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Effect of thyroid hormone-disrupting chemicals on swim bladder inflation and thyroid hormone-related gene expression in Japanese medaka and zebrafish

Short title: Thyroid hormone-disrupting chemicals suppress swim bladder inflation

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KEYWORDS: PFBA • T3 • TDCPP • *tsh β* • *tra* • *tr β*

Abstract (226 words)

We compared the influence of thyroid hormone–disrupting chemicals (heptafluorobutanoic acid, PFBA and tris(1,3-dichloro-2-propyl) phosphate, TDCPP), and thyroid hormone (3,3',5-triiodo-L-thyronine, T3) on swim bladder inflation and thyroid hormone–related gene expression in Japanese medaka and zebrafish. The swim bladder of most larvae had inflated at 4 hours post hatching (hph) in Japanese medaka and at 48 hph in zebrafish in controls. In both fish species, the swim bladder inflation was inhibited in larvae exposed to PFBA (lowest observed effect concentration (LOEC) in medaka: 40 mg/L; in zebrafish: 80 mg/L), TDCPP (LOEC in medaka: 1 mg/L; in zebrafish: 0.5 mg/L), and T3 (no inhibition in Japanese medaka; LOEC in zebrafish: 7.5 µg/L). We also examined the influence of PFBA, TDCPP, and T3 on the expression of thyroid stimulating hormone subunit beta (*tshβ*) or thyroid hormone receptor alpha (*trα*) and beta (*trβ*). No changes were observed in the expression of genes after PFBA and TDCPP exposure; however, T3 exposure upregulated *trα* and *trβ* expression in both fish species. When the results were compared between Japanese medaka and zebrafish, swim bladder inflation in both species was found to be inhibited by exposure to thyroid hormone–disrupting chemicals. Our results show that inhibition of the swim bladder inflation at 4 hph in Japanese medaka and 48 hph in zebrafish is a potential indicator of thyroid hormone–disturbing activity of chemicals.

Short abstract (55 words)

Thyroid hormone–disrupting chemicals, PFBA and TDCPP, inhibited swim bladder inflation in Japanese medaka and zebrafish larvae at 4 and 48 hph, respectively. Inhibition of swim bladder inflation can be used as a potential indicator for detecting thyroid hormone–disturbing activity of chemicals for 4 and 48 h exposure in Japanese medaka and zebrafish, respectively.

hph, hours post hatching; LOEC, lowest observed effect concentration; PFBA, heptafluorobutanoic acid; T3, 3,3',5-triiodo-L-thyronine; TDCPP, tris(1,3-dichloro-2-propyl) phosphate; *trα*, thyroid hormone receptor alpha; *trβ*, thyroid hormone receptor beta; *tshβ*, thyroid stimulating hormone subunit beta

1. Introduction

In vertebrates, thyroid hormone is secreted from the thyroid gland, and thyroid hormone receptors are expressed in most cells throughout the body (Ortiga-Carvalho et al., 2014). Thyroid hormone acts to increase cell metabolism in the body (Mullur et al., 2014). Thyroid hormone is regulated by thyroid stimulating hormone (TSH), which is secreted by the pituitary gland in the brain (Szkudlinski et al., 2002), and it functions via thyroid hormone receptors alpha ($Tr\alpha$) and beta ($Tr\beta$) (Ortiga-Carvalho et al., 2014). It is well-known that thyroid hormone promotes metamorphosis from tadpoles to frogs in amphibians (Brown and Cai, 2007; Thambirajah et al., 2019), and causes seasonal molting in birds (reviewed by Zimova et al., 2018).

In fish, thyroid hormone also plays important roles in maintaining normal physiological functions such as development, metabolism, and growth (Blanton and Specker, 2007; Vergauwen et al., 2018; Walpita et al., 2010), and adaption to seawater when descending to the sea in salmonids (Deal and Volkoff, 2020), therefore, chemicals that disrupt thyroid hormone activity could have marked negative impacts on fish populations. Two laboratory fish species often used in ecotoxicity tests to detect endocrine-disrupting chemicals are Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) (Dang and Kienzler, 2019). In zebrafish, knockdown of type 3 iodothyronine deiodinase, the primary inactivating deiodinase that terminates thyroid hormone action, has been shown to lead to abnormal swim bladder development, indicating that thyroid hormone is important for swim bladder development and inflation in this species (Heijlen et al., 2014). Spaan et al. (2019) have also reported that the thyroid hormone system controls the development of the swim bladder (Dumbarton et al., 2010; Lindsey et al., 2010; Robertson et al., 2007). These findings have led to swim bladder inflation being proposed as an indicator for detecting chemicals with thyroid hormone-disturbing activity (Dang et al., 2021). ERGO (EndoCRine in Guideline Optimisation) group in EURION (European Cluster to Improve Identification of Endocrine Disruptors) have been using this approach in addition to the eye and brain development in zebrafish to examine the effects of thyroid hormone disruption (Baumann et al., 2019; Holbech et al., 2020; Knapen et al., 2020). Godfrey et al. (2017, 2019) have reported that exposure of Japanese medaka and zebrafish to 3,3',5-triiodo-L-thyronine (T3), methimazole, tris(1,3-dichloro-2-propyl) phosphate (TDCPP), perfluorooctanoic acid, or heptafluorobutanoic acid (PFBA) induces absence of swim bladder inflation, though it should be noted that Godfrey et al. (2017) observed the presence or absence of swim bladder inflation in zebrafish at 6 days post fertilization and that they used only a single concentration of each test chemical. It is known that in zebrafish the swim bladder

becomes inflated at approximately 2 days after hatching (5 days after fertilization) (Horie et al., 2017a), meaning the effects of chemicals on swim bladder inflation can be investigated within 120 hours after fertilization. In Japanese medaka, however, it is currently unclear when the swim bladder becomes inflated.

Recently, Dang et al. (2021) reviewed the influence of thyroid hormone–disrupting chemicals on fish, including in Japanese medaka and zebrafish. They noted that studies have shown changes in the expression of thyroid-related genes, such as thyroid stimulating hormone subunit beta (*tshβ*), thyroid hormone receptor alpha (*trα*), and beta (*trβ*), after exposure to the following chemicals: 2,2',4,4'-tetrabromodiphenyl ether in zebrafish (Chan and Chan, 2012), TDCPP in Japanese medaka (Godfrey et al., 2019) and in zebrafish (Wang et al., 2013), and 2-ethylhexyl-4-methoxycinnamate (EHMC) in Japanese medaka (Lee et al., 2019) and in zebrafish (Chu et al., 2021). Although exposure to TDCPP and EHMC causes changes in the expression of thyroid-related genes in both Japanese medaka and zebrafish, the reports then specified that, after exposure to TDCPP, *trα* and *tshβ* are upregulated in Japanese medaka (Godfrey et al., 2019) and zebrafish (Wang et al., 2013), and after exposure to EHMC, *trα* and *trβ* are upregulated in Japanese medaka (Lee et al., 2019) but downregulated in zebrafish (Chu et al., 2021). These results indicate that our understanding of how thyroid hormone–disrupting chemicals change the expression of genes related to thyroid hormone in Japanese medaka and zebrafish remains incomplete. One of the reasons for this incomplete understanding is that various timings have been used in previous studies examining the expression of thyroid-related genes in both Japanese medaka (Godfrey et al., 2019: TDCPP, 10 days post fertilization; Lee et al., 2019: EHMC, adult fish) and in zebrafish (Wang et al., 2013: TDCPP, 6 days post fertilization; Chu et al., 2021: EHMC, adult fish). Thus, coordinated studies examining expression of thyroid-related genes at the time of swim bladder inflation. Then effects of thyroid hormone–disrupting chemicals on thyroid-related gene expression can be compared between Japanese medaka and zebrafish.

In the present study, we used the thyroid hormone–disrupting chemical substances, PFBA and TDCPP, which are reported to inhibit swim bladder inflation in both Japanese medaka and zebrafish, and T3, which is an active thyroid hormone, to investigate the following questions. First, at what stage does swim bladder inflation occur in Japanese medaka? Second, does exposure to PFBA, TDCPP, or T3 inhibit swim bladder inflation to the same degree in both Japanese medaka and zebrafish? Third, do the changes in expression of the thyroid-related genes *tshβ*, *trα*, and *trβ* differ between Japanese medaka and zebrafish?

2. Materials and methods

2.1. Test fish and test chemicals

In the present study, NIES-R strains (National Institute for Environmental Studies) of Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were used. Test fish were inbred at Akita Prefectural University (Akita, Japan) and Kobe University (Hyogo, Japan) from 2017 (water temperature, 25 ± 2 °C; 16-h light and 8-h dark). The fish used in the present study were handled according to the guidelines of Akita Prefectural University and Kobe University.

3,3',5-Triiodo-L-thyronine (T3, CAS No. 6893-02-3, purity >98.0%), tris(1,3-dichloro-2-propyl) phosphate (TDCPP, CAS No. 13674-87-8, purity >93.0%), and heptafluorobutanoic acid (PFBA, CAS No. 375-22-4, purity >98.0%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2. Timing of swim bladder inflation in Japanese medaka and zebrafish

Japanese medaka and zebrafish eggs were collected immediately after natural mating. Eggs were checked for fertilization under a stereomicroscope, and unfertilized or damaged eggs were removed. Fertilized eggs were transferred to 100 mL glass vessels at a density of 20 embryos per vessel and then cultured until hatching. Japanese medaka and zebrafish were checked every 30 min to observe if hatching had occurred, and the hatched individuals were transferred to a 500-mL beaker containing 400 mL of tap water. To clarify when the swim bladder inflates, we used a stereomicroscope (SZX16, Olympus, Tokyo, Japan) to observe the presence or absence of swim bladder inflation in Japanese medaka at just after hatching and at 0.5, 1, 2, 4, and 24 hours post hatching (hph) and in zebrafish at just after hatching and at 0.5, 24, 48, and 72 hph. In the present study, we defined the presence or absence of swim bladder inflation as follow; presence was presented in Figure 2D or Figure 3D and absence was as presented in Figure 2C or Figure 3C.

2.3. Effect of chemicals on swim bladder inflation in Japanese medaka and zebrafish

For all test substances, concentrations less than the water solubility were used. Water solubility of T3 is 3.96 mg/L (<https://www.nies.go.jp/kisplus/images/bunseki/pdfs/kurohon/2009/adoc2009-3-660.pdf>) in Japanese, TDCPP is 7 mg/L (<https://www.tcichemicals.com/JP/en/p/P0269>), and PFBA is completely miscible (<https://www.tcichemicals.com/JP/en/p/Q0054>), although stability of these chemicals during test period was unclear without chemical

[analysis in this study](#). The exposure concentrations were set to a level that induces non-inflation of the swim bladder or that is soluble in water. Nominal concentrations of each chemical were as follows: for T3, control, 0.12, 0.25, 0.5, and 1 mg/L in Japanese medaka and control, 1.7, 3.7, 7.5, 15, and 30 µg/L in zebrafish; for TDCPP, control, 0.12, 0.25, 0.5, 1, and 2 mg/L in both species; for PFBA, control, 5, 10, 20, 40, and 80 mg/L in both species.

Eggs both from Japanese medaka and zebrafish were checked under the stereomicroscope, and those that were unfertilized, damaged, or developmentally abnormal were removed. After this exclusion process, the remaining fertilized eggs were exposed to each chemical within 2 h after fertilization. After exposure (until at 4 hph for Japanese medaka and at 48 hph for zebrafish), 15 fertilized eggs at each concentration were distributed into 100-mL glass vessels (together with 60 mL of exposure liquid) and cultured until observation. For the test, four replicate 100-mL glass vessels were used at each concentration. Under the stereomicroscope, we observed the presence or absence of swim bladder inflation at 4 hph (i.e., total exposure time, 220 h) for Japanese medaka and at 48 hph (i.e., total exposure time, 120 h) for zebrafish. These times were selected based on the results of the experiment explained in Section 2.2. To reveal the expression of the thyroid hormone-related genes, *tshβ*, *tra*, and *trβ*, after their observation, larvae were stored in RNAlater® (Sigma-Aldrich, St. Louis, USA) and kept at 4 °C until RNA isolation (within 7 days).

2.4. Real-time quantitative polymerase chain reaction

We previously described our real-time quantitative polymerase chain reaction (PCR) methods (Horie et al., 2020). In short, total RNA was extracted from whole larvae using the RNeasy Mini Kit including an on-column RNase-free DNase treatment (Qiagen, Hilden, Germany). The concentration of RNA solutions was measured using a NanoDrop One Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, RNA was reverse-transcribed into cDNA using PrimeScript RT Master Mix (Perfect Real Time, Takara, Shiga, Japan), and the concentration of each cDNA solution was adjusted to 10 ng/µL. Real-time quantitative PCR was performed with a LightCycler 96 System (Roche, Basel, Schweiz) using a FastStart SYBR Green Master (Nippon Genetics Co., Ltd, Tokyo, Japan). Each reaction mixture (20 µL) contained 10 µL of PCR Master Mix (2×), 0.2 µL of each 20 µM primer, 1 µL of 10 ng/µL cDNA, and 8.6 µL of PCR grade water. Each sample for each target was run in duplicate. The primer sets used for each gene are shown in Table 1. The data were analyzed using the LightCycler® 96 SW 1.1 software (Roche) and exported to Microsoft Excel

(Microsoft, Redmond, WA, USA). The expression levels of *tsh β* , *tra*, and *tr β* were normalized to that of a housekeeping gene, which was elongation factor 1a (*ef1a*) for Japanese medaka and *β -actin* for zebrafish, by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

All data were analyzed in Microsoft Excel. Data are presented as mean \pm standard deviation. To analyze significant differences in gene expression compared with the control group, we used the open-source statistical software R (<http://www.R-project.org/>) and the package *Rcmdr* (Fox and Bouchet-Valat 2018) to test for homogeneity of variance using Bartlett's test (significance level, 5%). If the null hypothesis (i.e., the data are homoscedastic) was not rejected, we tested for differences among treatments using Dunnett's test; otherwise, we used Steel's test. To analyze significant differences in the number of individuals with an inflated swim bladder compared with the control group, the Chi-squared test was performed.

The lowest observed effect concentration (LOEC) is the lowest test concentration at which the substance is observed to have a statistically significant effect.

3. Results

3.1. Timing of swim bladder inflation in Japanese medaka and zebrafish

Figure 1 shows when the swim bladder became inflated in Japanese medaka (A) and in zebrafish (B). In the present study, the number of Japanese medaka larvae with an inflated swim bladder increased at each timepoint, with 1 of 50 individuals at 0.5 hph, 16 of 50 individuals at 1 hph, 38 of 50 individuals at 2 hph, 49 of 50 individuals at 4 hph, and all of the individuals at 24 hph. Figures 2A–H show representative images of fish at the various timepoints from just after hatching to 24 hph.

In zebrafish, inflated swim bladders were not observed until 24 hph (Fig. 1B and Fig. 3A, B). At 48 hph, most individuals (49 of 50) had inflated swim bladders (Fig. 3D), although a larva with edema that developed abnormally did not show swim bladder inflation (Fig. 3C). The swim bladder of the larva with edema had not inflated at 72 hph (Fig. 3E), although all normal larvae had swim bladder inflation (Fig. 3F).

3.2. Effect of PFBA, TDCPP, and T3 on swim bladder inflation in Japanese medaka and zebrafish

The effect of exposure to PFBA, TDCPP, and T3 on swim bladder inflation in Japanese medaka and zebrafish is shown in Figure 4. With PFBA, significantly less non-inflated

swim bladders were observed at 40 and 80 mg/L exposure in Japanese medaka (Fig. 4A) and at 80 mg/L exposure in zebrafish (Fig. 4D). With TDCPP, significantly less non-inflated swim bladders were observed at 1 and 2 mg/L exposure in Japanese medaka (Fig. 4B), and were also observed at 0.5, 1, and 2 mg/L exposure in zebrafish (Fig. 4E). With T3, no effect on swim bladder inflation was observed in Japanese medaka at any of the exposure levels (maximum exposure concentration was set at water solubility, 1 mg/L) (Fig. 4C), but a significant inhibition of swim bladder inflation was observed at 7.5, 15, and 30 µg/L exposure in zebrafish (Fig. 4F).

3.3. Effect of PFBA, TDCPP, and T3 on *tshβ*, *tra*, and *trβ* mRNA expression in Japanese medaka and zebrafish

After exposure to PFBA, *tshβ* and *tra* expression was not altered in Japanese medaka (Fig. 5A, B) or zebrafish (Fig. 5D, E). Expression of *trβ* was not changed in Japanese medaka (Fig. 5C), but it was significantly increased in zebrafish exposed to PFBA at 10, 40, and 80 mg/L compared with the control group (Fig. 5F).

After exposure to TDCPP, *tshβ* expression was not altered in Japanese medaka (Fig. 6A) or zebrafish (Fig. 6D). Expression of *tra* was not altered in Japanese medaka (Fig. 6B), but in zebrafish, the expression of this gene was significantly higher after exposure to TDCPP at 0.25 mg/L and significantly lower after exposure to TDCPP at 1 and 2 mg/L compared with the control group (Fig. 6E). Although *trβ* expression did not change in Japanese medaka (Fig. 6C), it was significantly increased in zebrafish exposed to TDCPP at 0.25, 1, and 2 mg/L compared with the control group (Fig. 6F).

After exposure to T3, although the *tshβ* expression was not altered in Japanese medaka (Fig. 7A), it was decreased in a dose-dependent manner in zebrafish and was significantly lower after exposure to T3 at 7.5, 15, and 30 µg/L compared with the control group (Fig. 7D). *Tra* expression was significantly increased in a dose-dependent manner in both Japanese medaka (Fig. 7B) and zebrafish (Fig. 7E) except at 0.25 mg/L in Japanese medaka. *Trβ* expression was significantly increased at all exposure levels compared with the control group in both Japanese medaka (Fig. 7C) and zebrafish (Fig. 7F).

3.4. Summary of the effect of PFBA, TDCPP, and T3 on swim bladder inflation and thyroid-related gene expression

Table 2 gives a summary of the effect of PFBA, TDCPP, and T3 on Japanese medaka and zebrafish. A comparison of the effects on Japanese medaka and zebrafish confirmed that swim bladder inflation was affected by PFBA and T3 exposure, albeit at different

concentrations. There was no uniform tendency in the changes in thyroid-related gene expression except after T3 exposure. In addition, no correlation was found between swim bladder inflation and changes in thyroid-related gene expression both in Japanese medaka and zebrafish exposed to PFBA, TDCPP, and T3 exposure.

4. Discussion

According to the integrated book for the biology of the Medaka (Iwamatsu, 2006, p.309, Japanese Language), it has been reported that the swim bladder became inflated shortly after hatching (stage 40; Iwamatsu, 2004) in Japanese medaka. The present study shows that swim bladder inflation is best observed at 4 hph (stage 40) or 48 hph in Japanese medaka or zebrafish. Our previous studies under the same laboratory conditions (e.g., fish strain, breeding water temperature, breeding density, water exchange frequency) showed that fertilized eggs hatched around 9 days post fertilization in Japanese medaka (Horie et al., 2017b) and 3 days post fertilization in zebrafish (Horie et al., 2017a), indicating that the time required to assess the influence of chemicals on swim bladder inflation is shorter in zebrafish than in Japanese medaka. In the present study, a zebrafish larva with morphologically abnormal development did not have an inflated swim bladder even at 72 hph, which suggests that abnormal larvae may not achieve swim bladder inflation. In zebrafish larvae, abnormal development is often induced not only by thyroid hormone-disrupting chemicals but also by exposure to various other chemicals, such as broflanilide (Duan et al., 2021), ethylparaben (Merola et al., 2020), fluxapyroxad (Lin et al., 2021), linuron (Maharaj et al., 2020), propylparaben (Perugini et al., 2019), 4-epianhydrotetracycline (Wang et al., 2020), and chlorinated anilines (Horie et al., 2017a), which induce edema, body curvature, or non-inflation of the swim bladder. Taken together with these studies, our results indicate that when zebrafish are used for *in vivo* assays to detect thyroid hormone-disrupting activity, the test period is shorter compared to the use of Japanese medaka, but to evaluate the effect of chemical substances on swim bladder inflation, it is necessary to determine whether the effect is due to thyroid disturbance or abnormal embryo development. Therefore, a zebrafish-based *in vivo* assay to detect thyroid hormone-disrupting activity will need to include not only non-inflation of the swim bladder, but also other biomarkers related to abnormal development. During our 7 years of laboratory experiments, chemical exposure did not often induce abnormal development in Japanese medaka (Supplementary Table 1). Therefore, when Japanese medaka are used for this type of assay, although the test period will be longer compared with that for zebrafish, when evaluating the effect of chemical substances on swim bladder inflation, morphological abnormalities will not need to be considered.

Thyroid hormone is known to be important for swim bladder development and inflation in zebrafish, because knockdown of Type 3 iodothyronine deiodinase, the primary inactivating deiodinase that terminates thyroid hormone action, leads to abnormal swim bladder development (Heijlen et al., 2014). The effect of chemicals on swim bladder inflation in fish was reviewed by Dang et al. (2021). Various chemicals affect swim bladder inflation in Japanese medaka and zebrafish, such as T3 (Godfrey et al., 2017, 2019), perfluorooctanesulfonic acid (Gaballah et al., 2020), PFBA (Godfrey et al., 2017, 2019), TDCPP (Godfrey et al., 2017, 2019), perfluorohexanesulfonic acid (Gaballah et al., 2020), 1,2,5,6-tetrabromocyclooctane (Van Essen et al., 2021), triphenyltin (Horie et al., 2021), and pentabromobenzene (Peng et al., 2020). The results in the present study that PFBA and TDCPP exposure both induced non-inflation of the swim bladder are consistent the findings from previous studies in Japanese medaka (Godfrey et al., 2019) and zebrafish (Godfrey et al., 2017). However, T3 exposure in the present study did not affect swim bladder inflation in Japanese medaka. In the future, it will be necessary to clarify the relationship between these thyroid hormone-disrupting chemicals and swim bladder inflation by using assays that include a range of exposures to the test chemicals.

Other reports have outlined the relationship between thyroid hormone-disrupting chemicals and the expression of thyroid hormone-related genes. In the present study, TDCPP exposure did not affect *tsh β* , *tra*, and *tr β* expression in Japanese medaka, although *tra* and *tr β* expression was upregulated in zebrafish. Previous studies report that TDCPP exposure upregulates *tra* in Japanese medaka (Godfrey et al., 2019) but that there is no change in *tsh β* , *tra*, and *tr β* expression in zebrafish (Godfrey et al., 2017), whereas Wang et al. (2013) and Liu et al. (2013) report that *tsh β* and *tra* are upregulated. In the present study, PFBA exposure upregulated *tr β* expression in zebrafish, but Godfrey et al. (2017, 2019) reported that there is no change in the expression of *tsh β* , *tra*, and *tr β* in Japanese medaka or in zebrafish. Our results show that T3 exposure downregulated *tsh β* expression in zebrafish and upregulated *tra* and *tr β* expression in both species. However, Godfrey et al. (2017, 2019) reported no change of *tsh β* , *tra*, and *tr β* expression in either species, except for *tra* in zebrafish. These results indicate that although thyroid hormone-disrupting chemicals affect the expression of thyroid hormone-related genes, the expression patterns are still not clear and vary in different fish species. In future, it will be necessary to reassess the relationship between thyroid hormone-disrupting chemicals and the expression of thyroid hormone-related genes in assays with increasing exposure to chemical substances.

This is the first study to compare the LOEC of thyroid hormone–disrupting chemicals for swim bladder inflation or the expression of thyroid hormone–related genes between Japanese medaka and zebrafish, although it should be noted that LOEC values were determined by using nominal concentrations. Similar effects on swim bladder inflation were observed in both species, although the LOEC of each was different and the species had varying sensitivity to different chemicals. Thyroid hormone–disrupting chemicals induced changes in the expression of thyroid hormone–related genes in Japanese medaka and zebrafish, although, again, the LOEC was not the same and the species had different levels of sensitivity. These results suggest that thyroid hormone–disrupting chemical substances may have induced the observed failure of swim bladders to inflate either directly or indirectly. However, the use of thyroid hormone–related gene expression as an indicator to detect thyroid hormone–disrupting activity has not been confirmed. To understand the wide variety in the effects of thyroid disruptors on thyroid hormone–related gene expression in aquatic species, the mechanisms through which these chemicals modulate these effects must be elucidated.

5. Conclusions

In the present study, we elucidated the timing of swim bladder inflation and the effect of thyroid hormone–disrupting chemical substances on swim bladder inflation and thyroid-related gene expression in Japanese medaka and zebrafish. First, inflation of the swim bladder was observed at around 4 hph in Japanese medaka and at around 48 hph in zebrafish. Second, non-inflation of the swim bladder was induced by exposure to PFBA and TDCPP exposure in both species. Third, changes in thyroid-related gene expression showed no clear trend, except after exposure to T3. The present results confirmed that thyroid hormone–disrupting chemicals induce failure of swim bladder inflation in zebrafish and Japanese medaka larvae, therefore, it is possibly be used for the short-term assay detecting thyroid hormone-disrupting chemicals using fertilized eggs of zebrafish (ca 5 days) and Japanese medaka (ca 10 days). The concomitant changes of thyroid hormone-related gene expression remain to be clarified.

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Conflicts of interest

The authors have no conflicts of interest related to this research.

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- 534

Figure Legends

Fig. 1 Timing of swim bladder inflation in Japanese medaka (A) and zebrafish (B). Numbers above each bar indicate the number of individuals with an inflated swim bladder.

Fig. 2 Representative images of Japanese medaka larvae with uninflated or inflated swim bladders at (A) just after hatching, (B) 0.5 hph, (C, D) 1 hph, (E, F) 2 hph, (G) 4 hph, and (H) 24 hph. Red arrow heads indicate inflated swim bladders.

Fig. 3 Representative images of zebrafish larvae with uninflated or inflated swim bladders at (A) just after hatching, (B) 24 hph, (C, D) 48 hph, and (E, F) 72 hph. Blue arrows indicate edema. Red arrow heads indicate inflated swim bladders.

Fig. 4 Effect of PFBA, TDCPP, and T3 on swim bladder inflation in Japanese medaka (A–C) and zebrafish (D–F) at 4 hph and 48 hph. Numbers above each bar indicate the number of individuals with an inflated swim bladder. Asterisks indicate values with significant difference compared with the control (Chi-squared test; $*P < 0.05$ vs. control group).

Fig. 5 Effect of PFBA on mRNA expression of *tsh β* , *tra*, and *tr β* in Japanese medaka at 4 hph (A–C) and zebrafish at 48 hph (D–F) as measured by real-time quantitative PCR analysis. The relative expression level describes the change in expression of the target gene relative to a control individual. Data are given as mean values and error bars (SD) (n = 6). *Values that are significantly different from control (Dunnett's test or Steel's test; $*P < 0.05$)

Fig. 6 Effect of TDCPP on mRNA expression of *tsh β* , *tra*, and *tr β* in Japanese medaka at 4 hph (A–C) and zebrafish at 48 hph (D–F) as measured by real-time quantitative PCR analysis. The relative expression level describes the change in expression of the target gene relative to a control individual. Data are given as mean values and error bars (SD) (n = 6). *Values that are significantly different from control (Dunnett's test or Steel's test; $*P < 0.05$)

Fig. 7 Effect of T3 on mRNA expression of *tsh β* , *tra*, and *tr β* in Japanese medaka at 4 hph (A–C) and zebrafish at 48 hph (D–F) as measured by real-time quantitative PCR analysis. The relative expression level describes the change in expression of the target gene relative to a control individual. Data are given as mean values and error bars (SD)

571 (n = 6). *Value significantly different from control (Dunnett's test or Steel's test; *P <
 572 0.05)

573

574 **Table 1** Primer sequences of the genes used for real-time quantitative PCR analysis

Species	Gene	Primer sequence (5'→3')	Accession number
Japanese medaka	<i>tshβ</i>	Forward: CAACAGGATTGGATGGCAAA	AB255697
		Reverse: TCGTCCTCGTCTTCCTCTCTTC	
	<i>tra</i>	Forward: TCTGAGCTGCCTTGTGAAGACC	AB114860
		Reverse: CAGCGTCAATGTTTCGCTCTC	
	<i>trβ</i>	Forward: GCTTTATGCGTGTGCAAGTT	NM_001104690
		Reverse: CGCGTACGAAGTCAAGGTTA	
	<i>ef-1α</i>	Forward: AGTACGCCTGGGTGTTGGAC	AB013606
		Reverse: AAACGGGCCTGGCTGTAAG	
	<i>tshβ</i>	Forward: AGGTTGCCGTGCCTATGTG	AY135147
		Reverse: GGACCCACCAACTCCTTTATGT	

	Forward:	
	GGCTCGGAGTGGTTTCTGA	
<i>tra</i>		NM_131396
	Reverse:	
	CTTGCGGTGGTTGATGTAGTG	
	Forward:	
	CACATGCTGTGTTGCAGCTT	
<i>trβ</i>		NM_131340
	Reverse:	
	TCATAAGAGCCAGAGCCCCT	
	Forward:	
	CGAGCAGGAGATGGGAACC	
<i>β-actin</i>		AF057040
	Reverse:	
	CAACGGAAACGCTCATTGC	

575

576 **Table 2** Comparison of the effect of different concentrations of PFBA, TDCPP, and T3
577 on inflation of the swim bladder and the expression of *tshβ*, *tra*, and *trβ* between Japanese
578 medaka and zebrafish.

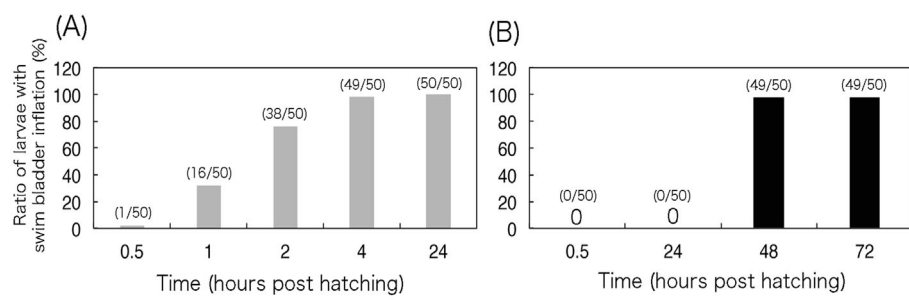
Chemical	Fish	Exposure concentration			
		Inflation of swim bladder	mRNA expression of <i>tshβ</i>	mRNA expression of <i>tra</i>	mRNA expression of <i>trβ</i>
PFBA	medaka	40 mg/L ↓	NE	NE	NE
	zebrafish	80 mg/L ↓	NE	NE	40 mg/L ↑
TDCPP	medaka	1 mg/L ↓	NE	NE	NE
	zebrafish	0.5 mg/L ↓	NE	0.25 ↑, 1 mg/L ↓	1 mg/L ↑
T3	medaka	NE	NE	0.5 mg/L ↑	0.12 mg/L ↑
	zebrafish	7.5 μg/L ↓	7.5 μg/L ↓	1.8 μg/L ↑	1.8 μg/L ↑

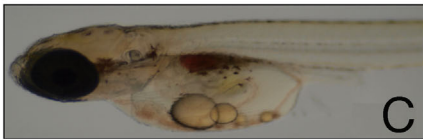
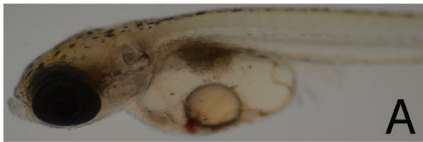
579 NE, No significant effect.

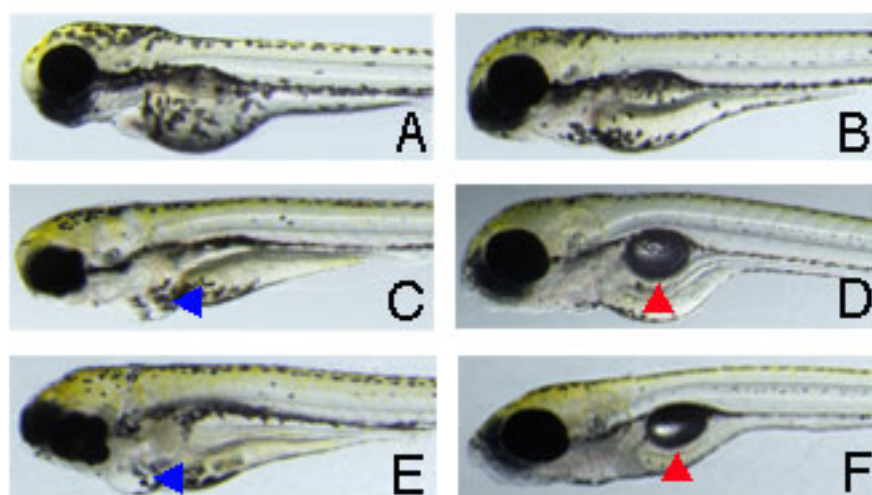
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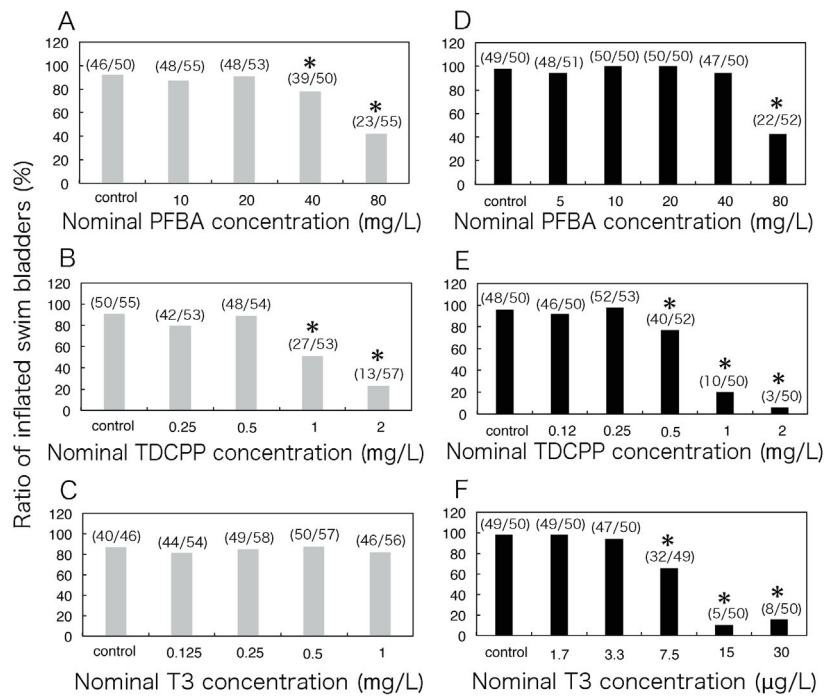
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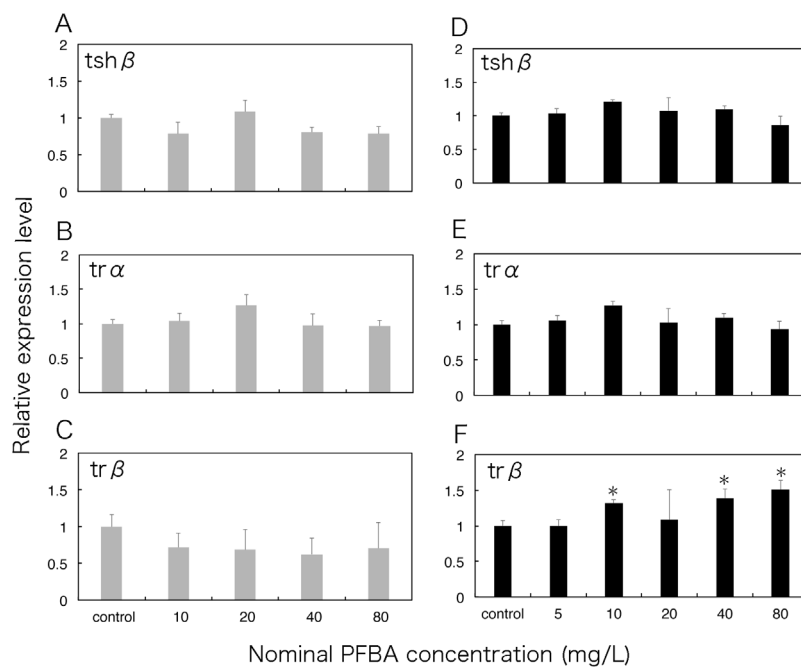
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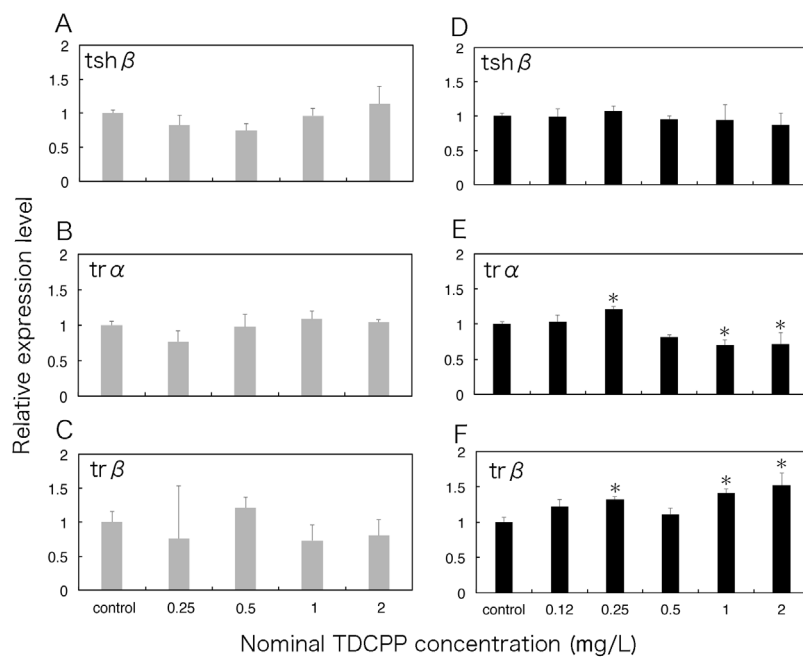


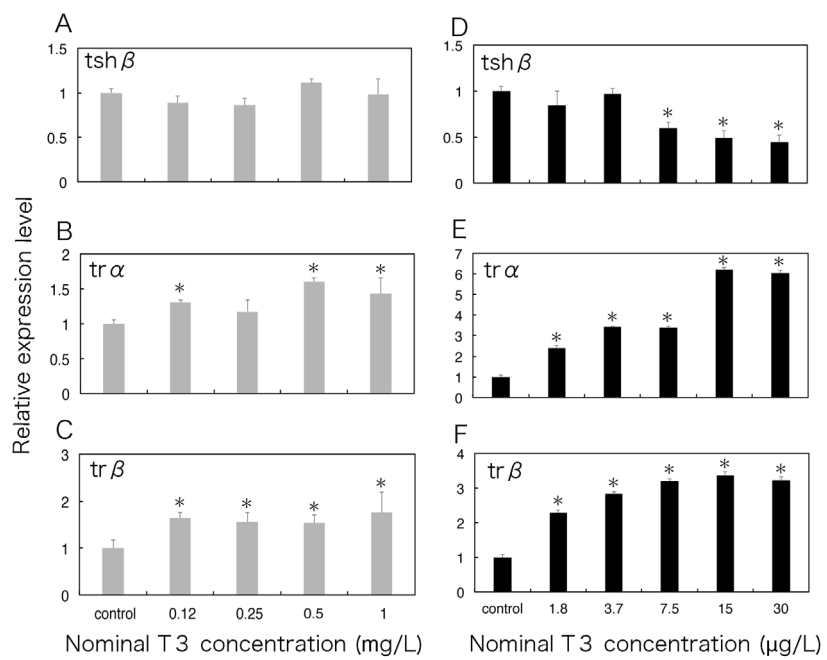












Supplementary Table 1. Presence or absence of induction of abnormal embryo development in Japanese medaka exposed to various chemicals in experiments conducted in our laboratory during the past 7 years.

Test chemical	Presence or absence of induction of abnormal embryo development	Reference
17 α -Methyltestosterone	Absence	Horie et al. (2016) ¹
Triphenyltin	Absence	Horie et al. (2017) ²
Triclosan	Absence	Horie et al. (2018) ³
Tributyltin	Presence (small eye or body malformation)	Horie et al. (2018) ⁴
Bisphenol A	Absence	Horie et al. (2020) ⁵
4-Nonylphenol	Absence	Horie et al. (2021) ⁶

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