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Adverse effects of thyroid-hormone-disrupting chemicals 6-propyl-2-thiouracil and tetrabromobisphenol A on Japanese medaka (*Oryzias latipes*)

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ABSTRACT

Thyroid-hormone-disrupting chemicals are increasingly attracting attention because of their potential harmful effects on animal health, including on fishes. Here, we investigated the effects of exposure to the thyroid-hormone-disrupting chemicals 6-propyl-2-thiouracil (PTU) and tetrabromobisphenol A (TBBPA) on swim bladder inflation, eye development, growth, swimming performance, and the expression of thyroid-related genes in Japanese medaka (*Oryzias latipes*). PTU exposure resulted in reductions in eye size, growth, and swim bladder inflation, and these effects led to poorer swimming performance. These phenotypic effects were accompanied by increased expression of the thyroid-stimulating hormone subunit beta (*tshβ*) paralog *tshβ-like*, but there were no significant changes in expression for *tshβ*, deiodinase 1 (*dio1*), deiodinase 2 (*dio2*), and thyroid hormone receptor alpha (*trα*) and beta (*trβ*). For PTU exposure, we identified the key event (swim bladder inflation reduction) and an adverse outcome (swimming performance reduction). No significant effects from TBBPA exposure were seen on swim bladder inflation, eye development, growth, or swimming performance. However, expression of *tshβ-like* and *tshβ* (significantly enhanced) and *trα* and *trβ* (significantly reduced) were affected by TBBPA exposure albeit not in dose-dependent manners. There were no effects of TBBPA on the expression of *dio1* and *dio2*. We thus show that the two thyroid-hormone-disrupting chemicals PTU and TBBPA differ in their effect profiles with comparable effects on the studied phenotypes and thyroid-related gene expression to those reported in zebrafish.

1. Introduction

Much attention has been directed toward the effects of endocrine-disrupting chemicals, principally (anti-) oestrogens and (anti-) androgens on fish growth (reviewed by Celino-Brady et al., 2021), fertility (Onishi et al., 2021; Kawashima et al., 2022), and sexual development, including secondary sexual characteristics (Horie et al., 2021, 2022a). Recently, however, a growing body of research has focused on the effects of thyroid-hormone-disrupting chemicals (TDCs), motivated partly by European Union (EU) legislation regulating industrial chemicals (Registration, Evaluation, Authorization and Restriction of Chemicals [REACH], EC, 1907/2006), plant protection products (Regulation, EC, 1107/2009), and biocide products (Regulation, 528/2012, EC, 2017a). In vertebrates, thyroid hormone, generally secreted from the thyroid gland, regulates the body's metabolism and is involved in growth (Mullur et al., 2014). Thyroid hormone promotes metamorphosis from tadpoles to frogs in amphibians (Brown and Cai, 2007; Thambirajah et al., 2019) and causes seasonal molting in birds (Zimova et al., 2018). In the case of fishes, thyroid hormone is involved not only in metabolism and growth, but also osmoregulation (adaptation to salt) and development of the swim bladder (Blanton and Specker, 2007; Vergauwen et al., 2018; Deal and Volkoff, 2020).

Thyroid hormone is regulated by thyroid-stimulating hormone (TSH)

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Abbreviations

dio	deiodinase
dpf	days post fertilization
dph	day post hatching
EU	European Union
hpf	hours post fertilization
MMI	methimazole
NIES	National Institute for Environmental Studies
OECD	Organisation for Economic Co-operation and Development
PFOA	perfluorooctanoic acid
PTU	6-propyl-2-thiouracil
RT-qPCR	real-time quantitative polymerase chain reaction
TBBPA	tetrabromobisphenol A
TDCs	thyroid-hormone-disrupting chemicals
TDCPP	tris(1,3-dichloro-2-propyl)phosphate
tshβ	thyroid-stimulating hormone subunit beta
tr	thyroid hormone receptor

secreted by the pituitary gland and this acts via two thyroid hormone receptors (*trα* and *trβ*) (Szkudlinski et al., 2002; Ortiga-Carvalho et al., 2014). Thyroid hormone is secreted from the thyroid gland as prohormone T4 (thyroxin) and converted to T3 (3,5,3′-triiodothyronine), which has strong physiological activity in the liver and muscles (reviewed by Deal and Volkoff, 2020). The T4-T3 converting enzyme is called iodothyronine deiodinase and is found in two types (Dio1 and Dio2) that have different localizations and regulation (reviewed by Deal and Volkoff, 2020). Dio1 is highly expressed in the liver and converts T4 to T3 (reviewed by Deal and Volkoff, 2020). Parsons et al. (2020) showed widespread and highly dynamic tissue expression of key genes (tsh subunit *β* [*tshβ*], *trα*, *trβ*, *dio1*, *dio2*) in the hypothalamus-pituitary-thyroid (HPT) axis in zebrafish (*Danio rerio*) embryo-larvae, supporting their roles in multiple developmental processes. The responsiveness of these genes to T3 suggests a high vulnerability of thyroid hormone-dependent tissues and physiological processes during early developmental windows to altered thyroid hormone signaling and potential mechanisms of action for TDCs.

Recently, Dang et al. (2021) reviewed fish toxicity testing for TDCs, most of which have been conducted using zebrafish. These studies have shown that TDCs, thyroid-peroxidase inhibitor chemicals, and sodium-iodide-symporter inhibitor chemicals all disrupt thyroid-related gene expression including *tshβ*, *trα*, *trβ*, *dio1*, and *dio2* (Li et al., 2012; Baumann et al., 2016; Parsons et al., 2019). These chemicals also alter swim bladder inflation (Godfrey et al., 2017; Stinckens et al., 2020; Horie et al., 2022b), eye development (Baumann et al., 2016), and growth (Baumann et al., 2016) in zebrafish. The Organisation for Economic Co-operation and Development (OECD) has established toxicity-testing guidelines for endocrine-disrupting chemicals on fishes and recommends the use of not only zebrafish but also Japanese medaka (*Oryzias latipes*) (https://www.oecd.org/), which shares many of the advantageous characteristics of zebrafish for use as a model species, including small genome size as well as small body size and short generation time for ease of maintenance in the laboratory.

Although many previous studies have reported the effects of TDCs on zebrafish, the influence of TDCs on Japanese medaka are still unclear. To our knowledge, only T3 (Godfrey et al., 2019; Horie et al., 2022b), TDCPP (Godfrey et al., 2019; Horie et al., 2022b), methimazole (MMI; Godfrey et al., 2019), perfluorooctanoic acid (PFOA; Godfrey et al., 2019), PFBA (Godfrey et al., 2019; Horie et al., 2022b), and 2-ethylhexyl-4-methoxycinnamate (Lee et al., 2019) have been found to affect thyroid-related gene expression or swim bladder inflation in Japanese medaka. In our recent studies on the impacts of T3, tris(1,3-dichloro-2-propyl)phosphate (TDCPP), and perfluorobutyric acid (PFBA) on swim bladder inflation and expression of thyroid-related genes *tshβ*, *trα*, and *trβ*, both compounds produced larvae with uninflated swim bladders in both zebrafish and medaka. We also identified changes in expression of thyroid-related genes (*tshβ*, *trα*, and *trβ*), but these effects were different for the different chemicals and between the zebrafish and medaka (Horie et al., 2022b).

In this study, we focused on the TDCs 6-propyl-2-thiouracil (PTU) and tetrabromobisphenol A (TBBPA), which have been reported to inhibit swim bladder inflation and disrupt expression of thyroid-related genes in zebrafish. First, we evaluated whether the two chemicals affect eye development, growth, swim bladder inflation, and expression of thyroid-related genes in Japanese medaka. We then compared their toxicity in Japanese medaka against those previously reported for zebrafish.

2. Materials and methods

2.1. Test fish and test chemicals

The Japanese medaka (*O. latipes*) used in this study were of the R strain from the National Institute for Environmental Studies (NIES). Test fish were supplied from NIES and bred at Kobe University (Hyogo, Japan) (water temperature, 25 ± 2 °C; 16-h light and 8-h dark). All animal experiments were conducted in accordance with the relevant national guidelines (Act on Welfare and Management of Animals, Ministry of the Environment, Japan), and all fish used in this study were handled in accordance with the animal care and use guidelines of Kobe University. All animal experiments were approved by the institutional animal care and use committee of the Research Center for Inland Sea, Kobe University (Permission number, 2021-04). Our research was also performed in accordance with the ARRIVE guidelines.

PTU (CAS No. 51-52-5, purity >99.0 %) and TBBPA (CAS No. 79-94-7, purity >98.0%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2. Test concentrations and exposure test method

The highest nominal exposure concentration was set to a concentration that was lower than the water solubility but high enough to affect swim bladder inflation or growth (total body length). The nominal exposure concentrations for PTU were control (0), 32, 100, 320, and 1000 mg/L and for TBBPA were 0, 32, 100, 320, and 1000 μg/L. To prepare an aqueous stock solution at the highest test concentration for each test chemical, the appropriate mass of PTU (1000 mg) and TBBPA (1 mg) were placed in a 1-L glass bottle and dissolved in 1 L of dechlorinated tap water by sonicating for 60 min in an ultrasonic bath. This stock solution was diluted with dechlorinated tap water to the exposure concentration for each group. The test solutions were renewed every 2 days. Water samples were collected during each test solution renewal, and were measured for chemical analysis.

Eggs were obtained from 10 pairs of parent medaka. Fertilized eggs, selected using a stereomicroscope, were exposed to each treatment concentration within 4 h post fertilization (hpf). Exposures were conducted in 100-mL glass vessels filled with 60 mL of exposure liquid. Twenty fertilized eggs were placed in each vessel, and 4 replicate vessels were used for each treatment concentration (i.e., 80 fertilized eggs per treatment). Just after hatching, the hatched larvae were counted, grouped by exposure concentration into 500-mL glass beakers, and assessed for swim bladder inflation, total body length, eye size, thyroid-related gene expression, and swimming performance. The exposure period was from 4 hpf to 1 day post hatching (dph; corresponding to around 10 days post fertilization [dpf] for PTU [average hatching day was 9] and 9 dpf for TBBPA [average hatching day was 8]). Therefore, the total exposure period was around 9–10 days. The exposure end time of 1 dph was selected based on a previous study (Horie et al., 2022b), i.

Table 1
Nominal and measured concentration of PTU and TBBPA.

PTU concentrations		TBBPA concentrations	
Nominal (mg/L)	Measured (mg/L)	Nominal (μg/L)	Measured (μg/L)
Control	ND	Control	ND
32	29.3	32	23.3
100	88.4	100	62.5
320	257	320	278
1000	873	1000	793

ND denotes that the concentration measured was lower than the limit of detection.

e., the number of Japanese medaka larvae with inflated swim bladders increased until 1 dph. At 1 dph, we evaluated swim bladder inflation, total body length, eye size, thyroid-related gene expression, and swimming performance. In addition, average hatching day, hatching rate, and frequency of abnormal development were also calculated.

2.3. Chemical analysis

To measure the PTU and TBBPA exposure concentration, samples of test solutions were first adequately diluted with acetonitrile:water 1:1 (v:v) solution. PTU concentrations were measured using LC–MS/MS (ACQUITY UPLC, Waters, Milford, MA, USA; QTRAP 6500, AB Sciex, Framingham, MA, USA). TBBPA concentrations were quantitatively determined by liquid chromatography tandem mass spectrometry, LC–MS/MS (1260 Infinity II and 6470 Triple Quadrupole LC/MS, Agilent, Santa Clara, CA, USA). In the analyses, the limits of detection and determination were 0.00061 and 0.0016 mg/L, respectively, for PTU and 0.087 and 0.23 μg/L, respectively, for TBBPA. Measured concentrations of PTU and TBBPA in the test solutions are shown in Table 1. The operating conditions in the analyses are provided in Supplementary Table 1.

2.4. Swim bladder inflation, total body length, and eye size

Swim bladder inflation, total body length, and eye size were evaluated in 50 larvae selected at random. The larvae were photographed at 1 dph with a stereomicroscope (SZX 16, OLYMPUS, Tokyo, Japan) and fitted camera (Visualix V900FL, Visualix, Kobe, Japan), and total body length and eye size were measured by using ImageJ software (Fig. 1).

2.5. Thyroid-related gene expression

Our real-time quantitative polymerase chain reaction (RT-qPCR) procedure was as described previously (Horie et al., 2022a, 2022b) (Fig. 1). At 1 dph, larvae were stored in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) and maintained at 4 °C. The next day, total RNA was extracted from each selected larva using an RNeasy Mini Kit including an on-column RNase-free DNase treatment (Qiagen, Hilden, Germany). Nine larvae were selected for total RNA extraction for each treatment concentration group (i.e., a total of 45 larvae per exposure chemical). After total RNA was extracted, the RNA extraction concentration was measured using a NanoDrop One Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Next, RNA was reverse-transcribed into cDNA by using PrimeScript RT Master Mix (Perfect Real Time, Takara, Shiga, Japan), and the concentration of each cDNA solution was adjusted to 10 ng/μL and maintained at −30 °C until RT-qPCR.

We investigated the expression levels of the following six thyroid-related genes: *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ*. Sequences for each primer are shown in Supplementary Table 2. RT-qPCR was performed using the Light Cycler 96 System (Roche, Basel, Switzerland) with 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 data were analyzed using LightCycler 96 SW 1.1 software (Roche) and exported to Microsoft Excel (Microsoft, Redmond, WA, USA). The expression levels of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ* were normalized to that of the housekeeping gene elongation factor 1a (ef1α) by

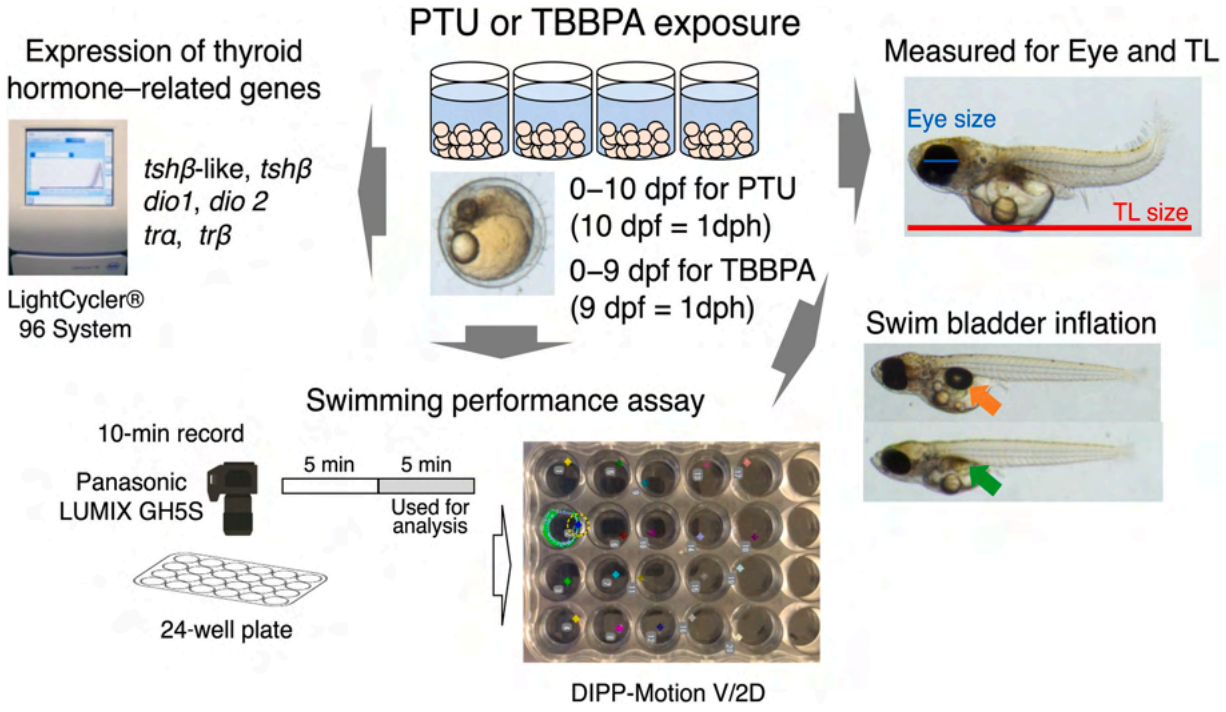

Fig. 1. Experimental flow chart. dpf, days post fertilization; dph, days post hatching; TL, total length. Orange arrow indicates inflated swim bladder. Green arrow indicates no inflation of swim bladder. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

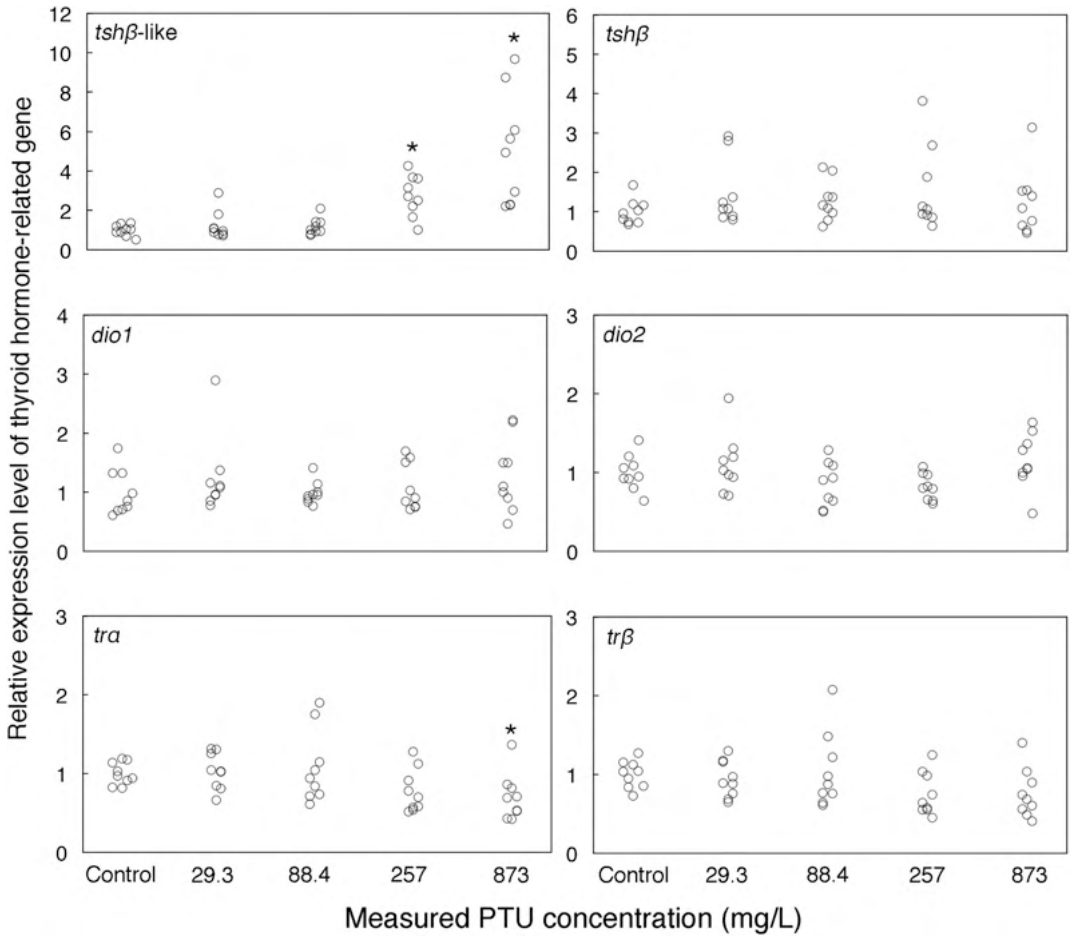

Fig. 2. Effect of PTU exposure on mRNA expression of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ* in Japanese medaka at 1 day post hatching as measured by real-time quantitative PCR analysis. The expression levels of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ* were normalized first against that of the *ef1α* housekeeping gene and then against controls. *Significantly different from control (Dunnett's test or Steel's test; $P < 0.05$).

using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Swimming performance

A flow chart summarizing the experimental procedure for the swimming performance assay is shown in Supplementary Fig. 1. Swimming performance assays were conducted for all treatment concentrations at 1 dph. Fish for assaying were placed individually into the wells of a 24-well microplate, with each microplate holding 4 individuals per treatment concentration. This was repeated 8 times, for a total *n* of 32 per treatment. Thirty-two larvae were selected at random by the naked eye to avoid artificial selection. Just after hatching, the larvae were transferred to a 24-well microplate before 10:00 a.m. and acclimatized to a water temperature of 25 ± 2 °C and a photoperiod of 16-h light, 8-h dark. After 24 h (i.e., 1 dph), swimming activity was recorded from 10:00 a.m. (first run) to 11:30 a.m. (end of the last run) under light conditions. A LUMIX GH5S camera (Panasonic, Osaka, Japan) was used to continuously record, from an aerial viewpoint, larval activity in each microplate at a rate of 60 frames/s for 10 min with a resolution of 1920 × 1080 pixels/mm. Total swimming distance over the last 5 min of video footage was used for assessing fish larval swimming performance with the video analysis conducted using DIPP-Motion V/2D (DITECT, Tokyo, Japan).

2.7. Statistical analysis

Statistical analyses were conducted as reported previously (Horie et al., 2022b). We first tested for homogeneity of variance of the data with Bartlett's test (significance level, 5 %) using the open-source statistical software R and the package Rcmdr (Fox and Bouchet-Valat, 2018). If homogeneity of variance was not rejected, we tested for differences among treatments by using Dunnett's test; otherwise, we used Steel's test. Statistical comparisons of swim bladder inflation among control and exposure groups were conducted by using the chi-squared test in Microsoft Excel.

3. Results

3.1. Effects of PTU and TBBPA exposure on thyroid-related gene expression (at 1 dph)

Exposure to PTU had a significant effect on *tshβ-like* expression in the 257 and 873 mg/L concentration groups as compared to the control. PTU exposure did not have a significant effect on expression of any of the other thyroid-function related genes (*tshβ*, *dio1*, *dio2*, *trα*, or *trβ*) as compared with the control (Fig. 2).

TBBPA exposure was associated with significantly higher *tshβ* expression in the 278 and 793 μg/L concentration groups and higher expression of *tshβ-like* in the 793 μg/L exposure group (Fig. 3). TBBPA exposure was also associated with lower *trα* expression in the 278 μg/L concentration group and lower *trβ* expression in the 62.5 and 278 μg/L exposure groups as compared to the control. The effects of TBBPA were not seen to be concentration dependent for *trα* and *trβ* expression. There were no significant effects of either substance on the expression of *dio1*

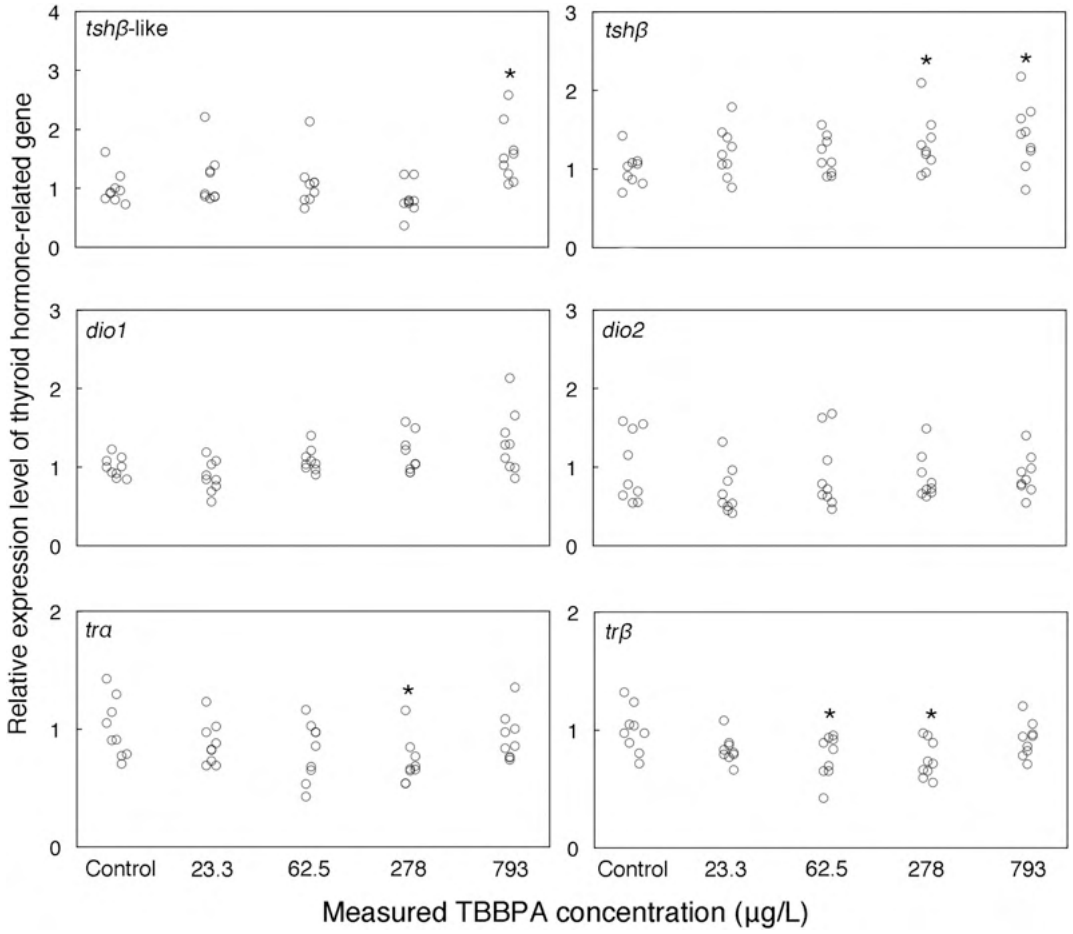

Fig. 3. Effect of TBBPA on mRNA expression of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ* in Japanese medaka at 1 day post hatching as measured by real-time quantitative PCR analysis. The expression levels of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ* were normalized first against that of the *ef1α* housekeeping gene and then against controls. *Significantly different from control (Dunnett's test or Steel's test; $P < 0.05$).

and *dio2*.

3.2. Effects of PTU and TBBPA exposure on swim bladder inflation and larval development (at 1 dph)

No effects of PTU were detected on average hatching day or on hatching rate, and there were no signs of any obvious abnormalities in embryo development (Supplementary Fig. 2). After hatching, however, bone dysplasia occurred and swim bladder inflation was absent in all individuals in the 873 mg/L concentration group (Fig. 4A–C). In the 257 mg/L concentration group, 8 of 50 larvae had no swim bladder inflation (Fig. 4A). PTU exposure was associated with a reduction in eye size in the 873 mg/L concentration group as compared to the control (Fig. 5B). In addition, total body length was smaller in the 88.4, 257, and 873 mg/L concentration groups as compared with the control (Fig. 5A).

There were no effects of TBBPA on average hatching day or on hatching rate (Supplementary Fig. 1). No effects were seen on swim bladder inflation or on larval development compared with the controls at any exposure concentration (Fig. 4D–F). We also observed no significant effects of TBBPA on eye size and total body length as compared with the controls (Fig. 5C, D).

3.3. Effects of PTU and TBBPA exposure on swimming performance (at 1 dph)

Total swimming distance over 5 min was reduced significantly after PTU exposure for all exposure concentration groups compared with the controls (Fig. 6A), but there were no effects on total swimming distance over 5 min in the TBBPA exposure groups (Fig. 6B).

4. Discussion

Recently, we reported the effects of TDCs on *tshβ* and *tr* expression and swim bladder inflation in Japanese medaka (Horie et al., 2022b). In the present study, we added to our previous work by measuring the effects of TDCs on the expression of *dio1* and *dio2*, eye development, and swimming activity. This information will help clarify the adverse outcome pathway network for TDCs in Japanese medaka.

In recent years, there has been increasing concern about the effects of TDCs on wildlife. For studies on the effects of TDCs in fish, much of the experimental laboratory work has been carried out on zebrafish (reviewed by Dang et al., 2021). Much of this work has been carried out on larval stages before the onset of feeding behavior (120 h post hatching for zebrafish, 2 dph for Japanese medaka), in part driven by the EU Directive 2010/63/EU for the protection of animals used for scientific purposes (EU, 2010), where fish larvae immediately post hatching and prior to feeding are exempt from animal welfare regulations, but also because thyroid hormones play fundamental roles in growth and development processes during early life stages.

Heijlen et al. (2014) reported that knockdown of type-3 iodothyronine deiodinase, the main inactivating deiodinase for thyroid hormone action, causes abnormal swim bladder inflation in zebrafish, suggesting that fish thyroid hormone is important for normal swim bladder inflation. Furthermore, in zebrafish, abnormal swim bladder inflation can be

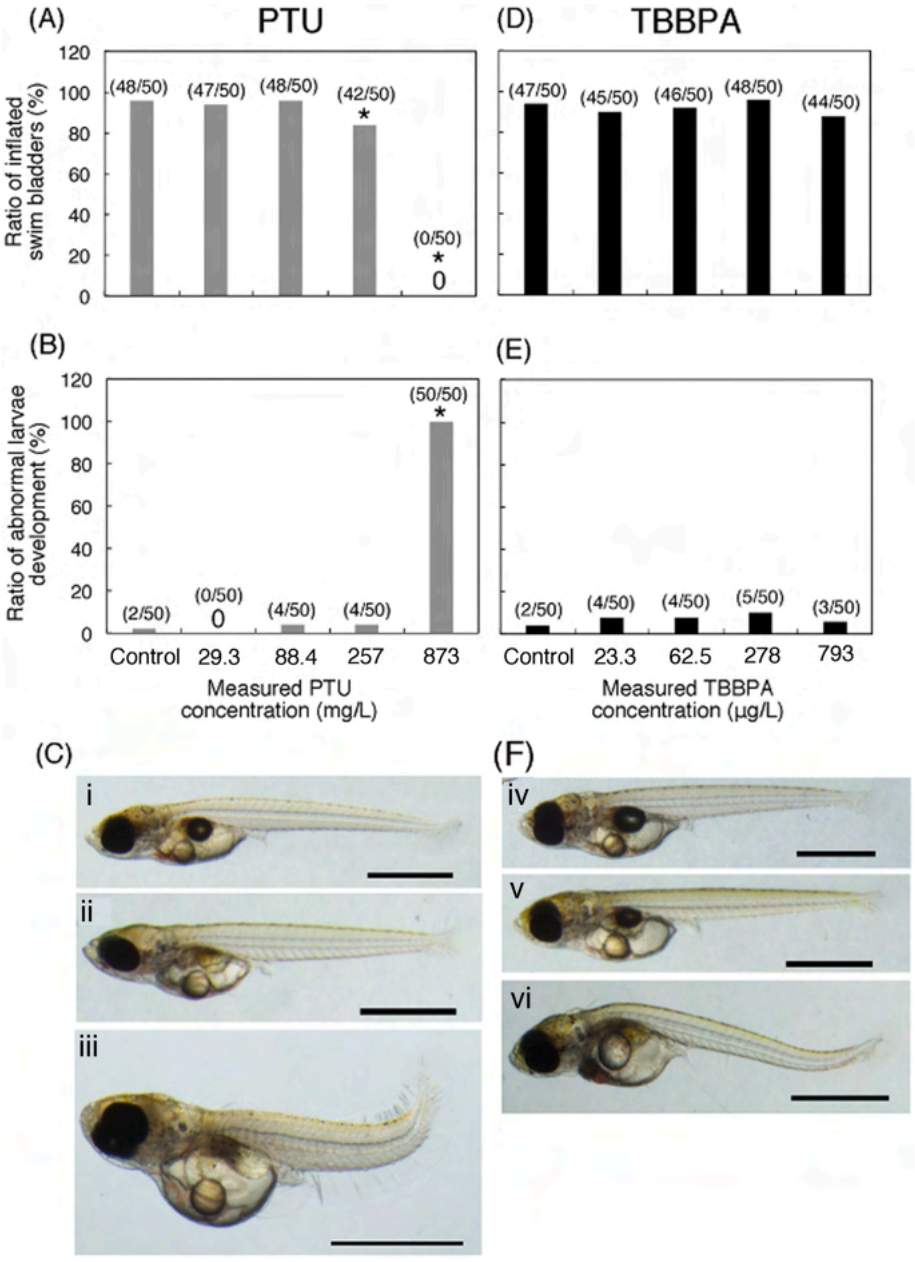

Fig. 4. Effects of PTU (A–C) and TBBPA (D–F) on swim bladder inflation (A and D) and abnormal larval development (B and E) in Japanese medaka at 1 day post hatching. Numbers above each bar indicate the number of individuals with inflated swim bladders or abnormal larval development. *Significantly different from control (Chi-squared test; $P < 0.05$). Representative images of larvae at 1 day post hatching after exposure to PTU (C) or TBBPA (F). Photographs show fish from the control group (i and iv), those exposed to 873 mg/L PTU (ii and iii), and those exposed to 793 µg/L TBBPA (v and vi). Blue arrows indicate inflated swim bladders. Red arrow arrows indicate abnormal larval development. Scale bars indicate 1 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

induced by exposure to T3 (Godfrey et al., 2017), PFOA (Godfrey et al., 2017), PFBA (Godfrey et al., 2017), perfluorooctane sulphonate potassium salt (Hagenaars et al., 2014), TDCPP (Godfrey et al., 2017), 2-mercaptobenzothiazole (Stinckens et al., 2016), MMI (Liu and Chan, 2002), and PTU (Stinckens et al., 2020). In Japanese medaka, abnormal swim bladder inflation is also known to be induced by exposure to MMI (Godfrey et al., 2019), TDCPP (Horie et al., 2022b), PFOA (Godfrey et al., 2019), and PFBA (Horie et al., 2022b). Our study shows that exposure to PTU also induces abnormal swim bladder inflation in Japanese medaka, adding to the research base showing that exposure to a wide range of TDCs in both zebrafish and Japanese medaka can lead to abnormal swim bladder inflation and furthermore that swim bladder inflation is a good indicator of TDC exposure.

In fish, thyroid hormone is essential for growth and skeletal development (Shkil et al., 2012). Furthermore, knockdown of deiodinase in zebrafish results in reduced growth (Bagci et al., 2015; Houbrechts et al., 2016). In our study, PTU exposure was associated with normal development of the embryo, but abnormalities were seen in skeletal development after hatching. PTU exposure also resulted in reduced growth rates in our study, which is consistent with results reported for zebrafish (Van Der Ven et al., 2006; Schmidt and Braunbeck, 2011), indicating that PTU inhibits growth in a variety of fishes. Growth inhibition in zebrafish has also been reported for exposure to other TDCs, such as PFOS (in males; Du et al., 2009), tris (2-butoxyethyl) phosphate (Zeng et al., 2018), and perfluorohexanoic acid (Zhang et al., 2022). By contrast, MMI exposure has been associated with a stimulatory effect on growth of fathead minnow (Crane et al., 2006). Taken together, these reports indicate that changes to growth could be a good indicator of TDC exposure. However, we found TBBPA exposure did not affect growth (to 1 dph). This, however, may simply relate to the very short exposure period adopted in our study. Use of the Fish Early-life Stage Toxicity Test (OECD TG 210) with Japanese medaka could help answer this question in the future.

Deficiency in dio2 in zebrafish can result in reduced eye size (Houbrechts et al., 2016), indicating the importance of thyroid hormone for eye development in fishes. To date, several studies in zebrafish have

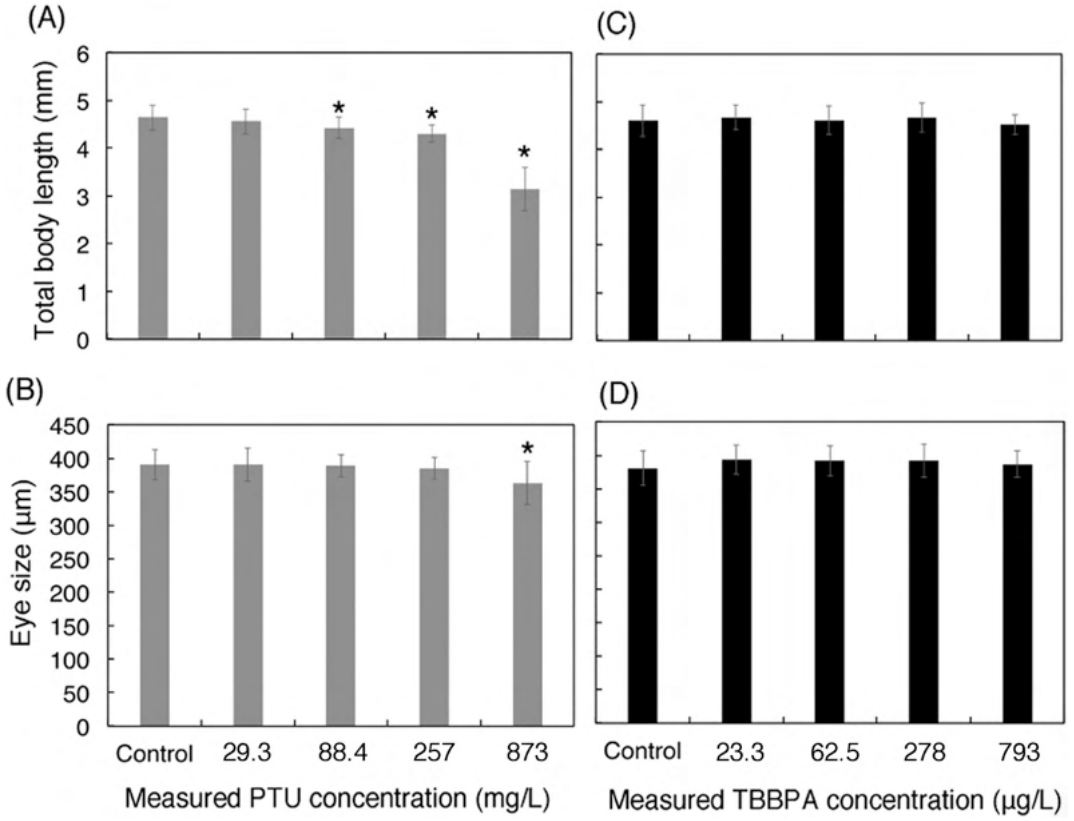

Fig. 5. Effects of PTU (A and B) and TBBPA (C and D) on total body length (A and C) and eye size (B and D) in Japanese medaka at 1 day post hatching. Columns indicate mean values, and error bars show ±SD ($n = 50$). *Significantly different from control (Dunnett's test or Steel's test; $P < 0.05$).

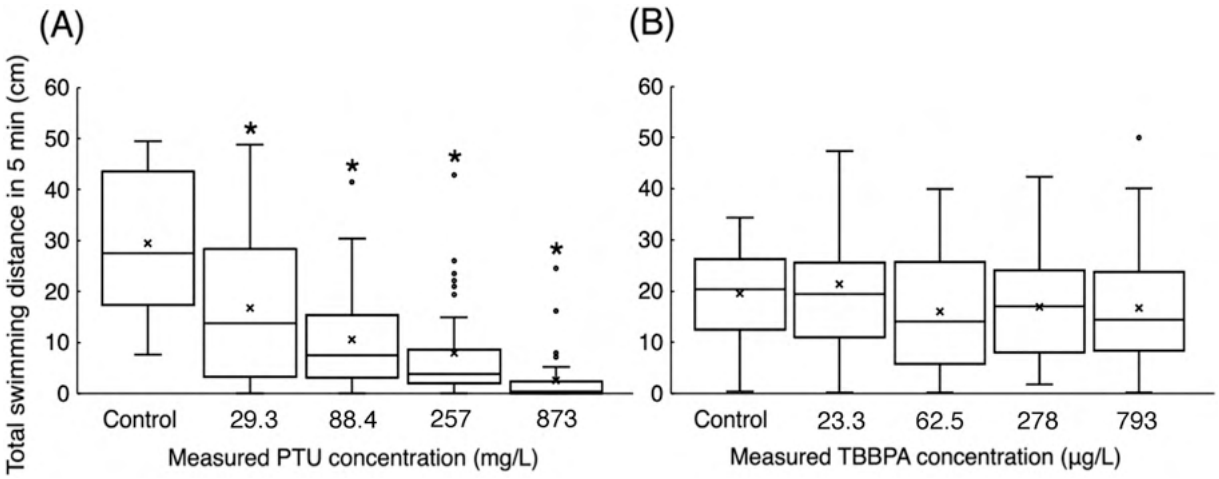

Fig. 6. Effects of PTU (A) and TBBPA (B) on swimming behavior in Japanese medaka at 1 day post hatching. Central horizontal bars indicate means and error bars show ±SD ($n = 32$). *Significantly different from control (Dunnett's test or Steel's test; $P < 0.05$).

identified relationships between reduced eye size and exposure to a range of TDCs, including PTU (Li et al., 2012; Baumann et al., 2016), MMI (Reider and Connaughton, 2014), and TBBPA (Baumann et al., 2016). In our study, PTU exposure was associated with a significant reduction in eye size in Japanese medaka, but TBBPA exposure had no significant effect on the eyes. This suggests that eye development in zebrafish and Japanese medaka responds differently to TDC exposure. Future studies on other TDCs such as PFOA, MMI, or PFBA in Japanese medaka would help clarify the relationship between TDCs and eye development in this species.

TSH-specific β subunit (tshβ) forms one of the two subunits that make up thyroid-stimulating hormone (TSH). There are two paralogs of *tshβ* (*tshβ*, XM_011477157; *tshβ-like*, XM_004068796) listed in the NCBI database for medaka (https://www.ncbi.nlm.nih.gov/) as reported for other teleost fishes (Maugars et al., 2016; Fleming et al., 2019), although the roles of the paralogs are unknown. Baumann et al. (2016) reported that in zebrafish, PTU exposure suppressed *trα* and *trβ* expression, enhanced *dio2* expression, and had no effects on *tsh* expression. In contrast, Liu et al. (2013) reported that PTU exposure had no effect on the expression of *tshβ*, *dio1*, or *dio2* in zebrafish, although the exposure concentrations of PTU were lower than those used by Baumann et al. (2016).

Table 2
Effects of exposure to PTU or TBBPA on swim bladder development, eye size, growth, swimming performance, and the expression of thyroid-related genes in Japanese medaka (this study) and zebrafish (previous studies).

Chemical	Fish	Exposure period (dpf)	Concentration	Swim bladder development	Eye size	Growth	Swimming performance	Expression of thyroid-hormone related genes	References
PTU	Japanese medaka	0–10 (1 dph)	0, 29.3, 88.4, 257, 873 mg/L	257 mg/L ↓	873 mg/L ↓	88.4 mg/L ↓	29.3 mg/L ↓	*tshβ-like* 257 mg/L↑, *tshβ*↔, *dio1*↔, *dio2*↔, *trα*↔, *trβ*↔	This study
	Zebrafish	0–5	0, 50, 100, 250 mg/L	NR	100 mg/L ↓	↓ #	NR	*tsh*↔, *dio1*↔, *dio2* 50 mg/L↑, *dio3* 50 mg/L↓, *trα* 50 mg/L↓, *trβ* 250 mg/L↓	Baumann et al. (2016)
		0–5	0, 0.3, 3, 30 mg/L	NR	NR	NR	NR	*tshβ*↔, *dio1*↔, *dio2*↔	Li et al. (2012)
		0–32	0, 37, 111 mg/L	37 mg/L ↓	NR	NR	111 mg/L ↓	NR	Stinckens et al. (2020)
TBBPA	Japanese medaka	0–9 (1 dph)	0, 23.3, 62.5, 278, 793 μg/L	↔	↔	↔	↔	*tshβ-like* 793 μg/L↑, *tshβ* 278 μg/L↑, *dio1*↔, *dio2*↔, trα 278 μg/L↓*, *trβ* 62.5, 278 μg/L↓*	This study
	Zebrafish	0–5	0, 100, 200, 300, 400 μg/L	NR	200 μg/L↓	↓ #	NR	*tsh*↔, *dio1*↔, *dio2*↔, *dio3*↔, trα 100 μg/L↑, *trβ* ↔	Baumann et al. (2016)
		0–5	0, 0.18, 0.46, 0.92, 1.38, 2.7 μM	NR	NR	NR	NR	*tshβ*↔, *tshr*↔, *dio1*↔, *dio2*↔, *dio3*↔, *trα*↔, *trβ*↔	Parsons et al. (2019)
		0–5	2 μM	NR	NR	NR	2 μM ↓	NR	Chen et al. (2021)

NR, no report; #, concentration not shown; *, not concentration-dependent; ↓, reduced; ↑, increased; ↔, no effect; dpf, days post fertilization; dph, days post hatching.

Our results show that TBBPA exposure increased *tshβ* and *tshβ-like* expression and reduced *trα* and *trβ* expression but did not affect the expression of *dio1* or *dio2*. However, the effects on *tshβ*, *tshβ-like*, *trα*, and *trβ* were not concentration-dependent. In zebrafish, Baumann et al. (2016) and Parsons et al. (2019) have reported that TBBPA exposure does not affect the expression of *tshβ*, *dio1*, *dio2*, *trα*, or *trβ*, whereas Zhu et al. (2018) reported that TBBPA exposure increases *tshβ* expression and suppresses *trβ* expression. Recently our research group reported that PFBA, TDCPP, and T3 exposure significantly affects the expression of *tshβ*, *trα*, and *trβ* in Japanese medaka, but PFBA and TDCPP exposure does not (Horie et al., 2022b). These results suggest that the effects of TDCs on thyroid-hormone related gene expression patterns are inconsistent. However, the reason for this inconsistency remains unexplained.

Our results show that PTU exposure significantly reduced larval swimming activity in Japanese medaka. Several studies have reported similar reductions in zebrafish swimming performance after exposure to TDCs. For example, TBBPA exposure significantly reduced average swimming speed (Zhu et al., 2018; Chen et al., 2021) and distance swum (Chen et al., 2021), and TDCPP exposure reduced swimming activity in zebrafish larvae (Dishaw et al., 2014). MMI, iopanoic acid, and PTU exposure have also all been shown to reduce swimming preforming in zebrafish (Stinckens et al., 2020). Our data suggest that swimming activity could be a useful indicator of TDC exposure, an observation that could be strengthened for Japanese medaka by examining effects on swimming of other TDCs such as PFOA, MMI, and PFBA.

In our study, swimming performance was reduced by PTU exposure even at the lowest and second-lowest exposure concentrations, where no effect on thyroid-related gene expression was detected. Thyroid hormones influence numerous neurodevelopmental processes including neurogenesis, synaptogenesis, and myelination (Bernal, 2022), and neuronal systems control swimming behavior in vertebrates (Mullins et al., 2011). This suggests that the neuronal system is also important for swimming activity. It is generally accepted that PTU shows anti-thyroid activity, leading to a reduction in circulating thyroid hormone levels, and in larval zebrafish, PTU inhibits mitosis of enteric neural crest cells and reduces the number of enteric neurons throughout the intestine (Wang et al., 2020). This indicates that PTU exposure at the lowest and second-lowest concentrations in medaka in our study might have reduced neuronal activity in the brain, which could have caused the observed reduction of swimming activity.

Table 2 shows a comparison of the effect concentrations of PTU and TBBPA for swim bladder inflation, eye development, growth, swimming performance, and the expression of thyroid-hormone related genes between Japanese medaka (the present study) and zebrafish (previous studies). PTU exposure reduced swim bladder inflation, eye development, growth, and swimming performance in both species, but the effect concentrations were higher in Japanese medaka. TBBPA exposure had no effect on swim bladder inflation, eye development, growth, or swimming performance in either Japanese medaka or zebrafish except in one study on zebrafish (Baumann et al., 2016).

The adverse outcome pathway framework, which consists of a molecular initiating event, key event, and adverse outcome, is well suited to the development of tiered testing approaches that seek to provide evidence for the association between perturbations of a toxicological pathway and downstream responses. Noyes et al. (2019) and Knapen et al. (2020) established adverse outcome pathways for TDCs in fishes, where improper inflation of the swim bladder (key event) leads to reduced swimming performance (adverse outcome). Our results on Japanese medaka exposed to PTU are largely consistent with this framework. However, we also observed reductions in swimming activity in exposure groups where improper swim bladder inflation was not observed. In this study, we only assessed the presence or absence of swim bladder inflation at 1 dph and assumed that swim bladder inflation at this time point was related to swimming performance. In the future, therefore, the relationship between the timing of swim bladder inflation and swimming performance after TDC exposure needs to be assessed.

5. Conclusions

Here, we evaluated the influence of two TDCs (PTU and TBBPA) on swim bladder inflation, eye development, growth, swimming behavior, and the expression of thyroid-related genes in Japanese medaka (*O. latipes*). PTU exposure induced changes in *tshβ-like* expression and caused reductions in eye size and growth, and disrupted swim bladder inflation and swimming activity. TBBPA exposure induced changes in the expression of *tshβ-like*, *tshβ*, *trα*, and *trβ*, but had no effect on swim bladder inflation, eye development, growth, or swimming behavior. For PTU exposure, we successfully identified the key event (swim bladder inflation reduction) and the adverse outcome (swimming behavior reduction) in Japanese medaka.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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