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

Yoshifumi Horie[a], Takahiro Yamagishi[b], Jun Yamamoto[c], Mayumi Suzuki[c], Yuta Onishi[c], Takashi Chiba[d], Shinichi Miyagawa[e], Anke Lange[f], Charles R. Tyler[f], Hideo Okamura[a], Taisen Iguchi[g]

[a] Research Center for Inland Seas (KURCIS), Kobe University, Fukaeminami-machi, Higashinada-ku, Kobe 658-0022, Japan
[b] Center for Health and Environmental Risk Research, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan
[c] Institute of Environmental Ecology, IDEA Consultants, Inc., Shizuoka 421-0212, Japan
[d] Department of Environmental and Symbiotic Science, Rakuno Gakuen University, 582 Bunkyodai Midorimachi, Ebetsu, Hokkaido 069-8501, Japan
[e] Department of Biological Science and Technology, Faculty of Advanced Engineering, Tokyo University of Science, Niijuku 6-3-1, Katsushika, Tokyo 125-8585, Japan
[f] Biosciences, College of Life and Environmental Sciences, University of Exeter, Stocker Road, Exeter EX4 4QD, UK
[g] Graudate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan

Correspondence: Yoshifumi Horie, Ph.D., Research Center for Inland Seas (KURCIS), Kobe University, Fukaeminami-machi, Higashinada-ku, Kobe 658-0022, Japan
Tel.: +81-78-431-6357
E-mail: horie@people.kobe-u.ac.jp

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.

Key words: dio, thyroid hormone receptor, thyroid-related gene, tshβ, swim bladder

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

1. Introduction

Much attention has been directed towards 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) 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

97 localizations and regulation (reviewed by Deal and Volkoff, 2020). Dio1 is highly
98 expressed in the liver and is the major converting enzyme that converts T4 to T3
99 (reviewed by Deal and Volkoff, 2020). Parsons et al. (2020) showed widespread and
100 highly dynamic tissue expression of key genes (tsh subunit *β* [*tshβ*], *trα*, *trβ*, *dio1*, *dio2*)
101 in the hypothalamus-pituitary-thyroid (HPT) axis in zebrafish (*Danio rerio*) embryo-
102 larvae, supporting their roles in multiple developmental processes. The responsiveness
103 of these genes to T3 suggests a high vulnerability of thyroid hormone-dependent tissues
104 and physiological processes during early developmental windows to altered thyroid
105 hormone signaling and potential mechanisms of action for TDCs.
106 Recently, Dang et al. (2021) reviewed fish toxicity testing for TDCs, most of
107 which have been conducted using zebrafish. These studies have shown that TDCs,
108 thyroid-peroxidase inhibitor chemicals, and sodium-iodide-symporter inhibitor
109 chemicals all disrupt thyroid-related gene expression including *tshβ*, *trα*, *trβ*, *dio1*, and
110 *dio2* (Li et al., 2012; Baumann et al., 2016; Parsons et al., 2019). These chemicals also
111 alter swim bladder inflation (Godfrey et al., 2017; Stinckens et al., 2020; Horie et al.,
112 2022b), eye development (Baumann et al., 2016), and growth (Baumann et al., 2016) in
113 zebrafish. The Organisation for Economic Co-operation and Development (OECD) has
114 established toxicity-testing guidelines for endocrine-disrupting chemicals on fishes and
115 recommends the use of not only zebrafish but also Japanese medaka (*Oryzias latipes*)
116 (https://www.oecd.org/), which shares many of the advantageous characteristics of
117 zebrafish for use as a model species, including small genome size as well as small body
118 size and short generation time for ease of maintenance in the laboratory.
119 Although many previous studies have reported the effects of TDCs on
120 zebrafish, the influence of TDCs on Japanese medaka are still unclear. To our

121 knowledge, only T3 (Godfrey et al., 2019; Horie et al., 2022b), TDCPP (Godfrey et al.,
122 2019; Horie et al., 2022b), methimazole (MMI; Godfrey et al., 2019), perfluorooctanoic
123 acid (PFOA; Godfrey et al., 2019), PFBA (Godfrey et al., 2019; Horie et al., 2022b),
124 and 2-ethylhexyl-4-methoxycinnamate (Lee et al., 2019) have been found to affect
125 thyroid-related gene expression or swim bladder inflation in Japanese medaka. In our
126 recent studies on the impacts of T3, tris(1,3-dichloro-2-propyl)phosphate (TDCPP), and
127 perfluorobutyric acid (PFBA) on swim bladder inflation and expression of thyroid-
128 related genes *tshβ*, *trα*, and *trβ,* both compounds produced larvae with uninflated swim
129 bladders in both zebrafish and medaka. We also identified changes in expression of
130 thyroid-related genes (*tshβ*, *trα*, and *trβ*), but these effects were different for the
131 different chemicals and between the zebrafish and medaka (Horie et al., 2022b).
132 In this study, we focused on the TDCs 6-propyl-2-thiouracil (PTU) and
133 tetrabromobisphenol A (TBBPA), which have been reported to inhibit swim bladder
134 inflation and disrupt expression of thyroid-related genes in zebrafish. First, we
135 evaluated whether the two chemicals affect eye development, growth, swim bladder
136 inflation, and expression of thyroid-related genes in Japanese medaka. We then
137 compared their toxicity in Japanese medaka against those previously reported for
138 zebrafish.
139
140 **2. Materials and methods**
141 2.1. Test fish and test chemicals
142 The Japanese medaka (*O. latipes*) used in this study were of the R strain from the
143 National Institute for Environmental Studies (NIES). Test fish were supplied from NIES
144 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 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. The residue was then 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.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

193 0.00061 and 0.0016 mg/L, respectively, for PTU and 0.087 and 0.23 μg/L, respectively,
194 for TBBPA. Measured concentrations of PTU and TBBPA in the test solutions are
195 shown in Table 1. The operating conditions in the analyses are provided in
196 Supplementary Table 1.
197
198 2.4. Swim bladder inflation, total body length, and eye size
199 Swim bladder inflation, total body length, and eye size were evaluated in 50 larvae
200 selected at random. The larvae were photographed at 1 dph with a stereomicroscope
201 (SZX 16, OLYMPUS, Tokyo, Japan) and fitted camera (Visualix V900FL, Visualix,
202 Kobe, Japan), and total body length and eye size were measured by using ImageJ
203 software (Schneider et al., 2012) (Fig. 1).
204
205 2.5. Thyroid-related gene expression
206 Our real-time quantitative polymerase chain reaction (RT-qPCR) procedure was as
207 described previously (Horie et al., 2020) (Fig. 1). At 1 dph, larvae were stored in
208 RNAlater (Sigma-Aldrich, St. Louis, MO, USA) and maintained at 4 ℃. The next day,
209 total RNA was extracted from each selected larva using an RNeasy Mini Kit including
210 an on-column RNase-free DNase treatment (Qiagen, Hilden, Germany). Nine larvae
211 were selected for total RNA extraction for each treatment concentration group (i.e., a
212 total of 45 larvae per exposure chemical). After total RNA was extracted, the RNA
213 extraction concentration was measured using a NanoDrop One Microvolume UV-Vis
214 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Next, RNA was
215 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 ℃ 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 by 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 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 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 (R Core Team, 2021) 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* 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 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 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).

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

382 medaka by examining effects on swimming of other TDCs such as PFOA, MMI, and
383 PFBA.
384 In our study, swimming performance was reduced by PTU exposure even at the
385 lowest and second-lowest exposure concentrations, where no effect on thyroid-related
386 gene expression was detected. Thyroid hormones influence numerous
387 neurodevelopmental processes including neurogenesis, synaptogenesis, and myelination
388 (Bernal, 2022), and neuronal systems control swimming behavior in vertebrates
389 (Mullins et al., 2011). This suggests that the neuronal system is also important for
390 swimming activity. It is generally accepted that PTU shows anti-thyroid activity,
391 leading to a reduction in circulating thyroid hormone levels, and in larval zebrafish,
392 PTU inhibits mitosis of enteric neural crest cells and reduces the number of enteric
393 neurons throughout the intestine (Wang et al., 2020). This indicates that PTU exposure
394 at the lowest and second-lowest concentrations in medaka in our study might have
395 reduced neuronal activity in the brain, which could have caused the observed reduction
396 of swimming activity.
397 Table 2 shows a comparison of the effect concentrations of PTU and TBBPA
398 for swim bladder inflation, eye development, growth, swimming performance, and the
399 expression of thyroid-hormone related genes between Japanese medaka (the present
400 study) and zebrafish (previous studies). PTU exposure reduced swim bladder inflation,
401 eye development, growth, and swimming performance in both species, but the effect
402 concentrations were higher in Japanese medaka. TBBPA exposure had no effect on
403 swim bladder inflation, eye development, growth, or swimming performance in either
404 Japanese medaka or zebrafish except in one study on zebrafish (Baumann et al., 2016).

405 The adverse outcome pathway framework, which consists of a molecular
406 initiating event, key event, and adverse outcome, is well suited to the development of
407 tiered testing approaches that seek to provide evidence for the association between
408 perturbations of a toxicological pathway and downstream responses. Noyes et al. (2019)
409 and Knapen et al. (2020) established adverse outcome pathways for TDCs in fishes,
410 where improper inflation of the swim bladder (key event) leads to reduced swimming
411 performance (adverse outcome). Our results on Japanese medaka exposed to PTU are
412 largely consistent with this framework. However, we also observed reductions in
413 swimming activity in exposure groups where improper swim bladder inflation was not
414 observed. In this study, we only assessed the presence or absence of swim bladder
415 inflation at 1 dph and assumed that swim bladder inflation at this time point was related
416 to swimming performance. In the future, therefore, the relationship between the timing
417 of swim bladder inflation and swimming performance after TDC exposure needs to be
418 assessed.
419
420 **5. Conclusions**
421 Here, we evaluated the influence of two TDCs (PTU and TBBPA) on swim bladder
422 inflation, eye development, growth, swimming behavior, and the expression of thyroid-
423 related genes in Japanese medaka (*O. latipes*). PTU exposure induced changes in *tshβ-*
424 *like* expression and caused reductions in eye size and growth, and disrupted swim
425 bladder inflation and swimming activity. TBBPA exposure induced changes in the
426 expression of *tshβ-like*, *tshβ*, *trα*, and *trβ*, but had no effect on swim bladder inflation,
427 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.

Acknowledgments

This study was supported by a grant for UK–Japan Research Cooperation from the Ministry of the Environment, Japan (T.I.). This project is part of the European Union's Horizon 2020 research and innovation program under grant agreement No. 825753 (ERGO). The viewpoints in this manuscript are solely the authors', and the European Union cannot be held responsible for any use that may be made of the information contained therein.

Conflicts of interest

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

References

Bagci, E., Heijlen, M., Vergauwen, L., Hagenaars, A., Houbrechts, A.M., Esguerra, C.V., Blust, R., Darras, V.M., Knapen, D., 2015. Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. PLoS One. 10(4), e0123285. https://doi.org/10.1371/journal.pone.0123285.

Baumann, L., Ros, A., Rehberger, K., Neuhauss, S.C., Segner, H., 2016. Thyroid disruption in zebrafish (*Danio rerio*) larvae: Different molecular response patterns lead to impaired eye development and visual functions. Aquat. Toxicol. 172, 44–55. https://doi.org/10.1016/j.aquatox.2015.12.015.

Bernal, J., 2022. Thyroid Hormones in Brain Development and Function. South Dartmouth (MA): MDText.com, Inc.; 2000-.

Blanton, M.L., Specker, J.L., 2007. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. Crit. Rev. Toxicol. 37, 97–115. https://doi.org/10.1080/10408440601123529.

Brown, D.D., Cai, L., 2007. Amphibian metamorphosis. Devel. Biol. 306 (1), 20–33. https://doi.org/10.1016/j.ydbio.2007.03.021.

Celino-Brady, F.T., Lerner, D.T., Seale, A.P., 2021. Experimental approaches for characterizing the endocrine-disrupting effects of environmental chemicals in fish. Front. Endocrinol. (Lausanne). 11, 619361. https://doi.org/10.3389/fendo.2020.619361.

Chen, J., Li, J., Jiang, H., Yu, J., Wang, H., Wang, N., Chen, S., Mo, W., Wang, P., Tanguay, R.L., Dong, Q., Huang, C., 2021. Developmental co-exposure of TBBPA

and titanium dioxide nanoparticle induced behavioral deficits in larval zebrafish. Ecotoxicol. Environ. Saf. 215, 112176. https://doi.org/ 10.1016/j.ecoenv.2021.112176.

Crane, H.M., Pickford, D.B., Hutchinson, T.H., Brown, J.A., 2006. The effects of methimazole on development of the fathead minnow, *Pimephales promelas*, from embryo to adult. Toxicol. Sci. 93(2), 278–285. htpps://doi.org/ 10.1093/toxsci/kfl063.

Dang, Z., Arena, M., Kienzler, A., 2021. Fish toxicity testing for identification of thyroid disrupting chemicals. Environ. Pollut. 284, 117374. https://doi.org/10.1016/j.envpol.2021.117374.

Deal, C.K., Volkoff, H., 2020. The role of the thyroid axis in fish. Front. Endocrinol. (Lausanne). 11, 596585. https://doi.org/10.3389/fendo.2020.596585.

Dishaw, L.V., Hunter, D.L., Padnos, B., Padilla, S., Stapleton, H.M., 2014. Developmental exposure to organophosphate flame retardants elicits overt toxicity and alters behavior in early life stage zebrafish (*Danio rerio*). Toxicol. Sci. 142(2), 445–454. https://doi.org/10.1093/toxsci/kfu194.

Du, Y., Shi, X., Liu, C., Yu, K., Zhou, B., 2009. Chronic effects of water-borne PFOS exposure on growth, survival and hepatotoxicity in zebrafish: a partial life-cycle test. Chemosphere 74(5), 723-729. https://doi.org/10.1016/j.chemosphere.2008.09.075.

EU, 2010. Directive 2010/63/EU of the European Parliament and Council of 22 September on the protection of animals used for scientific purposes. Off. J. Eur. Union L276, 33–79.

489 Fleming, M.S., Maugars, G., Lafont, A.G., Rancon, J., Fontaine, R., Nourizadeh-
490 Lillabadi, R., Weltzien, F.A., Yebra-Pimentel, E.S., Dirks, R., McCormick, S.D.,
491 Rousseau, K., Martin, P., Dufour, S., 2019. Functional divergence of thyrotropin
492 beta-subunit paralogs gives new insights into salmon smoltification metamorphosis.
493 Sci. Rep. 9(1), 4561. https://doi.org/10.1038/s41598-019-40019-5.
494 Fox, J., Bouchet-Valat, M., 2018. Rcmdr: R Commander. R package version 2.5-1.
495 Godfrey, A., Hooser, B., Abdelmoneim, A., Horzmann, K.A., Freemanc, J.L.,
496 Sepulveda, M.S., 2017. Thyroid disrupting effects of halogenated and next
497 generation chemicals on the swim bladder development of zebrafish. Aquat.
498 Toxicol. 193, 228–235. https://doi.org/10.1016/j.aquatox.2017.10.024.
499 Godfrey, A., Hooser, B., Abdelmoneim, A., Sepúlveda, M.S., 2019. Sex-specific
500 endocrine-disrupting effects of three halogenated chemicals in Japanese medaka. J.
501 Appl. Toxi. 39 (8), 1215–1223. https://doi.org/10.1002/jat.3807.
502 Hagenaars, A., Stinckens, E., Vergauwen, L., Bervoets, L., Knapen, D., 2014. PFOS
503 affects posterior swim bladder chamber inflation and swimming performance of
504 zebrafish larvae. Aquat. Toxicol. 157, 225–35.
505 https://doi.org/ 10.1016/j.aquatox.2014.10.017.
506 Heijlen, M., Houbrechts, A.M., Bagci, E., Van Herck, S.L., Kersseboom, S., Esguerra,
507 C.V., Blust, R., Visser, T.J., Knapen, D., Darras, V.M., 2014. Knockdown of type 3
508 iodothyronine deiodinase severely perturbs both embryonic and early larval
509 development in zebrafish. Endocrinology 155(4), 1547–1559.
510 https://doi.org/10.1210/en.2013-1660.
511 Horie, Y., Kanazawa, N., Takahashi, C., Tatarazako, N., Iguchi, T., 2021. Exposure to
512 4-nonylphenol induces a shift in the gene expression of gsdf and testis-ova

formation and sex reversal in Japanese medaka (*Oryzias latipes*). J. Appl. Toxicol. 41(3), 399–409. https://doi.org/10.1002/jat.4051.

Horie, Y., Kanazawa, N., Takahashi, C., Tatarazako, N., Iguchi, T., 2022a. *Gonadal soma-derived factor* expression is a potential biomarker for predicting the effects of endocrine-disrupting chemicals on gonadal differentiation in Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 41(8), 1875-1884. https://doi.org/10.1002/etc.5353.

Horie, Y., Nomura, M., Okamoto, K., Takahashi, C., Sato, T., Miyagawa, S., Okamura, H., Iguchi, T., 2022b. Effect of thyroid hormone-disrupting chemicals on swim bladder inflation and thyroid hormone-related gene expression in Japanese medaka and zebrafish. J. Appl. Toxicol. 42(8), 1385-1395. https://doi.org/10.1002/jat.4302.

Houbrechts, A.M., Delarue, J., Gabriëls, I.J., Sourbron, J., Darras, V.M., 2016. Permanent deiodinase type 2 deficiency strongly perturbs zebrafish development, growth, and fertility. Endocrinology 157(9), 3668–3681. https://doi.org/10.1210/en.2016-1077.

Kawashima, Y., Onishi, Y., Tatarazako, N., Yamamoto, H., Koshio, M., Oka, T., Horie, Y., Watanabe, H., Nakamoto, T., Yamamoto, J., Ishikawa, H., Sato, T., Yamazaki, K., Iguchi, T., 2022. Summary of 17 chemicals evaluated by OECD TG229 using Japanese Medaka, *Oryzias latipes* in EXTEND 2016. J. Appl. Toxicol. 42(5), 750–777. https://doi.org/10.1002/jat.4255.

Knapen, D., Stinckens, E., Cavallin, J.E., Ankley, G.T., Holbech, H., Villeneuve, D.L., Vergauwen, L., 2020. Toward an AOP network-based tiered testing strategy for the assessment of thyroid hormone disruption. Environ. Sci. Technol. 54(14), 8491–8499. https://doi.org/10.1021/acs.est.9b07205.

537 Lee, S., Kim, C., Shin, H., Kho, Y., Choi, K., 2019. Comparison of thyroid hormone
538 disruption potentials by bisphenols A, S, F, and Z in embryo-larval zebrafish.
539 Chemosphere 221, 115–123. https://doi.org/10.1016/j.chemosphere.2019.01.019.
540 Li, Z., Ptak, D., Zhang, L., Walls, E.K., Zhong, W., Leung, Y.F., 2012. Phenylthiourea
541 specifically reduces zebrafish eye size. PLoS One 7(6), e40132.
542 https://doi.org/10.1371/journal.pone.0040132.
543 Liu, Y.W., Chan, W.K., 2002. Thyroid hormones are important for embryonic to larval
544 transitory phase in zebrafish. Differentiation 70(1), 36–45.
545 https://doi.org/10.1046/j.1432-0436.2002.700104.x.
546 Liu, C., Yu, H., Zhang, X., 2013. Zebrafish embryos/larvae for rapid determination of
547 effects on hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-
548 interrenal (HPI) axis: mRNA expression. Chemosphere 93(10), 2327–2332.
549 https://doi.org/10.1016/j.chemosphere.2013.08.026.
550 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using
551 real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25 (4),
552 402–408. https://doi.org/10.1006/meth.2001.1262.
553 Maugars, G., Dufour, S., Cohen-Tannoudji, J., Quérat, B., 2016. Multiple thyrotropin β-
554 subunit and thyrotropin receptor-related genes arose during vertebrate evolution.
555 PLoS One 9(11), e111361. https://doi.org/10.1371/journal.pone.0111361.
556 Mullins, O.J., Hackett, J.T., Buchanan, J.T., Friesen, W.O., 2011. Neuronal control of
557 swimming behavior: comparison of vertebrate and invertebrate model systems.
558 Prog. Neurobiol. 93(2), 244–269. https://doi.org/ 10.1016/j.pneurobio.2010.11.001.
559 Mullur, R., Liu, Y.Y., Brent, G.A., 2014. Thyroid hormone regulation of metabolism.
560 Physiol. Rev. 94(2), 355–382. https://doi.org/10.1152/physrev.00030.2013.

561 Noyes, P.D., Friedman, K.P., Browne, P., Haselman, J.T., Gilbert, M.E., Hornung,
562 M.W., Barone, S.Jr., Crofton, K.M., Laws, S.C., Stoker, T.E., Simmons, S.O.,
563 Tietge, J.E., Degitz, S.J., 2019. Evaluating chemicals for thyroid disruption:
564 opportunities and challenges with in vitro testing and adverse outcome pathway
565 approaches. Environ. Health Perspect. 127(9), 95001.
566 https://doi.org/10.1289/EHP5297.
567 Onishi, Y., Tatarazako, N., Koshio, M., Watanabe, H., Okamura, T., Sawai, A.,
568 Yamamoto, J., Ishikawa, H., Sato, T., Kawashima, Y., Yamazaki, K. and Iguchi, T.
569 2021. Summary of reference chemicals evaluated by the Fish Short-Term
570 Reproduction Assay, OECD TG229, using Japanese medaka, *Oryzias latipes*. J.
571 Appl. Toxicol. 41(8), 1200-1221. https://doi.org/10.1002/jat.4104.
572 Ortiga-Carvalho, T.M., Sidhaye, A.R., Wondisford, F.E., 2014. Thyroid hormone
573 receptors and resistance to thyroid hormone disorders. Nat. Rev. Endocrinol. 10(10),
574 582–591. https://doi.org/10.1038/nrendo.2014.143.
575 Parsons, A., Lange, A., Hutchinson, T.H., Miyagawa, S., Iguchi, T., Kudoh, T., Tyler,
576 C.R., 2019. Molecular mechanisms and tissue targets of brominated flame
577 retardants, BDE-47 and TBBPA, in embryo-larval life stages of zebrafish (*Danio*
578 *rerio*). Aquat. Toxicol. 209, 99–112. https://doi.org/10.1016/j.aquatox.2019.01.022.
579 Parsons, A., Lange, A., Hutchinson, T.H., Miyagawa, S., Iguchi, T., Kudoh, T. and
580 Tyler, C.R. 2020. Expression dynamics of genes in the hypothalamic-pituitary-
581 thyroid (HPT) cascade and their responses to 3,5,3′-tri-iodothyronine highlights
582 potential vulnerability to thyroid-disrupting chemicals in zebrafish *(Danio rerio*)
583 embryo-larvae. Aquat. Toxicol. 225, 105547.
584 https://doi.org/10.1016/j.aquatox.2020.105547.

585 Reider, M., Connaughton, V.P., 2014. Effects of low-dose embryonic thyroid disruption
586 and rearing temperature on the development of the eye and retina in zebrafish. Birth
587 Defec. Res. B. Dev. Reprod. Toxicol. 101(5), 347–354.
588 https://doi.org/10.1002/bdrb.21118.
589 Schmidt, F., Braunbeck, T., 2011. Alterations along the hypothalamic-pituitary-thyroid
590 axis of the zebrafish (*Danio rerio*) after exposure to propylthiouracil. J. Thyroid
591 Res. 2011, 376243. https://doi.org/10.4061/2011/376243.
592 Shkil, F.N., Kapitanova, D.V., Borisov, V.B., Abdissa, B., Smirnov, S.V., 2012.
593 Thyroid hormone in skeletal development of cyprinids: effects and morphological
594 consequences: Thyroid hormone in skeletal development. J. Appl. Ichthyol. 28,
595 398–405. https://doi.org/10.1111/j.1439-0426.2012.01992.x
596 Stinckens, E., Vergauwen, L., Schroeder, A.L., Maho, W., Blackwell, B.R., Witters, H.,
597 Blust, R., Ankley, G.T., Covaci, A., Villeneuve, D.L., Knapen, D., 2016. Impaired
598 anterior swim bladder inflation following exposure to the thyroid peroxidase
599 inhibitor 2-mercaptobenzothiazole part II: Zebrafish. Aquat. Toxicol. 173, 204–217.
600 https://doi.org/10.1016/j.aquatox.2015.12.023.
601 Stinckens, E., Vergauwen, L., Blackwell, B.R., Ankley, G.T., Villeneuve, D.L.,
602 Knapen, D., 2020. Effect of thyroperoxidase and deiodinase inhibition on anterior
603 swim bladder inflation in the zebrafish. Environ. Sci. Technol. 54(10), 6213–6223.
604 https://doi.org/10.1021/acs.est.9b07204.
605 Szkudlinski, M.W., Fremont, V., Ronin, C., Weintraub, B.D., 2002. Thyroid-
606 stimulating hormone and thyroid-stimulating hormone receptor structure-function
607 relationships. Physiol. Rev. 82(2), 473–502.
608 https://doi.org/10.1152/physrev.00031.2001.

609 Thambirajah, A.A., Koide, E.M., Imbery, J.J., Helbing, C.C., 2019. Contaminant and
610 environmental influences on thyroid hormone action in amphibian metamorphosis.
611 Front. Endocrinol. (Lausanne), 10, 276. https://doi.org/10.3389/fendo.2019.00276.
612 Van, Der, Ven, L.T., van, Den, Brandhof, E.J., Vos, J.H., Power, D.M., Wester, P.W.,
613 2006. Effects of the antithyroid agent propylthiouracil in a partial life cycle assay
614 with zebrafish. Environ. Sci. Technol. 40(1), 74–81.
615 https://doi.org/10.1021/es050972c.
616 Vergauwen, L., Cavallin, J.E., Ankley, G.T., Bars, C., Gabriels, I.J., Michiels, E.D.G.,
617 Fitzpatrick, K.R., Periz-Stanacev, J., Randolph, E.C., Robinson, S.L., Saari, T.W.,
618 Schroeder, A.L., Stinckens, E., Swintek, J., Van Cruchten, S.J., Verbueken, E.,
619 Villeneuve, D.L., Knapen, D., 2018. Gene transcription ontogeny of hypothalamic-
620 pituitary-thyroid axis development in early-life stage fathead minnow and zebrafish.
621 Gen. Comp. Endocrinol. 266, 87–100. https:// doi.org/10.1016/j.ygcen.2018.05.001.
622 Wang, F., Jing, P., Zhan, P., Zhang, H., 2020. Thyroid hormone in the pathogenesis of
623 congenital intestinal dysganglionosis. Pediatr. Dev. Pathol. 23(4), 285–295.
624 https://doi.org/ 10.1177/1093526620908984.
625 Zeng, X., Sun, H., Huang, Y., Liu, J., Yu, L., Liu, C., Wang, J., 2018. Effects of
626 environmentally relevant concentrations of tris (2-butoxyethyl) phosphate on growth
627 and transcription of genes involved in the GH/IGF and HPT axes in zebrafish
628 (*Danio rerio*). Chemosphere 212, 376–384.
629 https://doi.org/10.1016/j.chemosphere.2018.08.102.
630 Zhang, S., Guo, X., Lu, S., He, J., Wu, Q., Liu, X., Han, Z., Xie, P., 2022.
631 Perfluorohexanoic acid caused disruption of the hypothalamus-pituitary-thyroid axis

in zebrafish larvae. Ecotoxicol. Environ. Saf. 232, 113283. https://doi.org/10.1016/j.ecoenv.2022.113283.

Zhu, B., Zhao, G., Yang, L., Zhou, B., 2018. Tetrabromobisphenol A caused neurodevelopmental toxicity via disrupting thyroid hormones in zebrafish larvae. Chemosphere 197, 353–361. https://doi.org/10.1016/j.chemosphere.2018.01.080.

Zimova, M., Hackländer, K., Good, J.M., Melo-Ferreira, J., Alves, P.C., Mills, L.S., 2018. Function and underlying mechanisms of seasonal colour moulting in mammals and birds: what keeps them changing in a warming world? Biol. Rev. Camb. Philos. Soc. 93(3), 1478–1498. https://doi.org/10.1111/brv.12405.

642 **Figures**

643 **Fig. 1.** Experimental flow chart. dpf, days post fertilization; dph, days post hatching;
644 TL, total length. Orange arrow indicates inflated swim bladder. Green arrow indicates
645 no inflation of swim bladder.
646
647 **Fig. 2.** Effect of PTU exposure on mRNA expression of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*,
648 and *trβ* in Japanese medaka at 1 day post hatching as measured by real-time quantitative
649 PCR analysis. The expression levels of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ* were
650 normalized first against that of the *ef1α* housekeeping gene and then against controls.
651 *Significantly different from control (Dunnett's test or Steel's test; $P < 0.05$).
652
653 **Fig. 3.** Effect of TBBPA on mRNA expression of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ*
654 in Japanese medaka at 1 day post hatching as measured by real-time quantitative PCR
655 analysis. The expression levels of *tshβ-like*, *tshβ*, *dio1*, *dio2*, *trα*, and *trβ* were
656 normalized first against that of the *ef1α* housekeeping gene and then against controls.
657 *Significantly different from control (Dunnett's test or Steel's test; $P < 0.05$).
658
659 **Fig. 4.** Effects of PTU (A–C) and TBBPA (D–F) on swim bladder inflation (A and D)
660 and abnormal larval development (B and E) in Japanese medaka at 1 day post hatching.
661 Numbers above each bar indicate the number of individuals with inflated swim bladders
662 or abnormal larval development. *Significantly different from control (Chi-squared test;
663 $P < 0.05$). Representative images of larvae at 1 day post hatching after exposure to PTU
664 (C) or TBBPA (F). Photographs show fish from the control group (i and iv), those
665 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.

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; $P < 0.05$).

Supplementary Fig. 1. Flow chart summarizing the experimental procedure for swimming behavior assay.

Supplementary Fig. 2. Effects of PTU (A) and TBBPA (B) on embryo development in Japanese medaka, as assessed by average hatching day (columns) and hatching rate (lines). Error bars show ± SD (n = 4).

685 **Table**

686 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

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

688 Table 2. Effects of exposure to PTU or TBBPA on swim bladder development, eye size, growth, swimming performance, and the

689 expression of thyroid-related genes in Japanese medaka (this study) and zebrafish (previous studies).

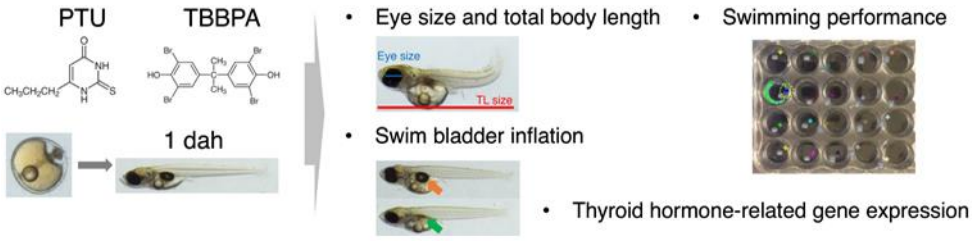
Chemical	Fish	Exposure period (dpf)	Concentration	Swim bladder development	Eye size	Growth	Swimming performance	Expression of thyroid-hormone related genes	References
PTU	Japanese medaka	0–10 (1 dph)	0, 29.3, 88.4, 257, 873 mg/L	257 mg/L ↓	873 mg/L ↓	88.4 mg/L ↓	29.3 mg/L ↓	*tshβ-like* 257 mg/L↑, *tshβ*↔, *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* 278µg/L↑, *dio1*↔, *dio2*↔, trα 278 µg/L↓*,	This study

							trβ 62.5, 278 μg/L↓*	
	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)
zebrafish	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)

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

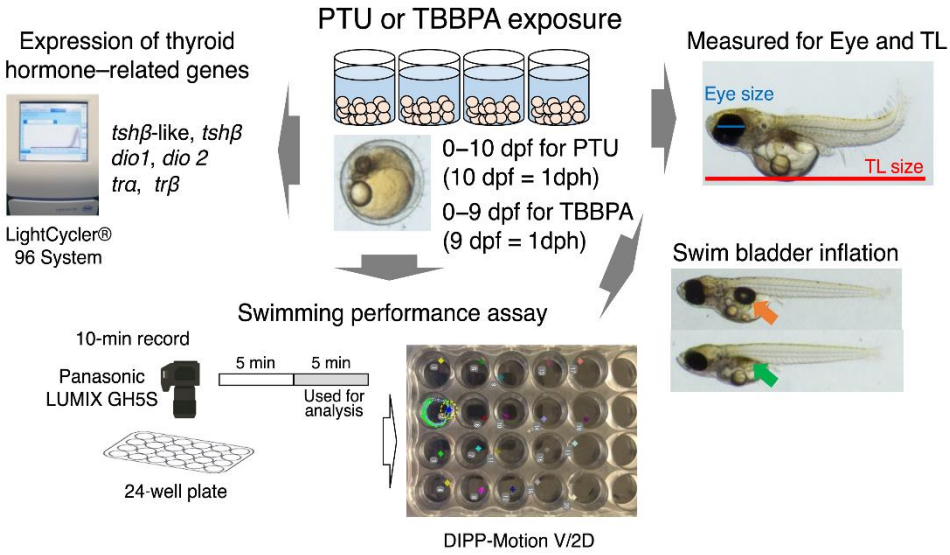
691 fertilization; dph, days post hatching

692 Graphical Abstract



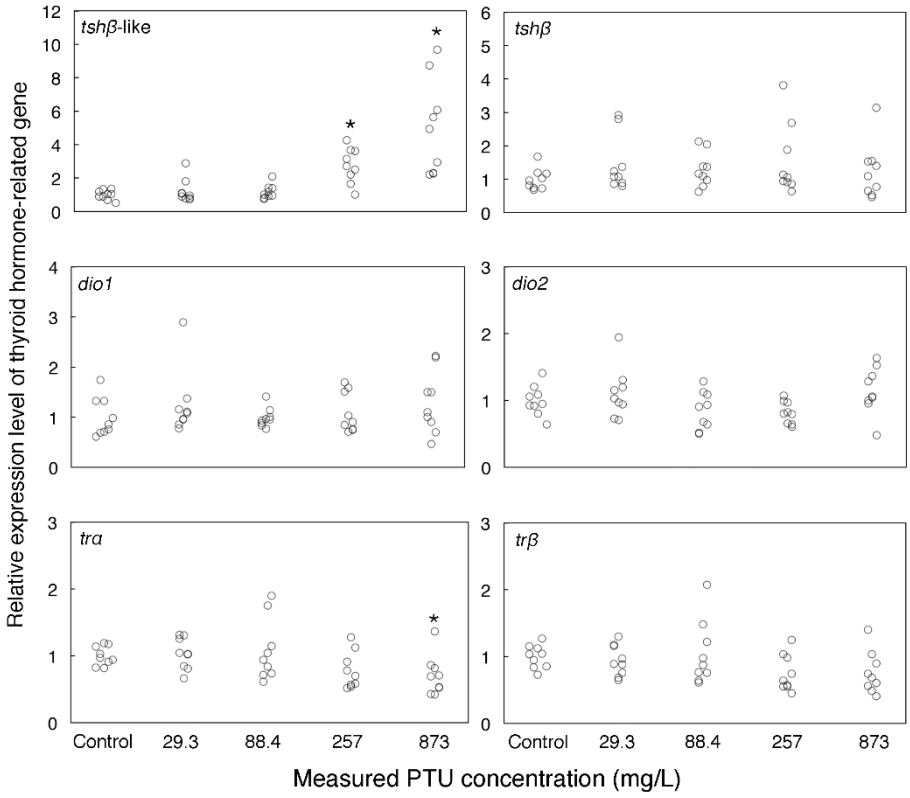
693

694 Figure 1



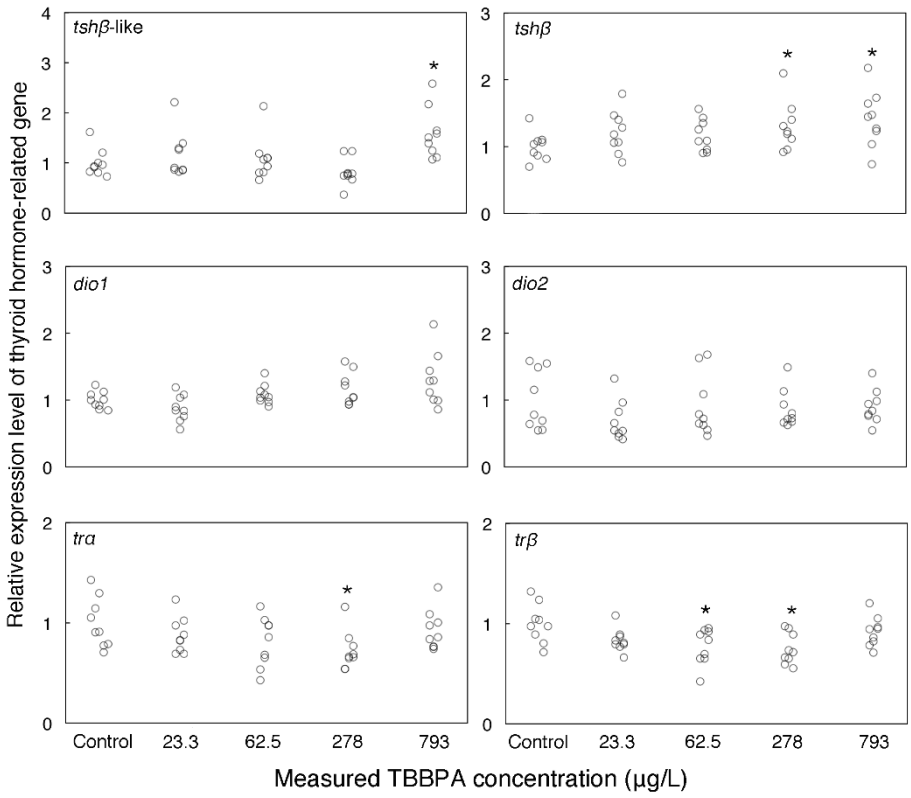
695

696 Figure 2



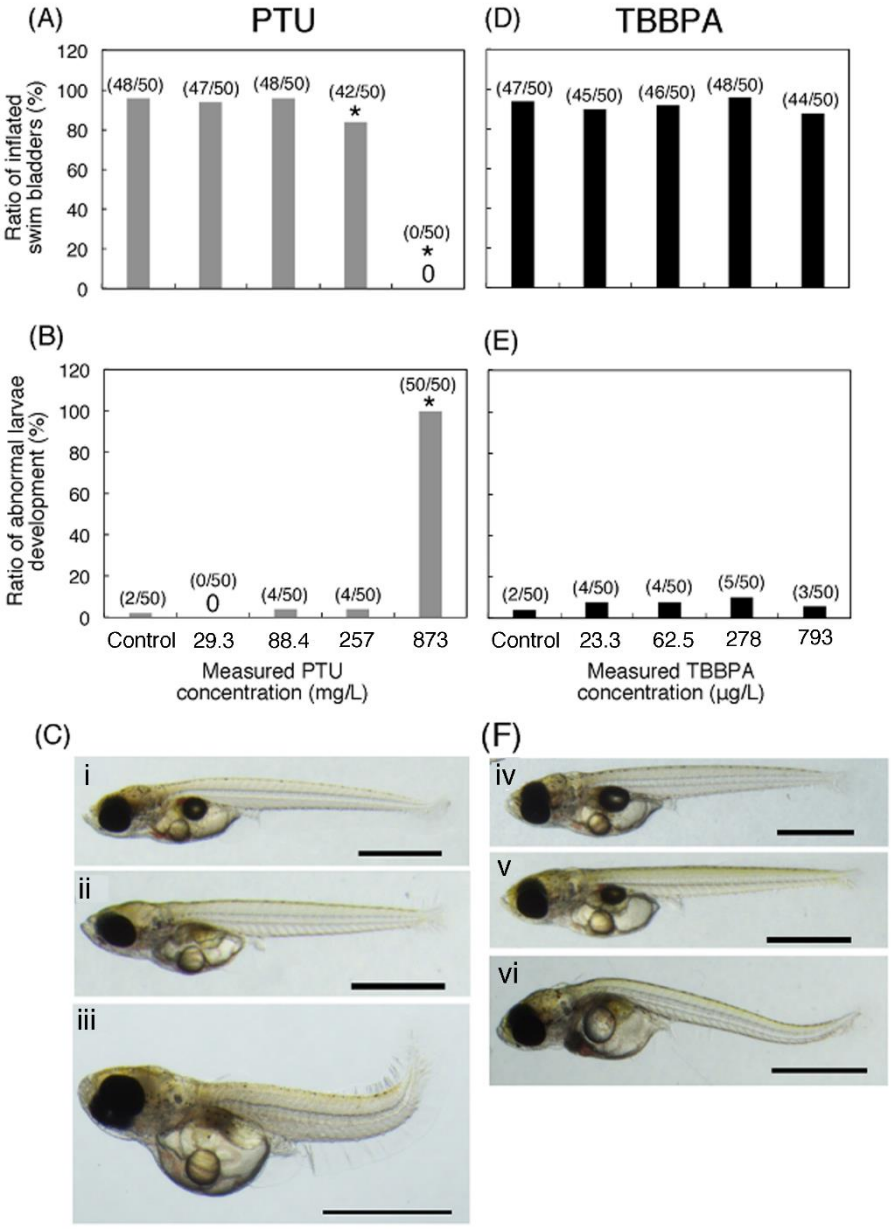
697

698 Figure 3



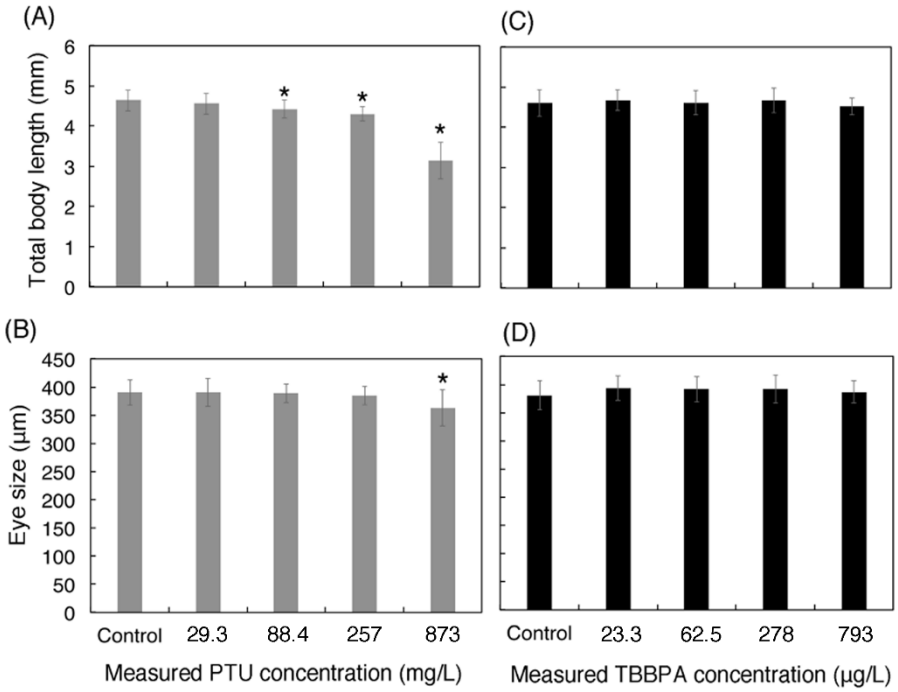
699

700 Figure 4

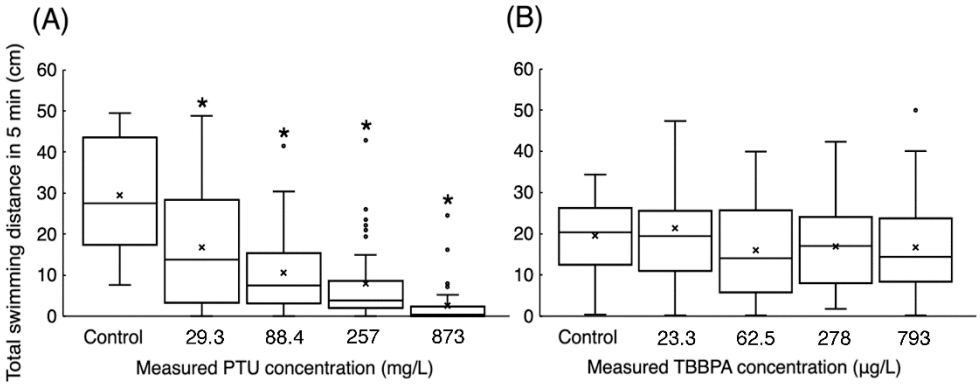


701

Figure 5

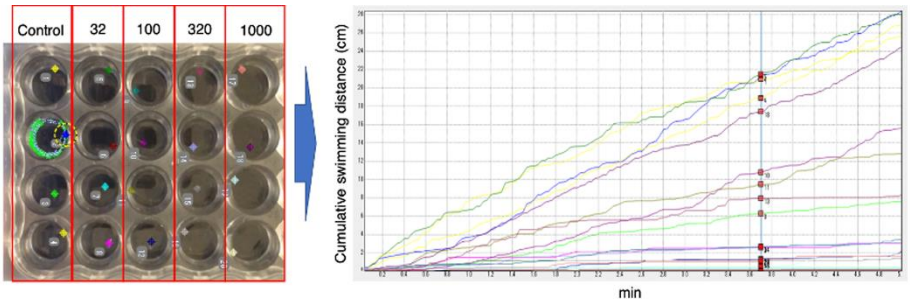

(A)
(C)
Total body length (mm)
0
1
2
3
4
5
6
*
*
*
(B)
(D)
Eye size (μm)
0
50
100
150
200
250
300
350
400
450
*
Control
29.3
88.4
257
873
Measured PTU concentration (mg/L)
Control
23.3
62.5
278
793
Measured TBBPA concentration (μg/L)

704 Figure 6



705

706 Supplementary Figure 1.



707

Supplementary Figure 2.