30 µg/L) of DCOIT. Test concentrations were chosen considering the highest concentration of DCOIT found in wastewater treatment plant effluents (Liu et al., 2017) and their toxic effects in our preliminary range-finding tests. Three biological replicates were used for each treatment group. Organism-level endpoints, namely embryo coagulation, malformation, hatchability, hatching time, and larval survival, were recorded daily. At the end of the 96 h exposure, body length (10 each larvae per replicates, n = 30) and wet weight (10 larvae pooling per replicates, n = 3) were measured. Moreover, larvae for transcriptional analysis were collected in groups of three replicates by pooling 10 larvae each.

Additional sets of experiments with same design were performed to evaluate thyroid hormones and miRNA expression. After 96 h of exposure, 150 larvae and 20 larvae were collected in triplicates from each treatment group to measure thyroid hormones and miRNA expression, respectively. All samples were stored in a -80°C freezer until further analysis.

2.2. Thyroid hormone analysis

After homogenizing the collected larvae in 250 µL of phosphate buffer saline, samples were centrifuged. The supernatant was collected and analyzed for T3 (Cat No. OKCA00348) and T4 (Cat No. OKCA00349) hormones with commercial enzyme-linked immunosorbent assay kits (Aviva System Biology, San Diego, USA). Detailed experiments were carried out following the guidance provided in the kits. The ratio of T3/T4, which is an indicator of hormonal balance, was calculated and normalized to the solvent control group.

2.3. Determination of gene transcription using real-time PCR

Genes related to the corticotropin releasing hormone (CRH) (*crh*), TRH (*trh* and *trhr1*), TSH (*tshβ* and *tshr*), thyroid hormone production (*tpo*, *ttr*, *tg*, and *nis*), thyroid hormone receptor (*traa* and *trβ*), and deiodination (*deio1, deio2*, *deio3a*, and *deio3b*) were analyzed using real-time polymerase chain reaction (PCR). The primer sequences and complete names of the analyzed genes are provided in Table S1. Detailed experiments were followed by the method described in Lee et al. (2022). Briefly, messenger RNA extraction and complementary DNA synthesis were performed using the RNeasy mini kit (QIAGEN) and the iScript™ cDNA Synthesis kit (BIORAD), respectively. ABI 7500 fast real-time PCR system (Applied Biosystem, Foster City, USA) was run with a 20 µL reaction mix composed of 10 µL of Power SYBR Green® PCR Master mix (Applied Biosystems), 1.8 µL of sense and antisense primer each, and 6.4 µL of diluted cDNA. The PCR reaction initiated with the first cycle at 50°C for 1 min, second cycle at 95°C for 10 min, and third cycles (×40) of 95°C for 15 s and 60°C for 1 min. Using the relative quantitation method (Livak and Schmittgen, 2001), the threshold cycle (Ct) of target gene was normalized to validated

housekeeping genes (*β-actin* and *rpl8*), then these values in target samples were compared to the reference samples.

2.4. Determination of miRNA expression using real-time PCR

Measurement of miRNA expression was conducted using a previously described method by Lee et al. (2018). Briefly, dre-miR-1, -193b, and − 499 were selected using TargetScanFish. The aforementioned miRNAs were chosen because they potentially regulate the transcription of *traa* and *trβ* genes. miRNA extraction, polyadenylation, and synthesis of complementary DNA were performed using the miRNeasy mini kit (QIAGEN) and the miRNA 1st -strand cDNA synthesis kit (Agilent Technologies). The method of running real-time PCR was the same as in transcriptional analysis except for the primer: miRNA-specific forward and universal RT primer were used. The Ct of target miRNA was normalized to small nuclear RNA U11, then these values in target samples were compared to the reference samples.

2.5. Statistical analyses

Statistical significance of treatment group compared to solvent controls was analyzed using Dunnett's one-way analysis of variance from the SPSS program (IBM Corp., New York, NY, USA). Linear regression analysis was conducted to assess dose-response relationship in organism-, hormonal-, genetic-, and miRNA-level endpoints. Spearman correlation analysis was conducted to investigate important molecular endpoints (hormone, gene, and miRNA) on larval survival and length using SAS program (version 9.2, SAS Institute, Cary, NA, USA). When the *p*-value was less than 0.05 in the statistical analysis, we considered it as statistically significant.

3. Results

3.1. Organism-level endpoints: developmental toxicity

Embryo coagulation was significantly elevated in fish exposed to 0.3, 3, and 30 μg/L DCOIT (Fig. 1). Hatching time was remarkably delayed and hatchability was significantly reduced in fish exposed to greater than 0.3 μg/L DCOIT (Fig. 1). Larval survival and length were much lower in fish exposed to 30 μg/L DCOIT (Fig. 1). DCOIT did not cause noteworthy effects in larval wet weight (Fig. 1).

The relationship between organism-level endpoints (larval survival and length) and other endpoints (production of T3 and T4, transcription of fifteen genes, and expression of three miRNAs) is shown in Table S3 (Supplementary data). Larval survival was positively related to the production of T3 and T4 as well as transcriptional changes in the genes associated with thyroid hormone production (*tpo*, *ttr*, *tg*, and *nis*), thyroid hormone receptors (*traa* and *trβ*), and deiodination (*deio1*, *deio2*, *deio3a*, and *deio3b*). On the other hand, it was negatively related to the transcriptional changes in the genes associated with CRH (*crh*), TRH (*trh* and *trhr1*), and TSH (*tshβ* and *tshr*), and two miRNAs (dre-miR-193b and − 499). Additionally, larval length was positively related to the production of T3 and T4 as well as transcriptional

changes of five genes (*traa*, *trβ*, *tg*, *nis*, and *deio2*), while it was negatively related to transcriptional changes in the five genes (*crh*, *trh*, *trhr1*, *tshβ*, and *tshr*) and one miRNA (dre-miR-499).

3.2. Hormonal-level endpoints: changes in T3 and T4

In larvae exposed to ≥ 3 μg/L DCOIT, the contents of T3 and T4 were significantly decreased (Fig. 2). The normalized ratio of T3/T4, which is an indicator of thyroid hormone balance, was remarkably lower in fish that were exposed to 30 μg/L DCOIT (Fig. 2).

3.3. Genetic-level endpoints: regulation of genes associated with the HPT axis

The transcriptional regulation of genes associated with the HPT axis is shown in Fig. 3. Following DCOIT treatment, transcription of *deio1* gene was significantly reduced at 30 μg/L. Furthermore, up-regulation of genes associated with CRH (*crh*), TRH (*trh*), and TSH (*tshr*), as well as down-regulation of genes associated with binding to thyroid hormone receptors (*traa* and *trβ*) and thyroid hormone production (*tpo* and *tg*) were noted in zebrafish exposed to greater than 3 μg/L DCOIT. In larvae exposed to ≥ 0.3 μg/L DCOIT, transcription of *tshβ* gene was significantly high, whereas that of *deio2* gene was remarkably low. Transcription of *trhr1* gene increased at 0.03, 0.3, and 30 μg/L DCOIT, compared to the solvent control group.

3.4. MiRNA-level endpoints: changes in miRNAs

Although the expression was slightly increased in dre-miR-193b, which regulates the transcription of *traa*, as well as dre-miR-499, which targets *traa* and *trβ*, statistical significance was not observed when compared to solvent control group (Fig. 4). No remarkable change was induced in dre-miR-1 (target on *trβ*) (Fig. 4).

4. Discussion

DCOIT has gained great attention because of its widespread use as an alternative to organotin compounds, based on its reduced half-life in seawater (Jacobson and Willingham, 2000). However, recent studies have reported high risks in marine environment based on the predicted no effect concentration of bivalves (dos Santos et al., 2020) and potential effects on reproduction (Wendt et al., 2016). The results of this study demonstrated that DCOIT reduced whole-body T3 and T4, modified the expression of mRNA and miRNA involved in the HPT axis, and affected the early development of zebrafish.

Organism-level endpoints including hatchability, survival, and somatic length are holistic indicators of thyroid endocrine disruption (Spaan et al., 2019). Zebrafish exposed to DCOIT above 0.3 μg/L experienced delayed hatching, reduced hatchability, and shortened body length. These results were in good agreement with those reported for other isothiazolinones, namely CMIT, MIT, and OIT, which adversely affect the development stages of zebrafish (Chatterjee et al., 2021; Lee et al., 2022). The no

observed effect concentration on acute mortality and development of DCOIT (0.03 μg/L) was 1,000 times lower than that of MIT (Lee et al., 2022) and similar to that of OIT (Lee et al., 2022).

The balance of thyroid hormones are critical for the embryonic development as well as larval growth of fish (Shkil et al., 2019). In this study, the contents of T3 and T4 were significantly reduced after exposure to 3 μg/L DCOIT, demonstrating that DCOIT could induce hypothyroidism in zebrafish embryo/larvae. Lee et al. (2022) concluded that the contents of T3 and T4 were remarkably decreased in zebrafish larvae after exposure to 30 μg/L MIT and 3 μg/L OIT, which was similar to the results of this study. Similarities in the thyroid endocrine disruption of isothiazolinone compounds could be explained by their similar chemical structure. The heterocyclic group containing sulfur and nitrogen atoms could be the key structural component responsible for the thyroid hormone disruption of isothiazolinone analogues. Through correlation analysis, it was confirmed that the decrease in T3 and T4 concentration was significantly related to the decline in the larval length.

TRH and CRH secreted from the hypothalamus and TSH secreted from the pituitary gland are the major regulators of the HPT axis in fish (Jia et al., 2016). A previous study showed that MIT and OIT upregulated the expression of mRNAs associated with CRH, TRH, and TSH (Lee et al., 2022). Current data demonstrated that genes associated with CRH (*crh*), TRH (*trh* and *trhr1*), and TSH (*tshβ* and *tshr*) were remarkably upregulated in zebrafish larvae exposed to DCOIT. These results suggest two possibilities: direct effect of DCOIT and indirect effect by a feedback mechanism to increase the production of both thyroid hormones. The results of our correlation analysis also support that the expression of mRNAs related to CRH, TRH, and TSH plays an essential role in controlling two thyroid hormones.

Thyroid hormone receptors (alpha and beta), get activated after binding to thyroid hormones (Essner et al., 1997). Thyroid peroxidase (tpo) aids in a chemical reaction that binds iodine to a protein called thyroglobulin (tg), which is an essential step in the production of thyroid hormones (Citterio et al., 2019). In the present study, significant reductions were observed in the transcription of *traa*, *trβ*, *tpo*, and *tg* genes, which revealed that DCOIT has the potential to inhibit the production of T3 and T4. Moreover, Lee et al. (2022) reported that the downregulation of *traa*, *trβ*, *tpo*, and *tg* genes was due to decreased secretion of T3 and T4. We examined the expression of dre-miR-1, -193b, and − 499 targeting the *traa* and *trβ* genes. It has been also reported that these miRNAs play a central part in the control of the thyroid hormone and development (Huang et al., 2017; Lee et al., 2022). An increase in dre-miR-499 has a significant correlation with the decrease in larvae length, reduction in T3 and T4 production, and decrease in transcription of *trβ* gene. These results support the miRNA-level mechanism of DCOIT in inducing hypothyroidism.

Transcription of three types of deiodinases genes, namely *deio1*, *deio2*, *deio3a*, and *deio3b* were analyzed in this study. Among them, type II deiodinase played the most important role in catalyzing the conversion of T4 to active T3 (Houbrechts et al., 2019). A decrease in T3 levels was accompanied by a decrease in the transcription of *deio2* gene in fish larvae exposed to ≥ 0.3 μg/L DCOIT. These results revealed that DCOIT had the potential to affect the production of local T3 and genes related to deiodinase activity.

The results of this study have important implications in the environmental risk assessment of DCOIT. Toxicity data were obtained from the ECOTOX Knowledgebase (Table S4). Moreover, the predicted no effect concentration (PNEC; 0.6 ng/L) was calculated from the lowest toxicity value (30 ng/L derived from this study) and an uncertainty factor (50) based on European Communities (2011). Using the value of PNEC (0.6 ng/L) and the maximum concentrations detected in wastewater treatment plant effluents (4.2 ng/L; Liu et al., 2017), the value of hazard quotient (7) was calculated. These results indicate that DCOIT could pose an environmental risk to freshwater organisms.

5. Conclusion

In conclusion, hypothyroidism induced by DCOIT exposure is potentially associated with genes related to the feedback regulatory circuits in the HPT axis and miRNA changes as well as changes in mRNA which eventually affect development. Furthermore, DCOIT-induced miRNA changes may affect the length of zebrafish larvae, thus providing clues to the miRNA-level mechanism of DCOIT toxicity. If current trends continue, the production and environmental release of DCOIT could increase. Considering that the logKow of DCOIT is larger than that of other isothiazolinones, more attention should be paid to its toxicological effects on the ecosystem.

Declarations

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Declaration of interest

There is no conflict of interest associated with this work.

Author contributions statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis, and writing the first draft of the manuscript were performed by Sujin Lee. Editing on previous version of the manuscript was conducted by Kyunghee Ji. All authors read and approved the final manuscript.

Ethics approval statement

This study was approved by the Institutional Animal Care and Use Committee of Yongin University, Korea (YUIACUC-2021-8).

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Figures

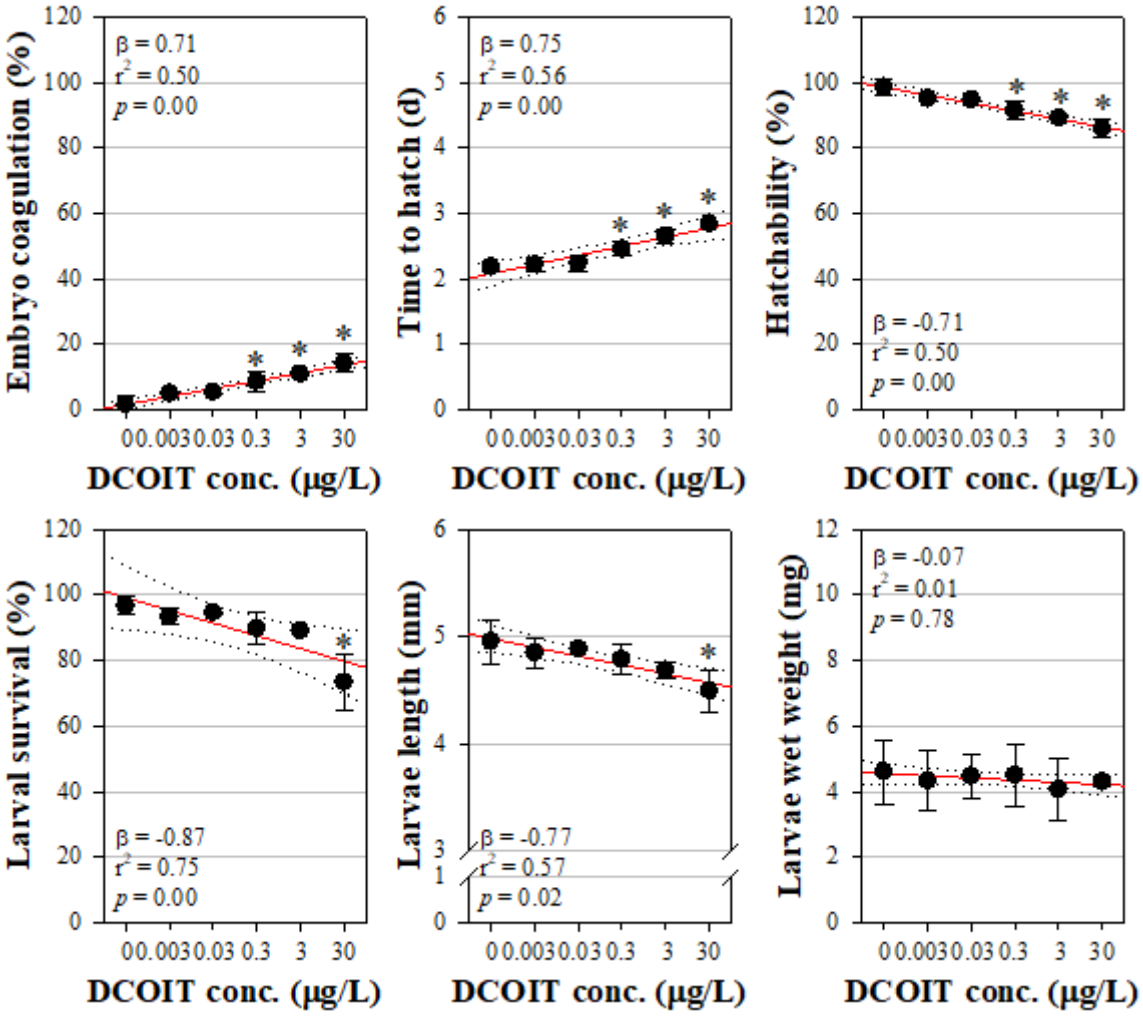

Figure 1

Organism-level effects of zebrafish embryo/larvae after exposure to dichlorooctylisothiazolinone (DCOIT). Embryo coagulation (%), time to hatch (d), hatchability (%), larval survival (%), larval length (mm), and larval weight (mg) were shown. The values represent mean ± standard deviation of three replicates. Asterisk indicates significant difference from solvent control (p<0.05).

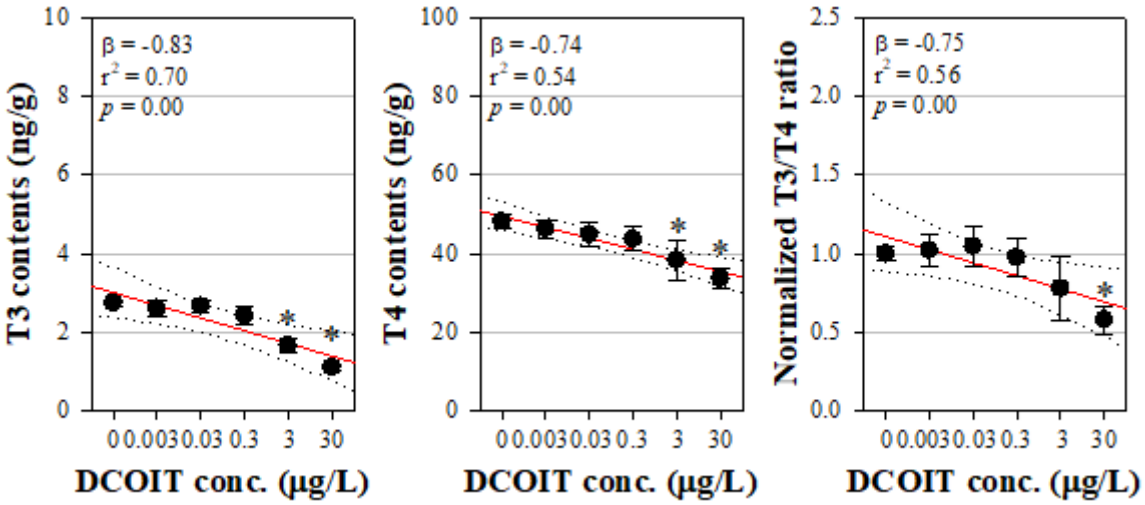

Figure 2

Hormone-level effects of zebrafish larvae after exposure to dichlorooctylisothiazolinone (DCOIT). Changes in triiodothyronine (T3) and thyroxine (T4) hormone were shown. The values represent mean ± standard deviation of three replicates. Asterisk indicates significant difference from solvent control (p<0.05).

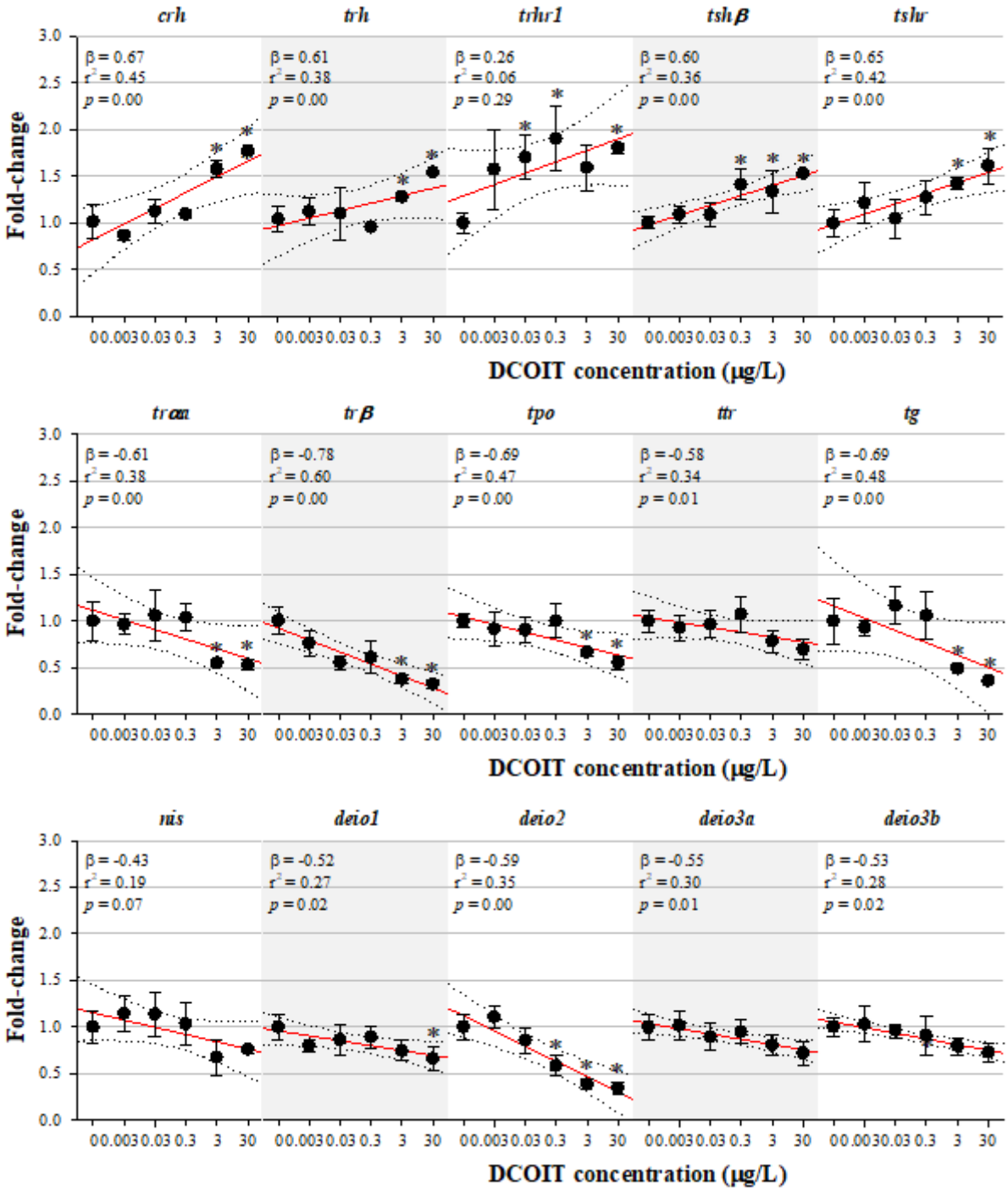

Figure 3

Transcriptional profiles of the genes related to the hypothalamus-pituitary-thyroid axis in fish exposed to dichlorooctylisothiazolinone (DCOIT) for 96 h. The values represent mean ± standard deviation of three replicates. Asterisk indicates significant difference from solvent control (p<0.05).

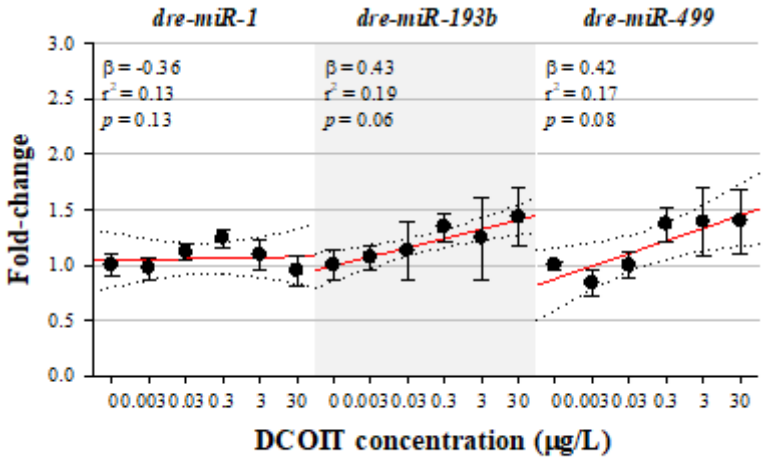

Figure 4

Expression of miRNAs in fish exposed to dichlorooctylisothiazolinone (DCOIT) for 96 h. The values represent mean ± standard deviation of three replicates. Asterisk indicates significant difference from solvent control (p<0.05).

Supplementary Files

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