Molecular mechanisms and tissue targets of brominated flame retardants, BDE-47 and TBBPA, in embryo-larval life stages of zebrafish (*Danio rerio*).

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Abstract

Brominated flame retardants are known to disrupt thyroid hormone (TH) homeostasis in several vertebrate species, but the molecular mechanisms underlying this process and their effects on TH-sensitive tissues during the stages of early development are not well characterised. In this study, we exposed zebrafish (*Danio rerio*) embryo-larvae to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and tetrabromobisphenol A (TBBPA) via the water for 96 h from fertilisation and assessed for lethality, effects on development and on the expression of a suite of genes in the hypothalamic-pituitary-thyroid (HPT) axis via both real time quantitative PCR (qRT-PCR) on whole body extracts and whole mount *in situ* hybridisation (WISH) to identify tissue targets. The 96-h lethal median concentration (96h-LC_{50}) for TBBPA was 0.9 μM and mortality was preceded by retardation of development (smaller animals) and morphological deformities including, oedemas in the pericardial region and tail, small heads, swollen yolk sac extension. Exposure to BDE-47 did not affect zebrafish embryo-larvae survival at any of the concentrations tested (1 – 100 μM) but caused yolk sac and craniofacial deformities, a curved spine and shorter tail at the highest exposure concentration. TBBPA exposure resulted in higher levels of mRNAs for genes encoding deiodinases (*dio1*), transport proteins (*ttr*), the thyroid follicle synthesis protein paired box 8 (*pax8*) and glucuronidation enzymes (*ugt1ab*) and lower levels of *dio3b* mRNAs in whole body extracts, with responses varying with developmental stage. BDE-47 exposure resulted in higher levels of *thrb, dio1, dio2, pax8* and *ugt1ab* mRNAs and lower levels of *ttr* mRNAs in whole body extracts. TBBPA and BDE-47 therefore appear to disrupt the TH system at multiple levels, increasing TH conjugation and clearance, disrupting thyroid follicle development and altering TH transport. Compensatory responses in TH production/ metabolism by deiodinases

were also evident. WISH analyses further revealed that both TBBPA and BDE-47 caused tissue-specific changes in thyroid receptor and deiodinase enzyme expression, with the brain, liver, pronephric ducts and craniofacial tissues appearing particularly responsive to altered TH signalling. Given the important role of TRs in mediating the actions of THs during key developmental processes and deiodinases in the control of peripheral TH levels, these transcriptional alterations may have implications for TH sensitive target genes involved in brain and skeletal development. These findings further highlight the potential vulnerability of the thyroid system to disruption by BFRs during early developmental windows.

Keywords: brominated flame retardant, thyroid, endocrine, fish, toxicology, development

1. Introduction

Over the last two decades, there has been growing concern over the levels of brominated flame retardants (BFRs) in the environment due to their ability to disrupt the thyroid hormone (TH) system [1]. THs play a key role in a wide range of vertebrate physiological functions both during early development and in adult life stages, including influencing the maturation of bones [2], the gonads [3] and the central nervous system [4]. Given the detrimental effects that can result from subtle changes in TH status, particularly during crucial developmental windows [5], even relatively low environmental levels of BFRs pose a potential risk to the health of humans and wildlife.

BFRs have been commercially important high production compounds since their introduction to the global market in the 1970s and are used routinely in industrial and consumer products in an effort to reduce fire-related injury and property damage [6]. Polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA) are amongst the most extensively used BFRs worldwide [6]. PBDEs are a family of 209 possible congeners that can be divided into 10 congener groups (mono- to decabromodiphenyl ethers). Commercial PBDE mixtures are made up of congeners with varying numbers of bromine atoms on their two phenyl rings and are classified according to their average bromine content; penta-, octa-, and deca-BDEs. The congener BDE-47 (2,2′,4,4′-tetrabromodiphenyl ether) was one of the main components of the now banned commercial penta-BDE mixtures, accounting for approximately 40% of the product by weight [6, 7].

Since PBDEs are blended physically rather than bonded chemically to the polymer, they subsequently migrate into the environment and over the past four decades they have become ubiquitous environmental contaminants. BDE-47 is often the most commonly detected PBDE congener in the aqueous phase of environmental water samples [8] and often dominates the PBDE profiles of human tissues, marine mammals, birds and birds eggs, invertebrates and fish [9-13]. The production and usage of penta-BDE commercial mixtures were prohibited globally in 2004 and officially labelled as Persistent Organic Pollutants (POPs) in 2009 [14]. Controls on the use of the penta-BDE products are only just beginning to yield declines in the lower brominated congener concentrations in environmental samples [15]. Nonetheless, global contamination occurs today as a result of the continued use and disposal of older products, manufactured prior to the restrictions, which still contain PBDEs. In 2016, over a decade since the introduction of restrictions, there is a continued presence of penta-BDE constituents in indoor air and dust [16] and they remain the dominant PBDEs detected in both human samples [up to 141 µg/kg lipid weight (lw) in placental tissue] and wildlife samples (up to 1 mg/kg lw in freshwater fish) [17-20].

TBBPA is used as a reactive and additive flame retardant in printed circuit boards and electronic enclosures, respectively. It is the most widely used BFR (representing approximately 60% of the total BFR market) [6], with global production estimated to be over 200,000 metric tonnes a year [21]. TBBPA has been identified in dust, sewage sludge, river sediments and the water phase of lakes and rivers [22-26]. Despite TBBPA's relatively short half-life [27] which suggests little potential for bioaccumulation, it has been detected globally, in some cases at high levels, in many

biotic samples including human breast milk and plasma (up to 37.3 μg/kg lw) [28], suggesting both recent and continuous use.

Several BFRs are structurally similar to thyroxine (T4), the precursor of the biologically active TH 3,3′,5-triiodo-L-thyronine (T3), and concerns have been raised about their effect on the thyroid system of both mammalian and non-mammalian vertebrates [1, 29]. Indeed, numerous *in vivo* studies have shown that both TBBPA and PBDEs can alter the circulating levels of both T4 and T3 in a range of vertebrate species, including fish [30, 31]. The thyroidal system is centrally driven by the hypothalamic-pituitary-thyroid (HPT) axis and under peripheral control in all vertebrates. The HPT axis is regulated as a negative feedback mechanism in which the level of thyroid-stimulating hormone (TSH) secreted by the pituitary controls the production and release of T4 by the thyroid follicles. In the peripheral system, TH activity is tightly regulated by the metabolising enzymes iodothyronine deiodinases, type I, II and III (D1, D2 and D3), which can modulate TH signalling in individual tissues as well as controlling serum TH concentrations. D2 catalyses the outer ring deiodination (ORD) of T4 to produce the bioactive T3. In contrast, D3 catalyses the inner ring deiodination (IRD) of T4 and T3 producing the inactive metabolites reverse T3 (rT3) and 3, 3′-diiodo-L-thyronine (T2), respectively. D1 is a kinetically inefficient enzyme that is capable of catalysing both IRD and ORD [32, 33].

Until recently, evaluating thyroid disruption by environmental chemicals mainly relied on measures of circulating TH levels, thyroid size or histopathology. It is important, however, to note that the thyroid system can maintain normal physiological functions in response to TH level perturbations by changing the production and/or metabolism

of THs by the thyroid gland itself, in target tissues or in the liver [34, 35]. Thyroid-disrupting chemicals (TDCs) may not cause obvious changes to TH levels, but may nonetheless alter TH homeostasis. Consequently, identifying tissue-specific responses to environmental contaminants is fundamental to building our understanding on the effect mechanisms of BFRs. Several studies have demonstrated that BFRs can elicit localised effects on the expression of thyroid related genes in adult fathead minnows (*Pimephales promelas*) and Chinese rare minnows (*Gobiocypris rarus)* [30, 36], however isolating organs and/or tissues of interest from embryos and larvae for studies on gene expression is more challenging. Whole mount *in situ* hybridisation (WISH), however, allows for sites of expression of target genes to be detected in whole zebrafish embryo-larvae and this approach has been used to illustrate a significant up-regulation of deiodinase encoding *dio1* mRNA levels in the periventricular region of the brain and *dio3b* mRNA in the pronephric ducts of zebrafish embryos exposed to the hydroxylated metabolite 6-OH-BDE-47 [37].

The overall aim of this study was to assess the toxicological effects of two important BFRs, TBBPA and BDE-47, on zebrafish during early development. The first objective was to examine the acute toxicity of both compounds on zebrafish embryo-larvae, in terms of induced mortalities and deformities, in order to calculate lethal and effect concentration (LC and EC values). The second objective was to examine the effect of these compounds on the expression of a suite of genes in the HPT axis, with the goal of highlighting potential effect mechanisms and target tissues of thyroid disruption. Given the plasticity of the TH system, we further undertook to examine the effects of TBBPA across different developmental life stages. We used a

combination of real time quantitative PCR (qRT-PCR) assays to quantify changes in gene transcript levels in whole body extracts and WISH to assess changes in tissue gene expression patterns.

2. Materials and Methods

2.1 Materials and reagents

Tetrabromobisphenol A (TBBPA; CAS 79-94-7; purity 97%) and 3,3',5-Triiodo-L-thyronine (T3; CAS 6893-02-3; purity ≥ 95%) were purchased from Sigma-Aldrich (Gillingham, UK). 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47; purity 99.8%) was provided by Ulrika Winnberg, Jorke Kamstra and Kees Swart on behalf of Dr. Juliette Legler from VU University Amsterdam, The Netherlands. Stock solutions of chemicals were prepared by dissolving them in dimethylsulfoxide (DMSO).

2.2 Zebrafish maintenance

Adult zebrafish [*casper* (*mitfa; roy*) mutant strain] were obtained from breeding stocks at the University of Exeter. The *casper* mutants lack melanocytes and iridophores, thus facilitating the visualisation of gene expression via WISH. Fish were maintained at 28 ± 1 °C in a 12:12 h light: dark cycle in a closed flow-through system. Embryos were collected approximately 1 hour post fertilisation (hpf) from breeding colonies, washed twice with embryo culture water with the addition of methylene blue (10^{-5} %) to prevent fungal growth [38]. We found no effects of methylene blue on thyroid signalling in fish reported in the literature. Eggs were incubated in culture water without methylene blue. Embryo culture water was aerated artificial freshwater made according to the ISO-7346/3 guidelines (ISO water

diluted 1:5, pH 6.5-7.5, air saturation 95-100%) [39]. Embryos were examined under a stereomicroscope and only those fertilised were selected for subsequent experimental work. All fish were maintained under approved protocols, according to the UK Home Office regulations for the use of animals in scientific procedures.

2.3 Acute toxicity of TBBPA and BDE-47

TBBPA exposures were carried out at 0, 0.18, 0.46, 0.92, 1.38 and 2.7 μM (in DMSO, not exceeding 0.01% of the culture medium). BDE-47 exposures were carried out at 0, 1, 5, 10, 50 and 100 μM (in DMSO, not exceeding 0.1% of the culture medium). Controls were incubated in DMSO at 0.01% (TBBPA) and 0.1% (BDE-47). BDE-47 has a low water solubility hence the requirement for a higher DMSO concentration. Twenty fertilised embryos were were randomly allocated into glass tanks containing 50 ml of each treatment concentration and half of the exposure solutions were replaced every 24 h with freshly prepared solutions. Exposures were conducted for 96 h starting from approximately 1-2 hpf. The number of dead embryo-larvae and phenotypic deformities (compared to normal development [40]) were recorded every 24 h before removing any dead embryo-larvae. Experiments were carried out in triplicate and repeated three times.

2.4 Effect of TBBPA and BDE-47 on gene transcripts in the HPT axis

TBBPA exposures were carried out at concentrations of 0, 0.04, 0.18 and 0.46 μM (in DMSO, not exceeding 0.01% of the culture medium) and BDE-47 exposures were carried out at concentrations of 0, 0.1, 1 and 10 μM (in DMSO, not exceeding 0.1% of the culture medium). Again, a higher DMSO concentration was used in the BDE-47 exposure due to its low water solubility. These concentrations were chosen

based on range-finding tests to determine concentrations that were sub-lethal and they are lower than, or similar to, the concentrations used in previous studies reported in the literature [41, 42]. Control groups were incubated in DMSO at 0.01% (TBBPA) and 0.1% (BDE-47). Fifty fertilised embryos were randomly allocated into glass tanks containing 50 ml of each treatment concentration and half of the exposure solutions were replaced every 24 h with freshly prepared solutions. TBBPA exposures were conducted for 48, 96 and 120 h from fertilisation. BDE-47 exposures were conducted for 96 h from fertilisation only, as this compound was obtainable in amounts sufficient for analyses at one life developmental stage only.

At the desired developmental stage for the TBBPA and BDE-47 exposures, 40 individuals from each treatment were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, washed and dechorionated in PBS and stored at -20 °C in 100% methanol for WISH experiments. The remaining 10 embryo-larvae from each treatment group were pooled, frozen in liquid nitrogen and stored at -80 °C for qRT-PCR analyses. Experiments were carried out in triplicate and repeated three times. An overview of the experimental design is provided in Figure 1.

2.5 Transcript profiling by quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to quantify the transcript profiles of several target genes in the HPT axis of zebrafish (whole body samples) including: thyroid receptors (*thraa* and *thrb*), thyroid-stimulating hormone (*tshb*), deiodinases type I, II and III (*dio1*, *dio2*, *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). Ribosomal protein l8 (*rpl8*) was used as a control gene for normalisation purposes and its stable

239 expression has been validated (Fig. S1). qRT-PCR assays for each target gene were
240 optimised as previously described [43] and detailed information for each assay is
241 provided in the supplementary information (Table S1). Results are expressed as
242 mean fold changes ± standard error of the mean.
243
244 **2.6 Whole mount *in situ* hybridisation (WISH)**
245 WISH was used to examine tissue-specific changes in gene expression for several
246 genes of interest in the HPT axis including: thyroid receptors (*thraa and thrb)* and
247 deiodinases *(dio1, dio2 and dio3b),* with modifications to the protocol of Thisse and
248 Thisse (2008) [44] For details on the methodologies for gene probe synthesis
249 (including vector information) and WISH are provided in the supplementary material
250 (Supplemental Material and Methods, Tables S2 & S3).
251
252 **2.7 Statistical analyses**
253 All statistical analyses were conducted in R (R Studio, 1.1.423) [45]. LC_x and EC_x
254 values and their 95% confidence intervals (CI), for TBBPA and BDE-47 were
255 calculated using generalised linear models (GLM) with binomial error structures and
256 probit links according to Finney, 1971 [46]. Concentrations were log transformed
257 (log_{10}) to linearise the data.
258
259 To examine the effect of TBBPA and BDE-47 exposures on gene transcripts in the
260 HPT axis, qRT-PCR data was analysed using general linear mixed models (GLMM)
261 with Gaussian error structures. GLMMs were performed with the *lme4* package
262 within R [47]. P values were obtained using maximum likelihood tests of the full
263 model (with BFR treatment incorporated as a fixed effect) against a reduced model

(without BFR treatment incorporated). BFR concentrations were incorporated as a fixed effect into the model. As a random effect, random intercepts for each experiment (since each experiment was repeated 3 times) were incorporated into the model. When an overall significant effect of the BFR concentrations was identified, pairwise comparisons to determine which groups differed were conducted using a multiple comparison analysis of means (Tukey contrasts) with the *multcomp* package within R [48]. Prior to analysis, gene expression data was scrutinised by Chauvenet's criterion to detect outliers for each gene and these were subsequently removed [49]. In addition, data were tested for equal variance and for normality using the Shapiro–Wilk test. Non-normal data were subjected to variance-stabilising log transformations. All statistical models were checked for homoscedasticity and normality of residuals. For all statistical analyses, differences were considered significant at $p<0.05$. All graphed data were plotted using the *ggplot2* R package [50].

3. Results

3.1. Acute toxicity of TBBPA and BDE-47

Exposure to TBBPA for 96 h led to a significant increase in mortalities and deformities of zebrafish embryo-larvae ($p<0.001$; Fig 2; Table S4). The 96h-LC_{50} with 95% confidence intervals for TBBPA was 0.9 µM (0.8 – 1 µM: Fig. 2A). The 96h-EC_{50} with 95% confidence intervals for TBBPA, based on deformities, was 0.7 µM (0.6 – 0.9 µM: Fig. 2B). At the higher TBBPA concentrations (>0.92 µM), mortality was preceded by retardation of development (smaller animals) and morphological deformities (oedemas in the pericardial region and tail, small heads, swollen yolk sac

289 extension; Fig. 2C). Deformities were observed in 86 ± 11% of surviving individuals
290 exposed to 0.92 µM TBBPA, including oedema, short body, curved spine, swollen
291 yolk sac, small eyes and craniofacial deformities (Fig. 2D).
292
293 Exposure to BDE-47 (1 - 100 µM) had no significant effect on the mortality of
294 zebrafish embryo-larvae compared with controls (Fig. 3A). Exposure to BDE-47 for
295 a 96-h period led to a significant increase in the proportion of zebrafish deformities
296 ($p<0.05$). The 96h-EC_{50} with 95% confidence intervals for BDE-47, based on
297 deformities, was 38.2 µM (10.4 – 475.9 µM: Fig. 3B). The mean number of
298 deformities were 42 ± 11%, 51 ± 10% and 68 ± 12.0% in groups exposed to BDE-47
299 at 10, 50 and 100 µM, respectively. At these higher BDE-47 concentrations,
300 deformities included oedema, short tail and head deformities in 24 hpf embryos (Fig.
301 3C). At 96 hpf, curved spines, yolk sac deformities and craniofacial deformities were
302 also observed (Fig. 3D). See Table 1 for the full list of LC_x and EC_x values for both
303 TBBPA and BDE-47.
304
305 **3.2. Effect of TBBPA on gene transcripts in HPT axis**

306 3.2.1. TBBPA: Whole body transcript levels (qRT-PCR)
307 The effect of TBBPA (pools of n=10 larvae) on the transcription of genes in the HPT
308 axis were quantified at 48, 96 and 120 hpf via qRT-PCR and results are shown in
309 Figure 4 (A-J). TBBPA had no effect on *thraa, thrb, dio2, crhb* and *tshb* mRNA levels
310 in whole body extracts at any of the developmental stages examined relative to
311 controls. There was no effect of TBBPA on the expression levels of *dio1* at 48 and
312 120 hpf in embryo-larvae samples. After 96 h, however, exposure to 0.18 µM TBBPA
313 resulted in a significantly higher (1.5-fold) level of *dio1* mRNA in compared with

controls ($p<0.01$). After 48- and 120-h exposures, TBBPA had no significant effect on the levels of *dio3b* at any of the concentrations tested. After 96 h, however, TBBPA exposure resulted in significantly reduced mRNA levels of *dio3b* in a concentration-dependent manner (1.7, 2.8- and 3.2-fold lower in 0.04, 0.18 and 0.46 µM treatment groups) compared to the control, respectively ($p<0.05$). Exposure to 0.46 µM TBBPA resulted in significantly higher levels of *ttr* after a 48-h exposure, with mRNA levels 2-fold higher compared with the control ($p<0.05$), but with no effects thereafter (96- and 120-h exposures). *Pax8* transcript levels were higher (1.5-fold) after a 48-h exposure to all TBBPA concentrations tested compared with the control ($p<0.01$), but this was not the case after 96- and 120-h exposures. *Ugt1ab* mRNA levels increased, in a concentration-dependent manner, following 48- and 96-h exposures to TBBPA ($p<0.05$). After 48 h, *ugt1ab* mRNA levels were 1.7- and 5.1-fold higher in the 0.18 and 0.46 µM TBBPA treatment groups, respectively, compared to controls and 2-fold higher in the 0.18 and 0.46 µM treatment groups compared with controls after 96 h. No significant effect of TBBPA exposure on *ugt1ab* transcription was detected after 120 h.

3.2.2. TBBPA: Tissue specific transcript expression (WISH)

Following a 48-h exposure to TBBPA, *thraa* expression appeared to be higher in the brain for all three concentrations and in the branchial arches for the 0.18 and 0.46 µM exposures, compared with controls (Fig. 5A-D). In 96 hpf larvae, exposure to 0.18 and 0.46 µM TBBPA resulted in higher (and concentration related) expression of *thrb* mRNA in the liver, brain, jaw cartilage and swim bladder. There was also an enhanced expression in the otic vesicles in animals treated with 0.46 µM TBBPA compared with the controls (Fig. 5E-H). *Thrb* expression in the pronephric ducts

appeared to be reduced in TBBPA treated individuals (Fig. 5E-H) with 56% of control larvae exhibiting *thrb* expression in the pronephric ducts compared with only 15% of larvae exposed to 0.46 μM TBBPA. TBBPA had no apparent effect of the expression pattern of *thraa* and *thrb* in 120 and 48 hpf embryos-larvae, respectively, compared with controls (Fig. S2A-H).

For all TBBPA exposure concentrations, WISH analysis illustrated that *dio1* expression was enhanced in the brain of TBBPA exposed animals compared with controls at 48 hpf (Fig. 6A-D). At 96 hpf, *dio1* expression was enhanced in the liver and intestinal bulb in TBBPA exposed larvae (Fig. 6E-H). After a 48-h exposure to TBBPA, a greater proportion of embryos displayed a detectable *dio2* expression in the pituitary gland; 52% in control versus 74% of embryos treated with 0.46 μM TBBPA (Fig. 6I-L). After 96 h, TBBPA exposure (0.04, 0.18 and 0.46 μM) resulted in enhanced *dio2* signalling in the liver, brain and intestinal bulb compared with control larvae (Fig. 6M-P). At 96 hpf, enhanced *dio2* signalling was also observed in the pituitary gland of TBBPA treated larvae; 17% in control versus 34% of larvae treated with 0.46 μM TBBPA (Fig. S3A-D).

Via WISH, no effect of TBBPA was seen on the expression pattern of *dio3b* in 48 hpf embryos (Fig. S3E-H) but in 96 hpf larvae expression of *dio3b* was reduced in the liver (0.46 μM; Fig. 7A-D).

3.3 Effect of BDE-47 on gene transcripts in HPT axis

3.3.1 BDE-47: Whole body transcript levels (qRT-PCR)

The effect of BDE-47 exposure on the transcription of genes in the HPT axis of whole body 96 hpf larval samples is shown in Figure 8 (A-J). BDE-47 had no significant effect on the expression of *thraa, crhb* and *tshb*. Compared with controls, exposure to BDE-47 significantly induced whole body transcript levels of *thrb* (1.9-fold at 10 µM; $p<0.001$), *dio1* (1.6-fold at 1 µM; $p<0.001$), *dio2* (1.4-fold at 1 µM; $p<0.01$), *dio3b* (1.5-fold at 10 µM; $p<0.01$), *pax8* (2.2- and 2.3-fold at 1 and 10 µM, respectively; $p<0.01$) and *ugt1ab* (2.1-fold at 1 µM; $p<0.01$). Transcript levels of *ttr* were significantly reduced in BDE-47 exposed larvae compared with controls (1.4-fold at 10 µM; $p<0.05$).

3.3.2. BDE-47: Tissue specific transcript expression (WISH)

Via WISH, enhanced *thraa* expression was observed in the brain, liver, pronephric ducts and branchial arches of larvae exposed to BDE-47 (1 and 10 µM) (Fig. 9A-D). *Thrb* expression on the other hand was seen to be supressed in the brain, otic vesicle, intestinal bulb and liver, while expression in the pronephric ducts was enhanced in larvae exposed to 1 and 10 µM BDE-47 (Fig. 9E-H).

Dio1 expression was enhanced in the pronephric ducts and liver of BDE-47 exposed larvae compared to controls (1 and 10 µM; Fig. 10A-D). A greater proportion of BDE-47-exposed larvae showed *dio3b* expression in the pronephric ducts compared to controls as determined by WISH; 28% and 7% of larvae exposed to 1 and 10 µM respectively versus none in controls (Fig. 10E-H).

4. Discussion

This study adopted zebrafish embryo-larvae as a biological model to evaluate TBBPA and BDE-47 induced developmental toxicity and gene expression changes in the HPT axis.

4.1. Acute toxicity of TBBPA and BDE-47

We show that exposure to high concentrations of TBBPA for 96-h was lethal to zebrafish embryo-larvae, with an LC_{50} value of 0.9 μM that is in general accordance with similar studies conducted previously [51, 52]. Neither of those studies however calculated an LC_{50} value to allow for direct toxicity comparisons with our findings. Lee *et al.,* (1993) reported an 96h-LC_{50} value of 3 mg TBBPA L^{-1} (5.5 μM) for adult zebrafish suggesting a greater sensitivity to TBBPA in early life stages [53]. Consistent with previous studies, here we also observed various morphological deformities for exposure to TBBPA, including pericardial and tail oedemas, small heads and swollen yolk sac extensions, which may be a result of apoptosis and/or oxidative damage [51, 52]. Based on deformities in surviving zebrafish, we estimated an 96h-EC_{50} of 0.7 μM for TBBPA, slightly lower than the 96h-EC_{50} (based on hatching) of 1.1 mg L^{-1} (2.0 μM) reported previously [41].

BDE-47 up to 100 μM was not lethal to zebrafish larvae in the 96-h exposures, consistent with the low acute toxicity of PBDEs observed in rodents [54]. There appear to be considerable differences in sensitivities between fish species with regards to lethal concentrations of BDE-47. In previous studies, zebrafish embryo-larvae showed significant mortalities after exposure to BDE-47 up to 5 mg L^{-1} (10.3 μM) albeit that the exposure period was longer (168 h) and the mortalities occurred

after 100 h [55]. In contrast, a relatively low 96h-LC_{50} of 14.13 µg L^{-1} (0.03 µM) has been reported for turbot (*Psetta maxima*) larvae [56], which may indicate considerable differences in sensitivities between fish species. We found, however, deformities such as short tails, oedemas, curved spines and craniofacial deformities for exposure to BDE-47, as also reported previously [55, 57] with a comparable 96h-EC_{50} for deformities (38.2 µM) with that reported for hatching (20.3 mg L^{-1} [41.8 µM]) [41].

4.2. Effects of TBBPA and BDE-47 on TR expression

Here we found that *thraa* mRNA levels in whole body extracts were unaffected by exposures to TBBPA and BDE-47 during early zebrafish development, comparable to previous studies with zebrafish larvae exposed to BDE-47 and DE-71 for 96 hpf and 14 dpf, respectively [41, 58]. In contrast, TRα transcript levels were enhanced in zebrafish larvae exposed to TBBPA for 96 hpf [41] as well as larvae exposed to TBBPA and BDE-47 later in development (from the point of hatching) [41]. Exposure to TBBPA did not alter *thrb* RNA levels at any of the developmental stages examined in the present study, consistent with previous finding with similar stage zebrafish embryo-larvae [41]. We did, however, observe that exposure to BDE-47 resulted in elevated *thrb* mRNA levels in 96 hpf larvae. While previous studies found no effect of BDE-47 or DE-71 on TRβ transcript levels in whole body zebrafish larvae samples exposed from fertilisation, others observed reduced mRNA levels in larvae exposed to BDE-47 from the point of hatching [41, 58]. Taken together, these results suggest that TR regulation by BFRs may vary depending on the specific developmental window in which exposure occurs [59] and may differ depending on the receptor and exposure compound in question.

Through WISH analyses, we observed tissue-specific alterations to both *thraa* and *thrb* expression in zebrafish embryo-larvae following exposure to TBBPA and BDE-47. Following a TBBPA exposure, *thraa* expression was higher in the brain and branchial arches of exposed 48 hpf embryos compared to controls, while *thrb* expression was enhanced in the liver, brain, jaw cartilage, otic vesicle and swim bladder of 96 hpf larvae. As far as we are aware, no study to date has examined tissue specific changes to TR gene expression in fish (either in adults via PCR or in larvae via WISH) exposed to TBBPA. Similar to that seen for the TBBPA exposures, *thraa* expression was found to be enhanced in the brain and branchial arches, as well as the liver and pronephric ducts, of 96 hpf larvae exposed to BDE-47. These results are consistent with the findings of increased *thra* transcript levels in the brain of adult fathead minnows following dietary exposure to BDE-47 and BDE-209 for 21 and 28 days, respectively [30, 60]. In contrast, *thrb* expression was suppressed in the liver, brain, intestinal bulb and otic vesicle of 96 hpf larvae exposed to BDE-47 in our study, though expression was induced in the pronephric ducts. Interestingly, a dietary exposure to BDE-47 reduced *thrb* mRNA levels in the brains of adult fathead minnows, but no changes were observed in the liver [61], whilst in a similar study with BDE-209 the opposite occurred [60]. TRs mediate the thyroid system's genomic control over several key developmental processes, including growth [62], auditory [63], eye [64], brain [65] and skeletal [66] development [67]. Whether the BFR-induced changes in TR transcripts observed here translate to altered TH-dependant developmental processes remains unclear. As far as we are aware, only one study has examined the effect of overexpression of TRα1 in zebrafish and report disrupted hindbrain patterning in embryos [68]. Based on the observed transcriptional changes

of TRs, our results suggest that TH signalling in the brain, as well as the liver, intestine and jaw cartilage are sensitive to TBBPA and BDE-47 during early zebrafish development.

The mechanisms by which TBBPA and BDE-47 altered both TR transcripts in this study are not fully understood. Changes may be a result of BFR-induced alterations in circulating and/or local TH levels or a result of a direct interaction with TRs or their co-repressors/activators (since the genes encoding TRs themselves contain TREs). Since several *in vitro* studies have demonstrated that BFRs have limited agonistic effects on TR-mediated gene transcription [69-71], it seems plausible that the increases in *thraa* and *thrb* transcription following TBBPA and BDE-47 exposures were a result of increased local T3 levels.

4.3. Effects of TBBPA and BDE-47 on deiodinase expression

Exposure to both TBBPA and BDE-47 resulted in higher levels of *dio1* transcripts in whole body samples of 96 hpf zebrafish larvae, in agreement with results observed in zebrafish larvae and adults exposed to DE-71, BDE-209 and BDE-47 [58, 72, 73]. In contrast, other studies found that *dio1* transcript levels were not altered in zebrafish juveniles exposed to BDE-47 or zebrafish larvae exposed to TBBPA, respectively, though the exposure period in the BDE-47 study began at a later stage in development [42, 74]. Interestingly, here TBBPA had no effect on *dio2* levels in whole zebrafish embryo-larvae at any stage examined, while exposure to BDE-47 led to higher *dio2* levels in 96 hpf larvae. These results are in line with previous studies, where no change in *dio2* levels were detected in zebrafish larvae exposed to TBBPA for 5 d from fertilisation [42], while increased *dio2* levels in larvae were

488 detected following exposure to BDE-47 and DE-71 for 14 d from fertilisation [58, 73].
489 Whilst D1 is capable of both activating and deactivating THs, in fish it has been
490 suggested that the former is its primarily function, in contrast to its regulation and
491 function in mammals [75, 76]. D2 is considered, however, to be the major TH-
492 activating enzyme. Therefore, the increased levels of *dio1* and *dio2* transcripts
493 observed here provide strong evidence of an induced systemic hypothyroid condition
494 in the TBBPA and BDE-47 exposed zebrafish larvae [76-78]. This likelihood is
495 supported by the reduced *dio3b* mRNA levels observed in whole larval (96 hpf)
496 samples exposed to TBBPA (0.18 and 0.46 µM groups), since D3 is the major
497 inactivating pathway of THs and thyroidal status often parallels hepatic D3 activity,
498 increasing during hyperthyroidism and decreasing during hypothyroidism [33, 79].
499
500 WISH analysis provided us with a more detailed insight into the tissue specific
501 changes and the mechanisms underlying thyroid disruption to *dio1*, *dio2* and *dio3b*
502 expression in zebrafish embryo-larvae following BFR exposure. We observed higher
503 *dio1* expression in the brain of embryos (48 hpf) and in the liver and intestinal bulb of
504 larvae (96 hpf) exposed to TBBPA, while higher levels of *dio2* expression were
505 observed in the pituitary of embryos (48 hpf) and in the brain, pituitary and intestinal
506 bulb of larvae (96 hpf). As far as we aware, no previous studies have examined
507 tissue specific changes in the expression of genes encoding the deiodinase
508 enzymes following exposure to TBBPA. The increased expression of *dio2* in the
509 pituitary of TBBPA exposed embryo-larvae is not fully understood. In fish under
510 normal physiological regulations, reduced circulating TH levels lead to increased
511 stimulation and secretion of TSH from the pituitary through the negative feedback
512 system of the HPT axis [35]. An increased conversion of T4 into T3 in the pituitary

513 (catalysed by increased D2 levels) may deplete local T4 levels, inducing the negative
514 feedback loop and increasing the production of THs by the thyroid follicles. In
515 support of the qPCR results, the higher expression of *dio1* and *dio2*, along with the
516 reduced expression of *dio3b*, in the liver of TBBPA exposed larvae provides strong
517 evidence of systemic hypothyroidism [76]. In addition, the elevated *dio1* and *dio2*
518 mRNA levels in the brain and intestinal bulb are likely associated with reduced local
519 TH levels and may be compensatory responses in order to maintain local TH
520 homeostasis during the development of neurological and gastrointestinal tissues. In
521 BDE-47 exposed larvae, enhanced *dio1* expression was observed in the pronephric
522 ducts and liver. This is in contrast to a previous study with zebrafish, which found no
523 significant changes in *dio1* transcript levels in the liver of adults exposed to BDE-47
524 for 180 days from fertilisation, though *dio2* levels were significantly increased [73].
525 Interestingly, we found that BDE-47 had no effect on *dio3b* expression in the liver but
526 higher levels were observed in the pronephric ducts. Increased D3 activity as a result
527 of increased *dio3b* expression in the pronephric ducts may reflect increased local T3
528 production (*dio1* expression also increased in pronephric ducts). Our results suggest
529 that altering the expression of deiodinase genes could be a potential mechanism by
530 which TBBPA and BDE-47 disrupt/correct TH homeostasis, though observed effects
531 vary depending on the life stage examined, exposure period and compound tested.
532
533 **4.4. Effects of TBBPA and BDE-47 on the expression of further genes in the**
534 **HPT Axis**
535 Elevated *ugt1ab* mRNA levels were detected in whole zebrafish samples exposed to
536 TBBPA (at 48 hpf and 96 hpf) and BDE-47 (at 96 hpf), similar to the effect observed
537 with zebrafish exposed to DE-71 [58]. UDPGTs play an important role in TH

538 metabolism in the liver and increased *ugt1ab* expression could lead to the increased
539 elimination of THs, a plausible explanation for the altered TH signalling observed
540 throughout the HPT axis here [80]. Previous findings in mammals and fish have
541 reported reduced circulating levels of T4, combined with increased UDPGT activity
542 and/or transcription, following exposure to BFRs [58, 81].
543
544 Elevated levels of *pax8* transcripts were observed here in zebrafish embryo-larvae
545 following exposures to TBBPA (at 48 hpf) and BDE-47 (at 96 hpf). Similar findings
546 have been reported in zebrafish embryos/larvae exposed to various other pollutants
547 [58, 82], although to our knowledge no studies have yet examined the effect of
548 TBBPA on *pax8* transcription. Pax8 proteins are essential for the late differentiation
549 of the thyroid follicular cells during development [83], therefore our results suggest
550 the promotion of thyroid primordium growth and possibly a compensation
551 mechanism for reduced TH levels, induced by the BRFs tested.
552
553 TTR is proposed to be the major TH carrier protein in fish [84] and plays a key role in
554 maintaining extra-thyroidal stores of TH and regulating its supply to various target
555 tissues [85]. Here we found that TBBPA significantly increased *ttr* transcripts levels
556 in 48 hpf embryos while BDE-47 significantly decreased *ttr* transcript levels in 96 hpf
557 larvae. Our results are consistent with previous studies which have found increased
558 and decreased *ttr* mRNA levels in zebrafish larvae following exposure to TBBPA [41]
559 and BDE-47, respectively [41, 73]. Altered levels of TTR proteins in exposed
560 individuals, may decrease or increase the amount of TTR available to bind free TH.
561 In the case of BDE-47, the reduced *ttr* levels may lead to excess unbound TH, which
562 would be more susceptible to hepatic catabolism, resulting in a greater clearance

563 rate and a decrease in circulating TH concentrations. The impacts of the increased
564 *ttr* levels in TBBPA exposed larvae may be more complex, since TBBPA has been
565 shown to potently compete with T3 for TTR binding sites. Increased TTR protein
566 levels could potentially lead to increased substrate available for TBBPA binding,
567 making the displaced and unbound THs in the plasma more susceptible to hepatic
568 metabolism and suppress circulating levels of T4/T3 [86]. The altered *ttr* mRNA
569 expression by TBBPA and BDE-47, suggest that while induction of UDPGTs may be
570 partly responsible for disrupting systemic TH levels, other mechanisms may also be
571 involved.
572
573 Corticotropin-releasing hormone (CRH) and thyroid-stimulating hormone (TSH)
574 regulate THs synthesis and secretion in fish through a negative feedback
575 mechanism within the HPT axis, triggered via alteration in circulating T4 levels [87].
576 Interestingly, in the present study, both TBBPA and BDE-47 had no effect on *tshb*
577 and *crhb* transcript levels in zebrafish embryo-larvae. This is in agreement with
578 previous studies that found no effect on *tshb* mRNA levels in 5 dpf zebrafish larvae
579 exposed to TBBPA and juvenile zebrafish exposed to BDE-47 for 40 days [42, 74].
580 However, the expression of *crhb* and *tshb* has been shown to be elevated in adult
581 fathead minnows exposed to BDE-47 [61] and both adult and larval zebrafish
582 exposed to various BFRs, including TBBPA and BDE-47 [41, 73]. Increased *crhb*
583 and *tshb* expression in these studies were accompanied by reduced T4 levels.
584 Interestingly, recent studies have reported lower *crhb* and/or *tshb* transcript levels in
585 zebrafish adults, embryos and larvae exposed to TBBPA or BDE-47 [41, 88],
586 associated with increased T4 and T3 levels [88]. From these studies, it is evident
587 that BFRs have varying effects on the expression of *crh* and *tsh* genes in fish,

588 highlighting the importance of examining the effect of TDC on multiple levels of the
589 HPT axis.
590
591 **4.5. Combining qRT-PCR and WISH to enhance our understanding of tissue**
592 **specific targets of BFRs.**
593 Given the wide range of tissues influenced by THs during early development and the
594 complex nature involved in regulating the TH system, it is important to assess the
595 effect of TDCs on the spatial expression of targeted thyroid-related genes. To date,
596 assessing transcriptional changes in fish embryo-larvae following exposure to BFRs
597 have largely relied on qRT-PCR, while tissue-specific effects have been limited to
598 adult studies where isolating organs/tissues of interest was relatively straightforward.
599 In the present study, we simultaneously examined the effects of TBBPA and BDE-47
600 on the transcription of several thyroid-related genes using WISH and qRT-PCR.
601 While qRT-PCR provides quantitative data on gene expression and is sensitive at
602 detecting even small changes in mRNA levels, when carried out on pooled
603 homogenates of whole embryo-larvae, transcriptional changes in small and/or
604 localised tissues may not be detected if diluted by non-responsive organ/tissues of
605 the whole animal. Only recently has WISH been used in ecotoxicology studies as a
606 means of examining gene expression changes on a spatial scale and identifying
607 tissue-specific markers of exposure during early stages of development [37, 89].
608 Interestingly here, the transcription of several genes in the HPT axis appeared
609 unaffected by BFR exposures when examined using qRT-PCR but tissue-specific
610 effects were observed using WISH. These included *thraa* (at 48 hpf; TBBPA and at
611 96 hpf; BDE-47) *dio1* (at 48 hpf; TBBPA), *dio2* (at 48 and 96 hpf; TBBPA) and *dio3b*
612 (at 96 hpf; BDE-47). Here we demonstrate the value of WISH as an approach, in

conjunction with qRT-PCR, to examine the mechanistic basis of the effects of TDCs on early-life stage zebrafish.

4.6. Developmental stage-specific effects

We also examined the effect of TBBPA exposure on thyroid gene transcripts at three early developmental stages and found that effects were developmental stage specific. TBBPA-induced alterations to *dio1* and *dio3b* transcription were observed in 96 hpf larvae while *ttr* and *pax8* mRNAs were altered in 48 hpf embryos only. Finally, *ugt1ab* expression was altered at 48 and 96 hpf. TBBPA had no effect on any of the genes examined following 120-h exposures. This study is one of only a few that examined the effect of BFRs on thyroid-related gene transcription at different life stages. Similar to our findings, several genes in the HPT axis were found to be transiently altered in the liver and/or brain of adult male fathead minnows exposed to BDE-209 [60]. The TH system is regulated by sophisticated compensatory mechanisms both centrally (negative feedback loop in HPT axis) and peripherally (by deiodinases), which, if adequate, can normalise both serum and peripheral TH concentrations [90]. These compensatory mechanisms may explain why no transcriptional effects were detected here in 120 hpf larvae. It is also important to note that increased endogenous T3 levels are associated with the embryo-larval transition of teleost fish, and this natural increase may intensify the effects of BFRs on the thyroid system at this life stage. We observed that zebrafish at 96 hpf, a period coinciding with increased TH levels, were particularly sensitive to TBBPA exposure.

Summary

In summary, the present study demonstrates that the BFRs, TBBPA and BDE-47, can disrupt the thyroid axis of zebrafish embryo-larvae at multiple levels by altering mRNA transcript levels of genes encoding deiodinases, thyroid synthesis proteins, transport proteins and glucuronidation enzymes. We have also shown that both compounds induced tissue-specific transcriptional changes for several genes in the HPT axis, with TH signalling in the brain, liver, pronephric duct and craniofacial tissues appearing particularly sensitive to TBBPA and BDE-47 exposures (Fig. 11). Furthermore, TH disruption by TBBPA appeared more pronounced in larvae at 96 hpf, compared to 48 and 120 hpf. These results demonstrate the effects of BFRs on the thyroid system should not be generalised across tissues or developmental stages in fish species. Future work should focus on understanding the consequences of chronic low level exposure to BFRs and examine whether the BFR-induced changes in TR transcript levels in the brain and skeleton as observed here translate into health consequences for wild fish populations and humans.

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References

1. Legler, J. and A. Brouwer, *Are brominated flame retardants endocrine disruptors?* Environment international, 2003. **29**(6): p. 879-885.
2. Williams, G.R., *Thyroid hormone actions in cartilage and bone.* European Thyroid Journal, 2013. **2**(1): p. 3-13.
3. Wagner, M.S., S.M. Wajner, and A.L. Maia, *The role of thyroid hormone in testicular development and function.* The Journal of Endocrinology, 2008. **199**(3): p. 351-365.
4. Chan, S. and M. Kilby, *Thyroid hormone and central nervous system development.* Journal of Endocrinology, 2000. **165**(1): p. 1-8.
5. Rovet, J.F., *The role of thyroid hormones for brain development and cognitive function*, in *Paediatric Thyroidology*. 2014, Karger Publishers. p. 26-43.
6. Alaee, M., et al., *An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release.* Environment International, 2003. **29**(6): p. 683-689.
7. La Guardia, M.J., R.C. Hale, and E. Harvey, *Detailed polybrominated diphenyl ether (PBDE) congener composition of the widely used penta-, octa-, and deca-PBDE technical flame-retardant mixtures.* Environmental Science & Technology, 2006. **40**(20): p. 6247-6254.
8. Luo, X., et al., *Distribution and partition of polybrominated diphenyl ethers (PBDEs) in water of the Zhujiang River Estuary.* Chinese Science Bulletin, 2008. **53**(4): p. 493-500.
9. Daniels, J.L., et al., *Individual characteristics associated with PBDE levels in U.S. human milk samples.* Environmental Health Perspectives, 2010. **118**(1): p. 155-60.
10. Law, R.J., et al., *Levels and trends of brominated diphenyl ethers in blubber of harbor porpoises (Phocoena phocoena) from the UK, 1992– 2008.* Environmental Science & Technology, 2010. **44**(12): p. 4447-4451.
11. Chen, D. and R.C. Hale, *A global review of polybrominated diphenyl ether flame retardant contamination in birds.* Environment International, 2010. **36**(7): p. 800-811.
12. Boon, J.P., et al., *Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North Sea food web.* Environmental Science & Technology, 2002. **36**(19): p. 4025-4032.
13. Xia, K., et al., *Polybrominated diphenyl ethers (PBDEs) in biota representing different trophic levels of the Hudson River, New York: from 1999 to 2005.* Environmental Science & Technology, 2008. **42**(12): p. 4331-4337.
14. U.N. United Nations. *The 9 new POPs under the Stockholm Convention.* 2009; Available from: http://chm.pops.int/Implementation/NewPOPs/TheNewPOPs/tabid/672/Default.aspx.
15. Law, R.J., et al., *Levels and trends of PBDEs and HBCDs in the global environment: Status at the end of 2012.* Environment International, 2014. **65**: p. 147-158.
16. Venier, M., et al., *Brominated flame retardants in the indoor environment — Comparative study of indoor contamination from three countries.* Environment International, 2016. **94**: p. 150-160.
17. Walker, L., et al., *Polybrominated diphenyl ethers (PBDEs) in Eurasian otters (Luta lutra) collected from Britain in 2010: A predatory bird monitoring scheme (PBMS) report.* 2012.
18. Abdallah, M.A.-E. and S. Harrad, *Polybrominated diphenyl ethers in UK human milk: Implications for infant exposure and relationship to external exposure.* Environment International, 2014. **63**: p. 130-136.
19. Hale, R.C., et al., *Polybrominated diphenyl ether flame retardants in virginia freshwater fishes (USA).* Environmental Science & Technology, 2001. **35**(23): p. 4585-4591.

20. Leonetti, C., et al., *Concentrations of polybrominated diphenyl ethers (PBDEs) and 2,4,6-tribromophenol in human placental tissues.* Environment international, 2016. **88**: p. 23-29.
21. Howard, P.H. and D.C.G. Muir, *Identifying new persistent and bioaccumulative organics among chemicals in commerce.* Environmental Science & Technology, 2010. **44**(7): p. 2277-2285.
22. Abb, M., B. Stahl, and W. Lorenz, *Analysis of brominated flame retardants in house dust.* Chemosphere, 2011. **85**(11): p. 1657-1663.
23. Gorga, M., et al., *Determination of PBDEs, HBB, PBEB, DBDPE, HBCD, TBBPA and related compounds in sewage sludge from Catalonia (Spain).* Science of The Total Environment, 2013. **444**(0): p. 51-59.
24. Zhang, X.-L., et al., *Spatial distribution and vertical profile of polybrominated diphenyl ethers, tetrabromobisphenol A, and decabromodiphenylethane in river sediment from an industrialized region of South China.* Environmental Pollution, 2009. **157**(6): p. 1917-1923.
25. Harrad, S., et al., *Current-use brominated flame retardants in water, sediment, and fish from English lakes.* Environmental Science & Technology, 2009. **43**(24): p. 9077-9083.
26. Labadie, P., et al., *Development of analytical procedures for trace-level determination of polybrominated diphenyl ethers and tetrabromobisphenol A in river water and sediment.* Analytical & Bioanalytical Chemistry, 2010. **396**(2): p. 865-875.
27. Morris, S., et al., *Distribution and fate of HBCD and TBBPA brominated flame retardants in north sea estuaries and aquatic food webs.* Environmental Science & Technology, 2004. **38**(21): p. 5497-5504.
28. Cariou, R., et al., *Exposure assessment of French women and their newborns to tetrabromobisphenol-A: Occurrence measurements in maternal adipose tissue, serum, breast milk and cord serum.* Chemosphere, 2008. **73**(7): p. 1036-1041.
29. Birnbaum, L.S. and D.F. Staskal, *Brominated flame retardants: Cause for concern?* Environmental Health Perspectives, 2004. **112**(1): p. 9-17.
30. Lema, S.C., et al., *Dietary exposure to 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47) alters thyroid status and thyroid hormone-regulated gene transcription in the pituitary and brain.* Environmental Health Perspectives, 2008. **116**(12): p. 1694-1699.
31. Kuiper, R.V., et al., *Long-term exposure of European flounder (Platichthys flesus) to the flame-retardants tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD).* Ecotoxicology & Environmental Safety, 2007. **67**(3): p. 349-360.
32. Bianco, A.C., et al., *Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases.* Endocrine Reviews, 2002. **23**(1): p. 38-89.
33. Bianco, A.C. and B.W. Kim, *Deiodinases: Implications of the local control of thyroid hormone action.* The Journal of Clinical Investigation, 2006. **116**(10): p. 2571-2579.
34. Sarne, D., *Effects of the environment, chemicals and drugs on thyroid function*, in *Endotext*, L.J. De Groot, et al., Editors. 2000, MDText.com, Inc.: South Dartmouth (MA).
35. Blanton, M.L. and J.L. Specker, *The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction.* Critical Reviews in Toxicology, 2007. **37**(1-2): p. 97-115.
36. Li, W., et al., *Effects of decabromodiphenyl ether (BDE-209) on mRNA transcription of thyroid hormone pathway and spermatogenesis associated genes in Chinese rare minnow (Gobiocypris rarus).* Environmental Toxicology, 2011. **29**(1): p. 1-9.
37. Dong, W., et al., *Using whole mount in situ hybridization to examine thyroid hormone deiodinase expression in embryonic and larval zebrafish: A tool for examining OH-BDE toxicity to early life stages.* Aquatic Toxicology, 2013. **132–133**: p. 190-199.
38. Nusslein-Volhard, C. and R. Dahm, *Zebrafish: A practical approach*. 2002: Oxford University Press.
39. ISO. International Organization for Standardization, *Water quality -- Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-*

Buchanan (Teleostei, Cyprinidae)] -- Part 1: Static method. Flow-Through Method, Part 3. 1996.
40. Kimmel, C.B., et al., *Stages of embryonic development of the zebrafish.* Developmental Dynamics, 1995. **203**(3): p. 253-310.
41. Chan, W.K. and K.M. Chan, *Disruption of the hypothalamic-pituitary-thyroid axis in zebrafish embryo–larvae following waterborne exposure to BDE-47, TBBPA and BPA.* Aquatic Toxicology, 2012. **108**(0): p. 106-111.
42. Baumann, L., et al., *Thyroid disruption in zebrafish (Danio rerio) larvae: Different molecular response patterns lead to impaired eye development and visual functions.* Aquatic Toxicology, 2016. **172**: p. 44-55.
43. Lange, A., et al., *Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring.* Aquatic Toxicology, 2012. **109**: p. 250-258.
44. Thisse, C. and B. Thisse, *High-resolution in situ hybridization to whole-mount zebrafish embryos.* Nature Protocols, 2008. **3**(1): p. 59-69.
45. Team, R., *RStudio: Integrated Development Environment for R*. 2016, RStudio, Inc.
46. Finney, D.J., *Probit analysis*. 3rd ed. 1971: Cambridge University Press.
47. Bates, D., et al., *Fitting Linear Mixed-Effects Models Using lme4.* Journal of Statistical Software, 2015. **67**(1): p. 1-48.
48. Hothorn, T., F. Bretz, and P. Westfall, *Simultaneous Inference in General Parametric Models.* Biometrical Journal, 2008. **50**(3): p. 346-363.
49. Chauvenet, W.A., *Manual of spherical and practical astronomy: 2: Theory and use of astronomical instruments*. 1863: Lippincott.
50. Wickham, H., *ggplot2: Elegant Graphics for Data Analysis*. 2009: Springer-Verlag New York.
51. Hu, J., et al., *Assessing the toxicity of TBBPA and HBCD by zebrafish embryo toxicity assay and biomarker analysis.* Environmental Toxicology, 2009. **24**(4): p. 334-342.
52. Wu, S., et al., *TBBPA induces developmental toxicity, oxidative stress, and apoptosis in embryos and zebrafish larvae (Danio rerio).* Environmental Toxicology, 2016. **31**(10): p. 1241-9.
53. Lee, S.K., et al., *Effects of dissolved humic materials on acute toxicity of some organic chemicals to aquatic organisms.* Water Research, 1993. **27**(2): p. 199-204.
54. Darnerud, P.O., et al., *Polybrominated diphenyl ethers: Occurrence, dietary exposure, and toxicology.* Environmental Health Perspectives, 2001. **109**(Suppl 1): p. 49.
55. Lema, S.C., et al., *Neural defects and cardiac arrhythmia in fish larvae following embryonic exposure to 2,2′,4,4′-tetrabromodiphenyl ether (PBDE 47).* Aquatic Toxicology, 2007. **82**(4): p. 296-307.
56. Mhadhbi, L., et al., *Acute toxicity of polybrominated diphenyl ethers (PBDEs) for turbot (Psetta maxima) early life stages (ELS).* Environmental Science & Pollution Research, 2012. **19**(3): p. 708-717.
57. Liu, H., et al., *Bioaccumulation, biotransformation, and toxicity of BDE-47, 6-OH-BDE-47, and 6-MeO-BDE-47 in early life-stages of zebrafish (Danio rerio).* Environmental Science & Technology, 2015. **49**(3): p. 1823-1833.
58. Yu, L., et al., *Exposure to DE-71 alters thyroid hormone levels and gene transcription in the hypothalamic–pituitary–thyroid axis of zebrafish larvae.* Aquatic Toxicology, 2010. **97**(3): p. 226-233.
59. Fini, J.B., et al., *Thyroid hormone signaling in the Xenopus laevis embryo is functional and susceptible to endocrine disruption.* Endocrinology, 2012. **153**(10): p. 5068-5081.
60. Noyes, P.D., et al., *Low level exposure to the flame retardant BDE-209 reduces thyroid hormone levels and disrupts thyroid signaling in fathead minnows.* Environmental Science & Technology, 2013. **47**(17): p. 10012-10021.

61. Lema, S.C., et al., *Dietary exposure to 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47) alters thyroid status and thyroid hormone-regulated gene transcription in the pituitary and brain.* Environmental Health Perspectives, 2008. **116**(12): p. 1694-1699.
62. Forhead, A.J. and A.L. Fowden, *Thyroid hormones in fetal growth and prepartum maturation.* Journal of Endocrinology, 2014. **221**(3): p. R87-R103.
63. Forrest, D., et al., *Thyroid hormone receptor beta is essential for development of auditory function.* Nature Genetics, 1996. **13**(3): p. 354-7.
64. Ng, L., et al., *A thyroid hormone receptor that is required for the development of green cone photoreceptors.* Nature Genetics, 2001. **27**(1): p. 94-8.
65. Rivas, M. and J.R. Naranjo, *Thyroid hormones, learning and memory.* Genes, Brain & Behavior, 2007. **6 Suppl 1**: p. 40-4.
66. Bassett, J.H.D., et al., *Thyroid status during skeletal development determines adult bone structure and mineralization.* Molecular Endocrinology, 2007. **21**(8): p. 1893-1904.
67. Tsai, M. and B.W. O'Malley, *Molecular mechanisms of action of steroid/thyroid receptor superfamily members.* Annual review of biochemistry, 1994. **63**(1): p. 451-486.
68. Essner, J.J., R.G. Johnson, and P.B. Hackett, *Overexpression of thyroid hormone receptor α1 during zebrafish embryogenesis disrupts hindbrain patterning and implicates retinoic acid receptors in the control of hox gene expression.* Differentiation, 1999. **65**(1): p. 1-11.
69. Oka, T., et al., *Establishment of transactivation assay systems using fish, amphibian, reptilian and human thyroid hormone receptors.* Journal of Applied Toxicology, 2013. **33**(9): p. 991-1000.
70. Kitamura, S., et al., *Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds.* Toxicological Sciences, 2005. **84**(2): p. 249-259.
71. Kojima, H., et al., *Nuclear hormone receptor activity of polybrominated diphenyl ethers and their hydroxylated and methoxylated metabolites in transactivation assays using Chinese hamster ovary cells.* Environmental Health Perspectives, 2009. **117**(8): p. 1210.
72. Chen, Q., et al., *Bioconcentration and metabolism of decabromodiphenyl ether (BDE-209) result in thyroid endocrine disruption in zebrafish larvae.* Aquatic Toxicology, 2012. **110-111**: p. 141-8.
73. Zhao, X., et al., *Life-cycle exposure to BDE-47 results in thyroid endocrine disruption to adults and offsprings of zebrafish (Danio rerio).* Environmental Toxicology & Pharmacology, 2016. **48**: p. 157-167.
74. Chen, T.-H., et al., *Growth and transcriptional effect of dietary 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47) exposure in developing zebrafish (Danio rerio).* Ecotoxicology & Environmental Safety, 2010. **73**(3): p. 377-383.
75. Berry, M.J., L. Banu, and P.R. Larsen, *Type I iodothyronine deiodinase is a selenocysteine-containing enzyme.* Nature, 1991. **349**(6308): p. 438-440.
76. Van der Geyten, S., et al., *Hypothyroidism induces type I iodothyronine deiodinase expression in tilapia liver.* General & Comparative Endocrinology, 2001. **124**(3): p. 333-342.
77. Burmeister, L.A., J. Pachucki, and D.L.S. Germain, *Thyroid hormones inhibit type 2 iodothyronine deiodinase in the rat cerebral cortex by both pre- and posttranslational mechanisms.* Endocrinology, 1997. **138**(12): p. 5231-5237.
78. St. Germain, D.L., *The effects and interactions of substrates, inhibitors, and the cellular thiol-disulfide balance on the regulation of type II iodothyronine 5′-deiodinase.* Endocrinology, 1988. **122**(5): p. 1860-1868.
79. Walter, K.M., et al., *Effects of thyroid hormone disruption on the ontogenetic expression of thyroid hormone signaling genes in developing zebrafish (Danio rerio).* General and Comparative Endocrinology, 2018.
80. Clarke, D.J. and B. Burchell, *The uridine diphosphate glucuronosyltransferase multigene family: Function and regulation*, in *Conjugation—Deconjugation Reactions in Drug*

Metabolism and Toxicity, F.C. Kauffman, Editor. 1994, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 3-43.
81. Richardson, V.M., et al., *Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener.* Toxicology & Applied Pharmacology, 2008. **226**(3): p. 244-250.
82. Wang, Q., et al., *Exposure of zebrafish embryos/larvae to TDCPP alters concentrations of thyroid hormones and transcriptions of genes involved in the hypothalamic-pituitary-thyroid axis.* Aquatic Toxicology, 2013. **126**: p. 207-13.
83. Wendl, T., et al., *pax2.1 is required for the development of thyroid follicles in zebrafish.* Development, 2002. **129**(15): p. 3751-3760.
84. Yamauchi, K., et al., *Purification and characterization of thyroid-hormone-binding protein from masu salmon serum. A homolog of higher-vertebrate transthyretin.* European Journal of Biochemistry, 1999. **265**(3): p. 944-9.
85. Power, D.M., et al., *Evolution of the thyroid hormone-binding protein, transthyretin.* General & Comparative Endocrinology, 2000. **119**(3): p. 241-55.
86. Miyata, K. and K. Ose, *Thyroid hormone-disrupting effects and the amphibian metamorphosis assay.* Journal of Toxicologic Pathology, 2012. **25**(1): p. 1-9.
87. Seasholtz, A., R. Valverde, and R. Denver, *Corticotropin-releasing hormone-binding protein: biochemistry and function from fishes to mammals.* Journal of Endocrinology, 2002. **175**(1): p. 89-97.
88. Yu, L., et al., *Parental transfer of polybrominated diphenyl ethers (PBDEs) and thyroid endocrine disruption in zebrafish.* Environmental Science & Technology, 2011. **45**(24): p. 10652-10659.
89. Cavallin, J.E., et al., *Evaluation of whole-mount in situ hybridization as a tool for pathway-based toxicological research with early-life stage fathead minnows.* Aquatic Toxicology, 2015. **169**: p. 19-26.
90. McClain, R.M., et al., *The effect of phenobarbital on the metabolism and excretion of thyroxine in rats.* Toxicology & Applied Pharmacology, 1989. **99**(2): p. 216-228.

Figures

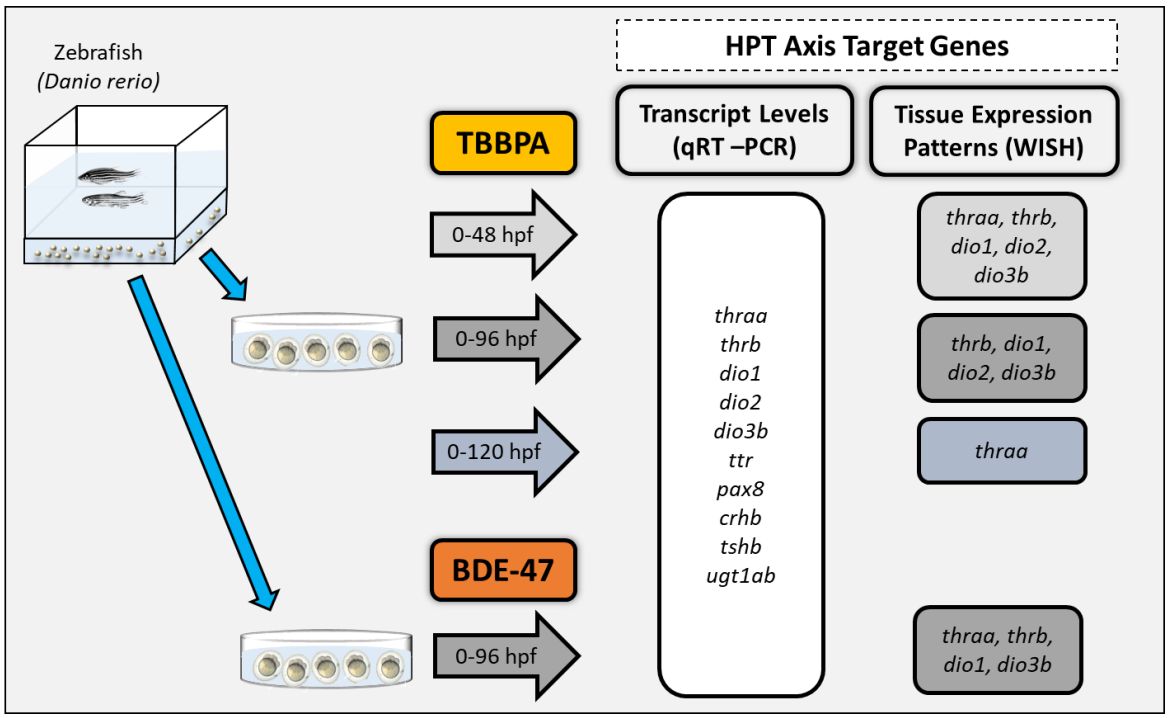


Figure 1. Overview of the experimental design of this study, showing the HPT axis target genes analysed with WISH and qRT-PCR assays following exposures of zebrafish embryo-larvae to TBBPA or BDE-47 exposures.

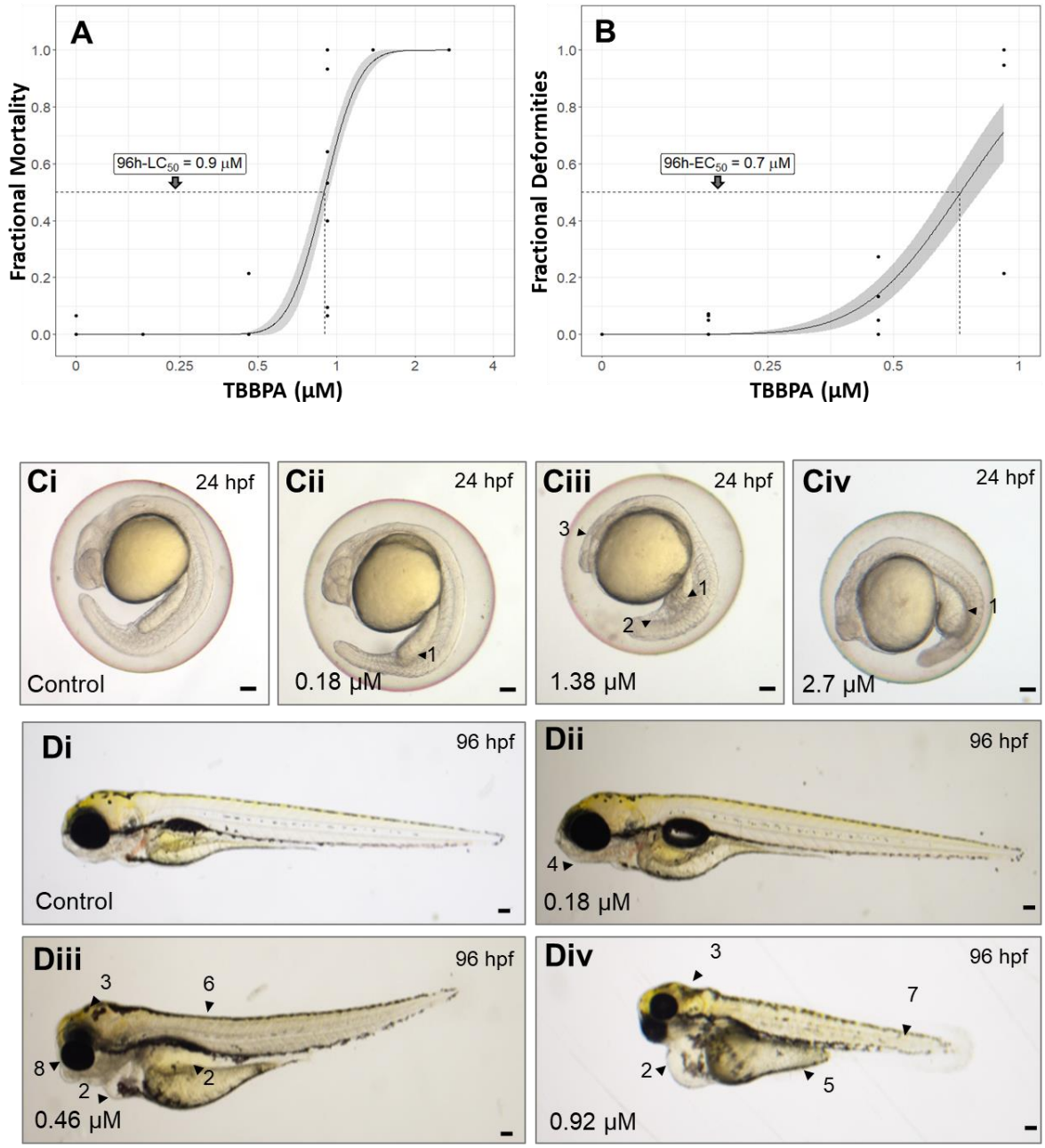
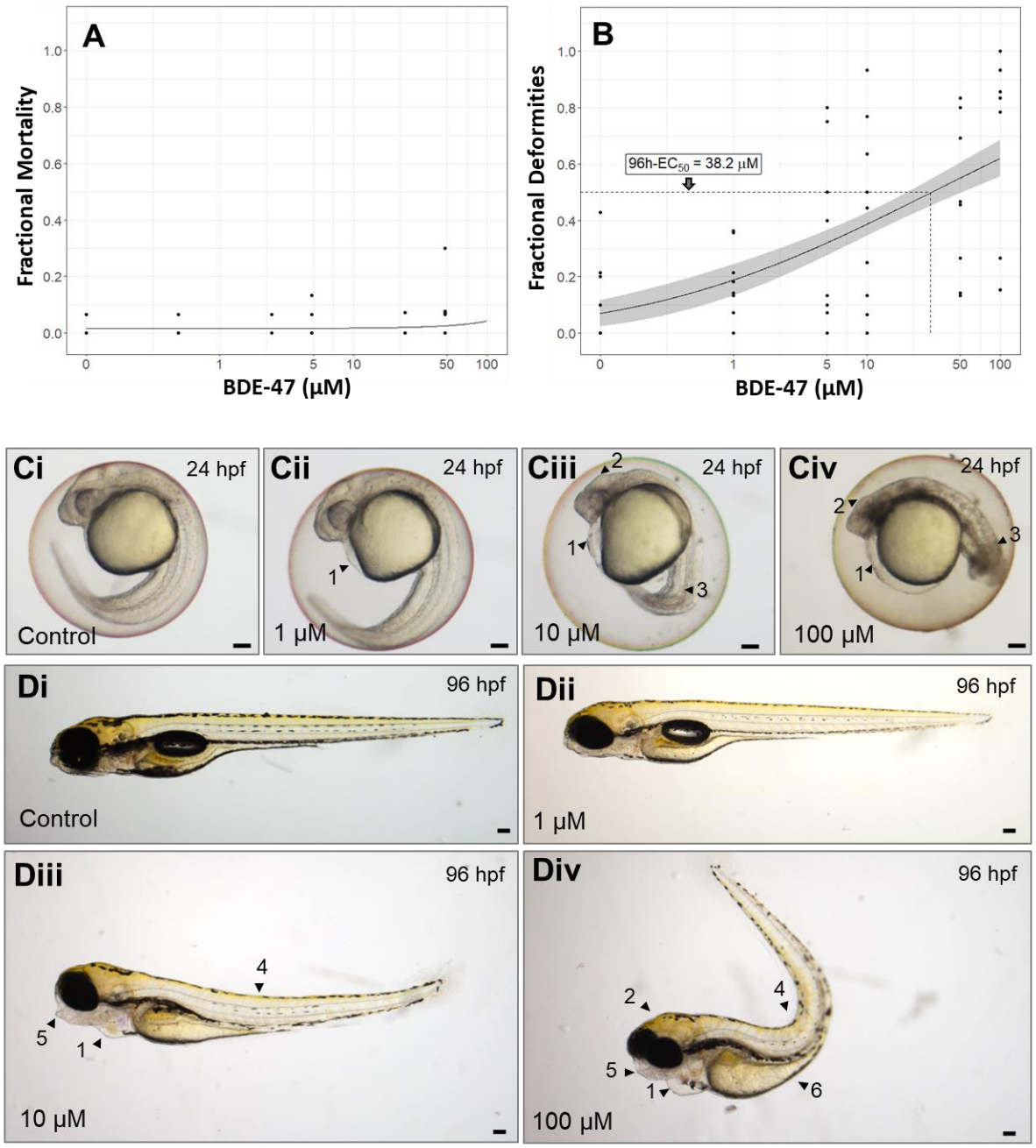


Figure 2. Effects of TBBPA on developing zebrafish embryo-larvae. Dose response curves showing (A) mortality and (B) deformities in zebrafish embryo-larvae after 96-h exposures to TBBPA (at nominal concentrations between 0.18 and 2.7 µM). Each point on the graphs represents the relative mortality and relative deformities in an individual replicate glass dish containing 15-20 individuals and the lines represent the best-fit model for the data, calculated using generalised linear models in R (model output summarised in Table S5A). Representative images of zebrafish embryos at 24 hpf exposed to (Ci) Control (Cii) 0.18 µM, (Ciii) 1.38 µM and (Civ) 2.7

903 µM and at 96 hpf exposed to (Di) Control (Dii) 0.18 µM, (Diii) 0.46 µM and (Div) 0.92
904 µM showing- 1. swollen yolk sac extension, 2. oedema, 3. head deformity, 4. lower
905 jaw deformity, 5. yolk sac deformity, 6. bent spine, 7. short tail and 8. smaller than
906 normal eyes. Scale bar=100 µm.



907

908 Figure 3. Effects of BDE-47 on developing zebrafish embryo-larvae. Dose response
909 curves showing (A) mortality and (B) deformities in zebrafish embryo-larvae after 96-
910 h exposures to BDE-47 (at nominal concentrations between 1 and 100 µM). Each
911 point on the graphs represents the relative mortality and relative deformities in an
912 individual replicate glass dish containing 15-20 individuals and the lines represent
913 the best-fit model for the data, calculated using generalised linear models in R
914 (model output summarised in Table S5B). Representative images of zebrafish

915 embryo-larvae at (C) 24 hpf and (D) 96 hpf following exposure to (i) Control (ii) 1 µM,
916 (iii) 10 µM and (iv) 100 µM showing— 1. oedema, 2. head deformity, 3. short tail, 4.
917 bent spine, 5. lower jaw deformity and 6. yolk sac deformity. Scale bar=100 µm.

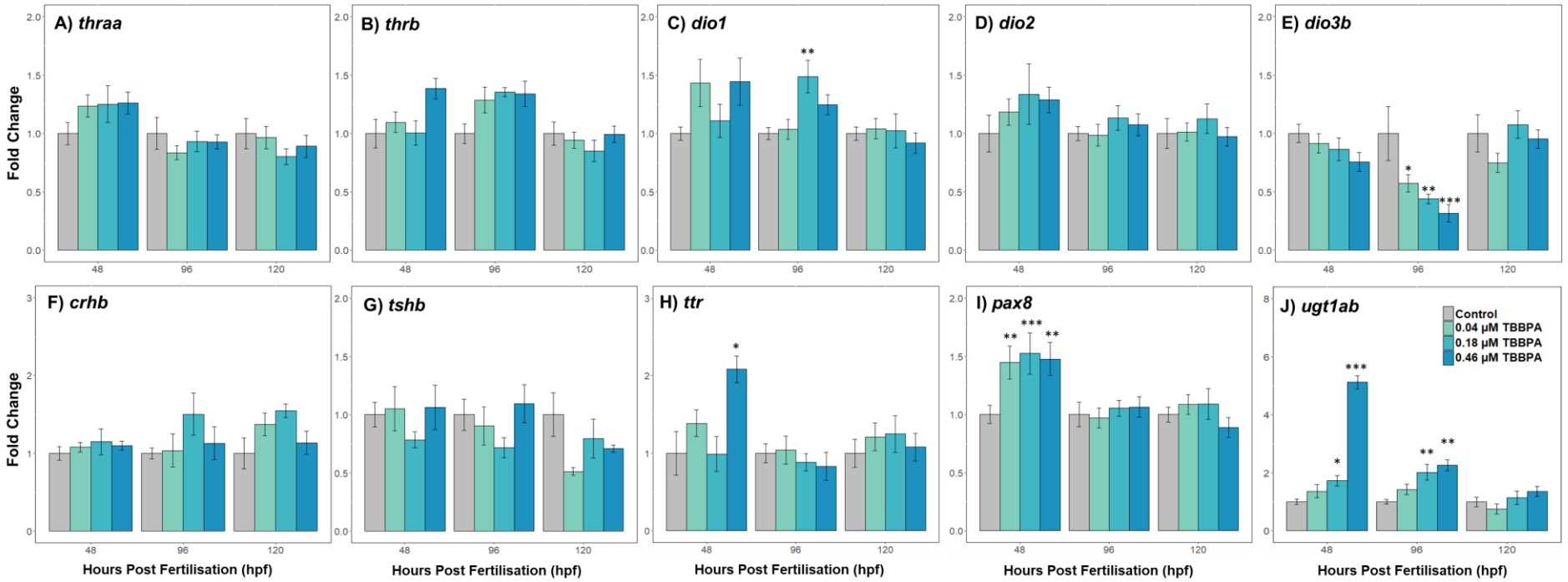


Figure 4. Transcript profile of genes in the HPT axis of zebrafish embryo-larvae exposed to TBBPA. Changes in (A) thyroid receptor alpha (*thraa*), (B) thyroid receptor beta (*thrb*), (C) deiodinase type I (*dio1*), (D) deiodinase type II (*dio2*), (E) deiodinase type III (*dio3b*), (F) corticotropin-releasing hormone (*crhb),* (G) thyroid-stimulating hormone (*tshb)*, (H) transthyretin (*ttr*), (I) paired box 8 (*pax8)* and (J) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* mRNA levels in whole zebrafish following exposure to TBBPA (0, 0.04, 0.18 and 0.46 µM) for up to 120 hpf. Transcript profiles were determined using qRT-PCR and the relationship between transcript expression and TBBPA concentration was assesses using linear mixed models (model output summarised in Table S6).

925 Plotted data are presented as mean fold changes (normalised against the expression of the control gene *rpl8*) ± SEM compared to
926 the corresponding control group. Outliers, identified as described in the text, were excluded from the analysis, resulting in a
927 replication of n= 6-9 samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

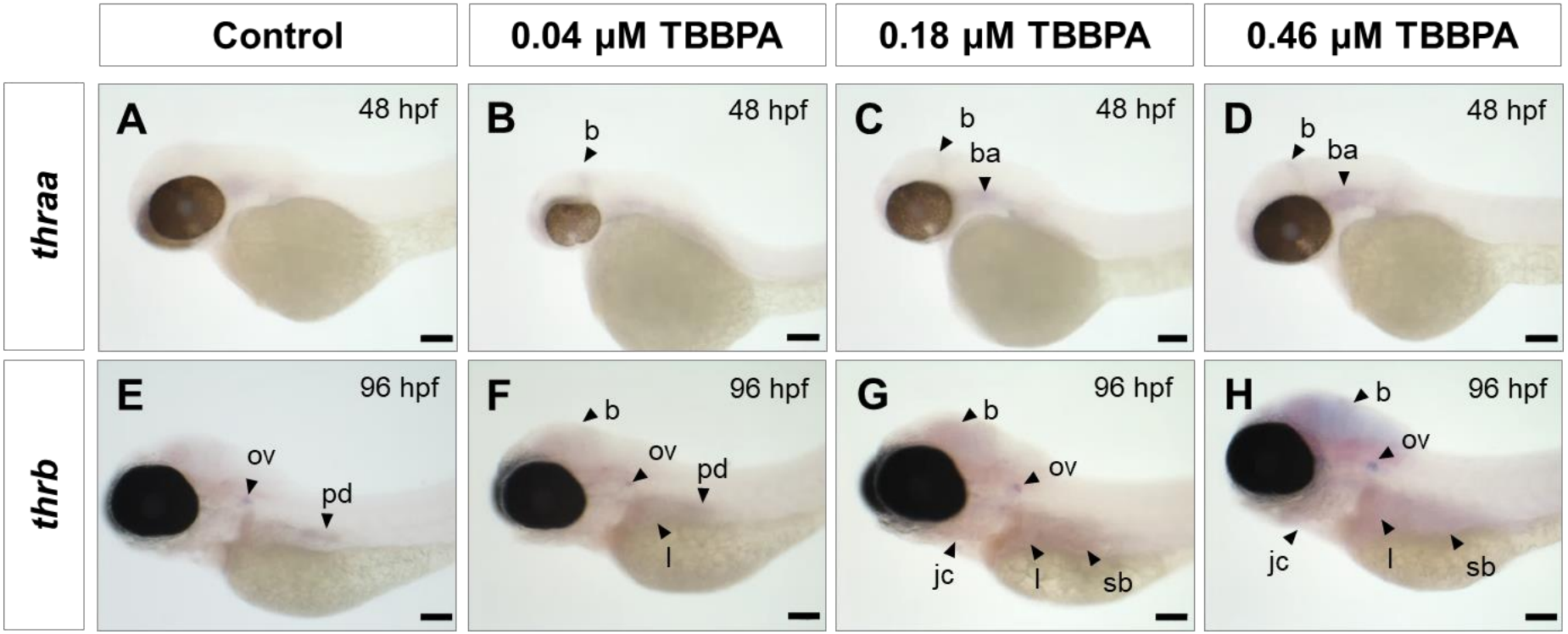
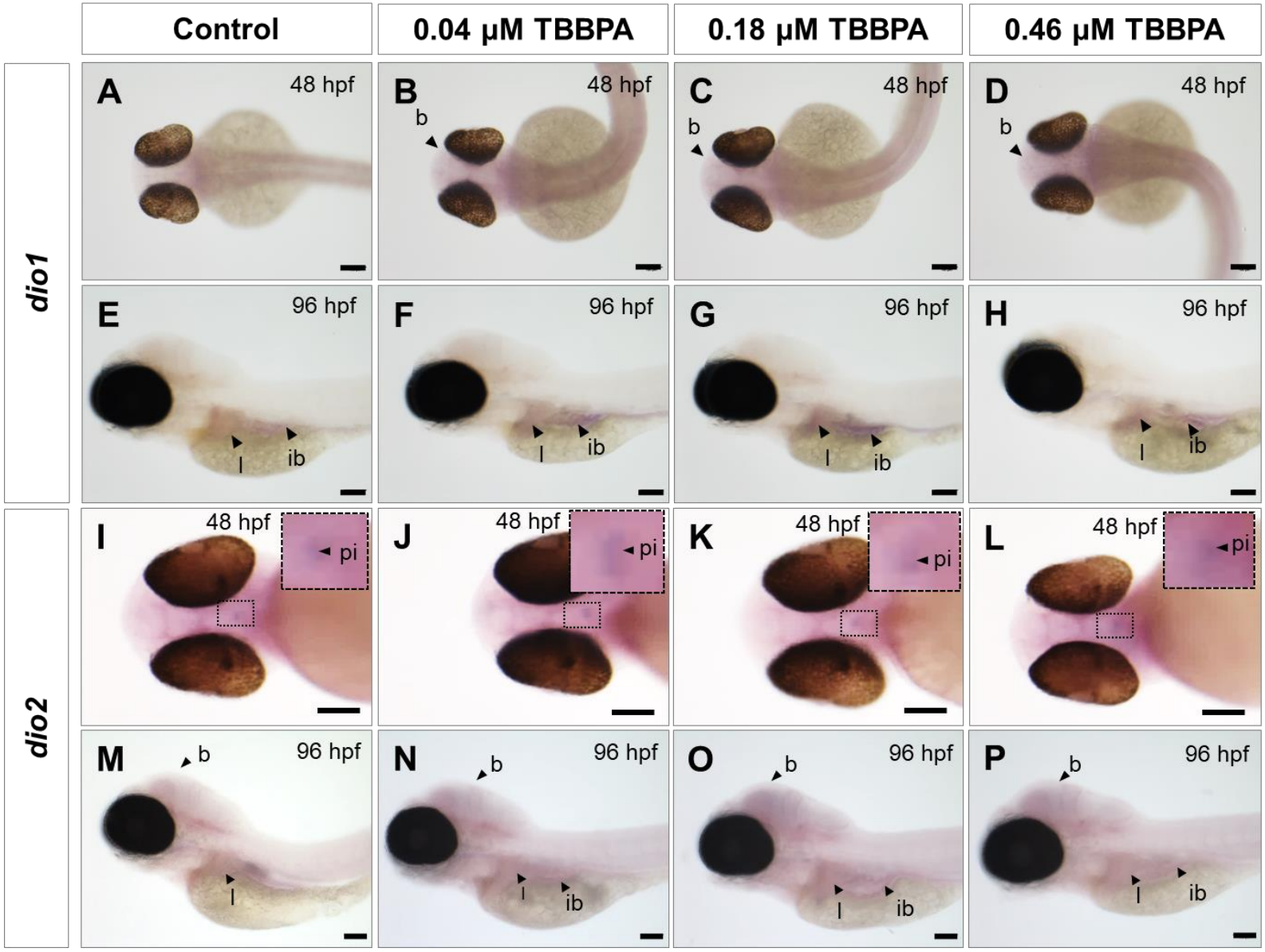


Figure 5. WISH images of TR mRNA expression patterns in zebrafish embryo larvae following exposure to TBBPA. Representative images of (A-D) *thraa* mRNA expression patterns in zebrafish embryos at 48 hpf and (E-H) *thrb* mRNA expression patterns in zebrafish larvae at 96 hpf treated with TBBPA. Lateral (A-D) and dorsal (E-H) views of whole embryo-larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, ba=branchial arches, ov=otic vesicle, l=liver, sb=swim bladder, pd=pronephric duct, jc=jaw cartilage. Scale bar=100 µm. A summary of the variability in mRNA expression in the different zebrafish tissues in provided in Table S7.


Control
0.04 µM TBBPA
0.18 µM TBBPA
0.46 µM TBBPA
dio1
dio2
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
48 hpf
96 hpf
b
l
ib
pi

935

Figure 6. WISH images of deiodinase type I and type II mRNA expression patterns in zebrafish embryo larvae following exposure to TBBPA. Representative images of mRNA expression patterns in zebrafish embryo-larvae treated with TBBPA showing (A-D) *dio1* at 48 hpf, (E-H) *dio1* at 96 hpf, (I-L) *dio2* at 48 hpf and (M-P) *dio2* at 96 hpf. Dorsal (A-D), lateral (E-H, M-P) and ventral (I-L) views of whole embryo-larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. l=liver, ib=intestinal bulb, b=brain, pi=pituitary. Scale bar=100 µm. A summary of the variability in mRNA expression in the different zebrafish tissues in provided in Table S8.

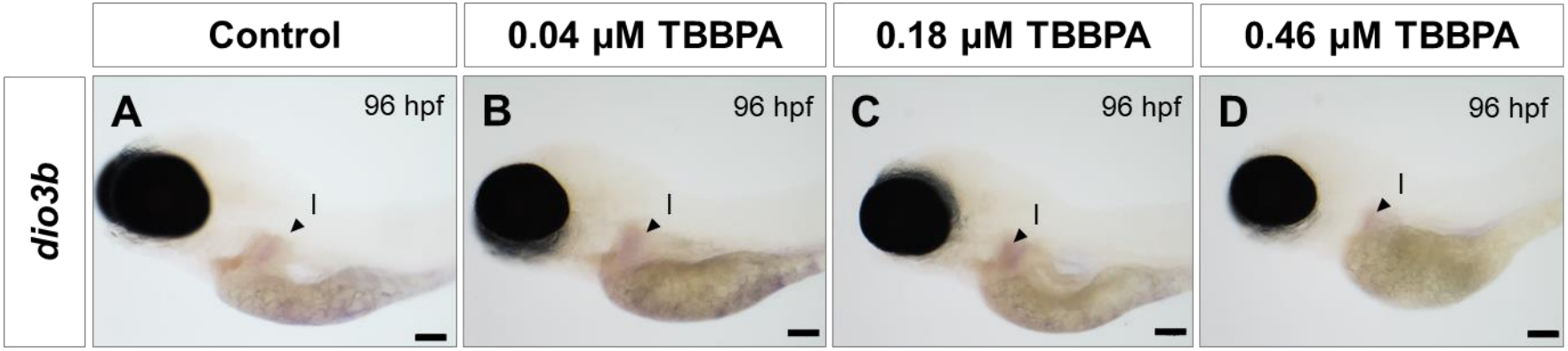


Figure 7. WISH images of deiodinase type III mRNA expression patterns in zebrafish larvae following exposure to TBBPA. (A-D) Representative images of *dio3b* mRNA expression patterns in 96 hpf zebrafish larvae treated with TBBPA. Lateral views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. l=liver. Scale bar=100 µm. A summary of the variability in mRNA expression in the different zebrafish tissues in provided in Table S8.

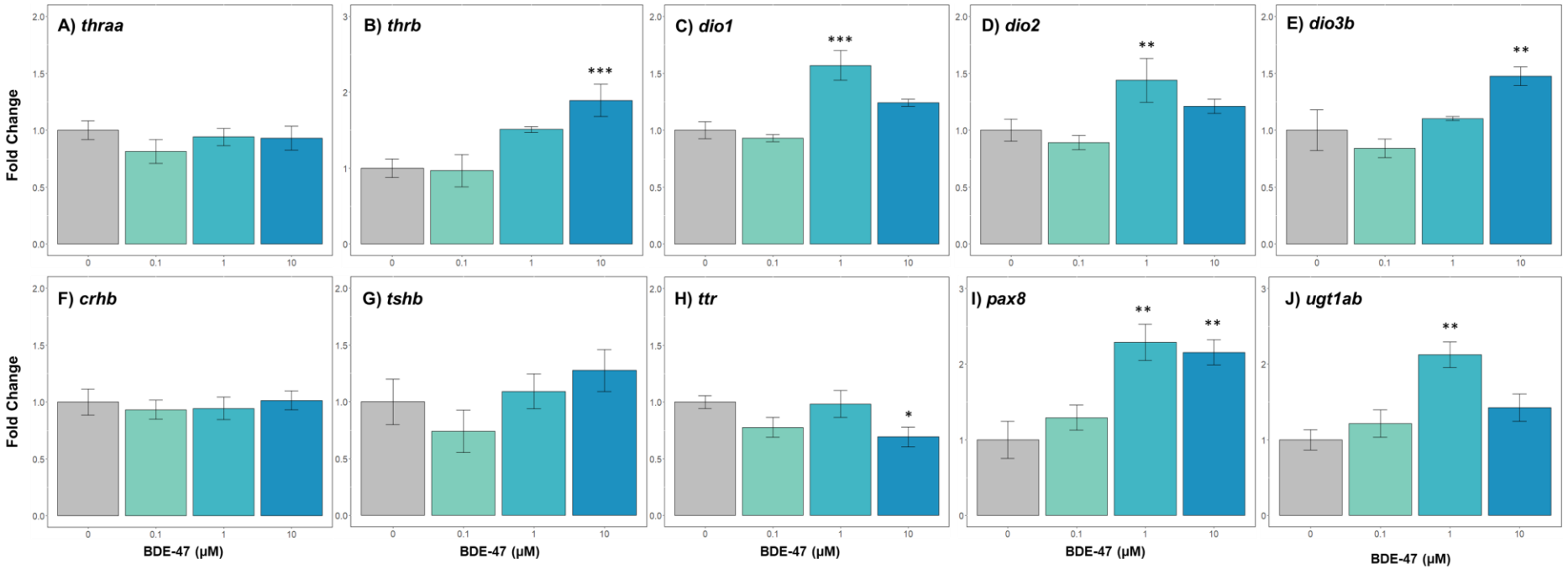


Figure 8. Transcript profile of genes in the HPT axis of zebrafish larvae exposed to BDE-47. Changes in (A) thyroid receptor alpha (*thraa*), (B) thyroid receptor beta (*thrb*), (C) deiodinase type I (*dio1*), (D) deiodinase type II (*dio2*), (E) deiodinase type III (*dio3b*), (F) corticotropin-releasing hormone (*crhb),* (G) thyroid-stimulating hormone (*tshb)*, (H) transthyretin (*ttr*), (I) paired box 8 (*pax8)* and (J) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* mRNA levels in whole zebrafish following exposure to BDE-47 (0, 0.1, 1 and 10 µM) for 96 hpf. Transcript profiles were determined using qRT-PCR and the relationship between transcript expression and TBBPA concentration was assesses using linear mixed models (model output summarised in Table S9). Plotted data are presented as mean fold changes (normalised against the expression of the control gene *rpl8*) ± SEM compared to the corresponding control

group. Outliers, identified as described in the text, were excluded from the analysis, resulting in a replication of n= 5-6 samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

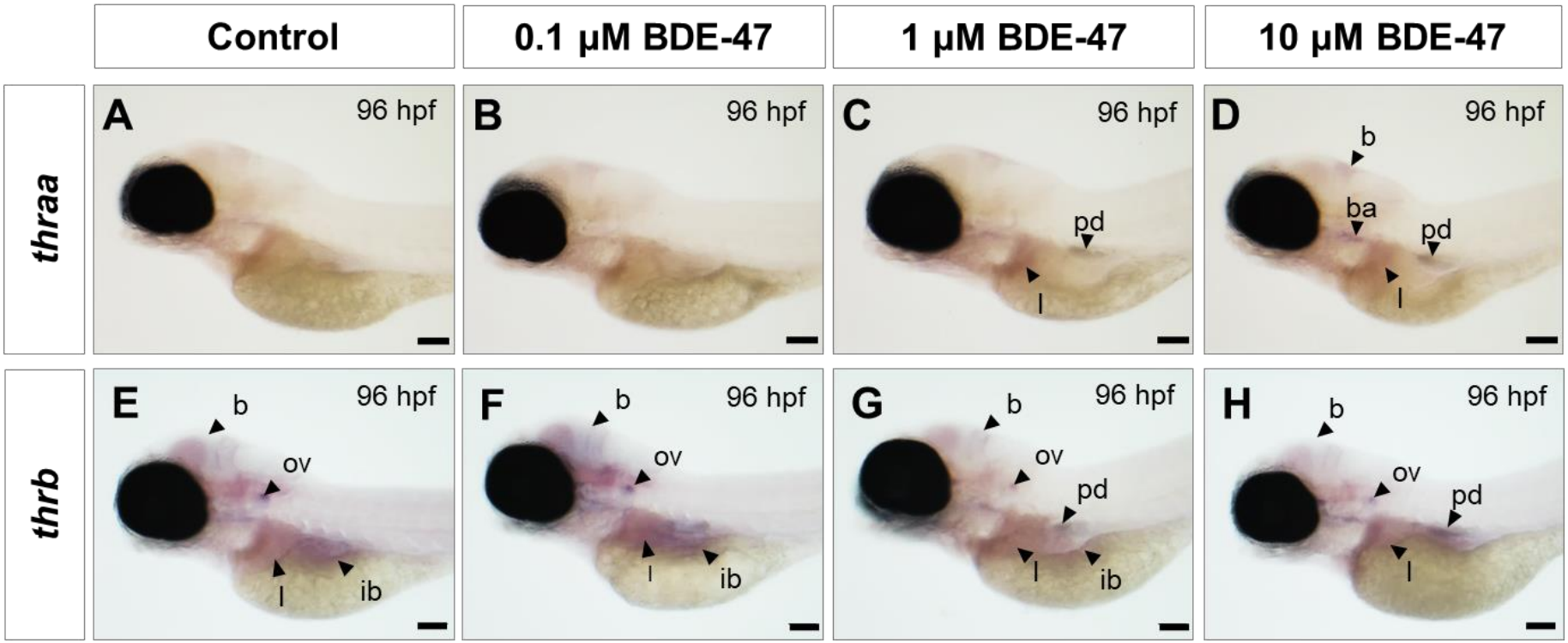


Figure 9. WISH images of TR mRNA expression patterns in zebrafish larvae following exposure to BDE-47. Representative images of (A-D) *thraa* and (E-H) *thrb* mRNA expression patterns in 96 hpf zebrafish larvae treated with BDE-47 (0.1, 1 and 10 µM). Lateral views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, ba=branchial arches, pd=pronephric ducts, l=liver, ov=otic vesicle, ib=intestinal bulb. Scale bar=100 µm. A summary of the variability in mRNA expression in the different zebrafish tissues in provided in Table S10.

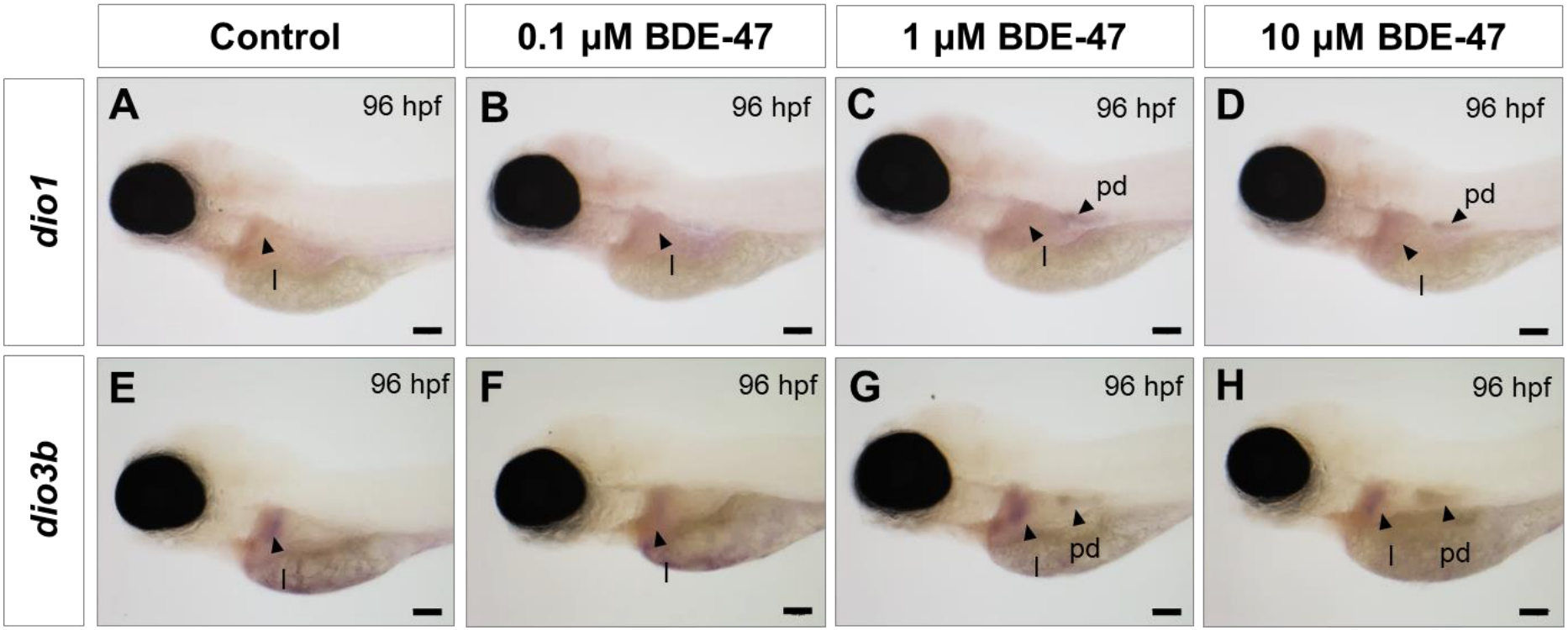
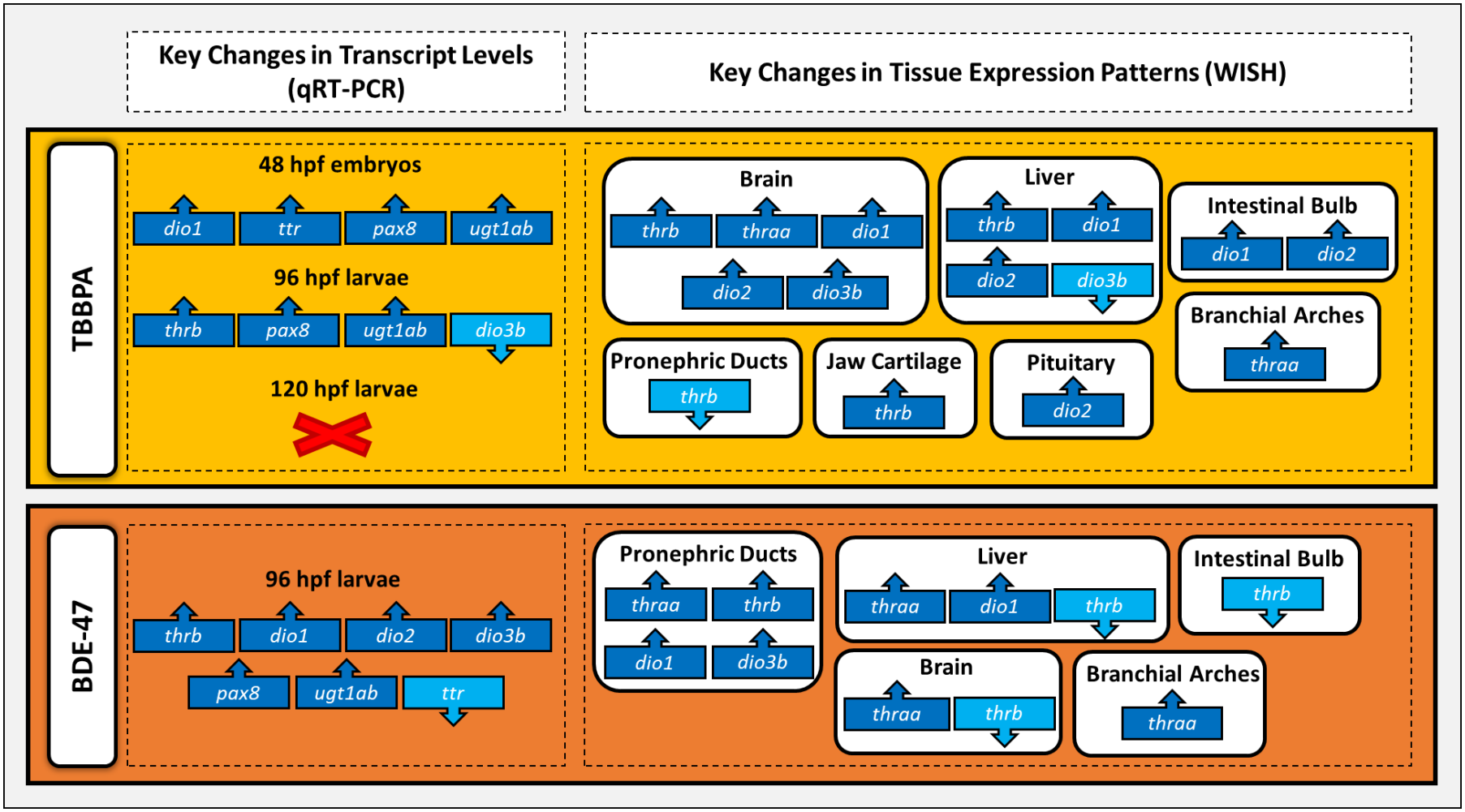


Figure 10. WISH images of deiodinase type I and type III mRNA expression patterns in zebrafish larvae following exposure to BDE-47. Representative images of (A-D) *dio1* and (E-H) *dio3b* mRNA expression patterns in zebrafish larvae at 96 hpf treated with BDE-47 (0.1, 1 and 10 µM). Lateral views of whole larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, pd=pronephric ducts and l=liver. Scale bar=100 µm. A summary of the variability in mRNA expression in the different zebrafish tissues in provided in Table S11.


Key Changes in Transcript Levels (qRT-PCR)
Key Changes in Tissue Expression Patterns (WISH)
TBBPA
48 hpf embryos
dio1
ttr
pax8
ugt1ab
96 hpf larvae
thrb
pax8
ugt1ab
dio3b
120 hpf larvae
Brain
thrb
thraa
dio1
dio2
dio3b
Liver
thrb
dio1
dio2
dio3b
Intestinal Bulb
dio1
dio2
Branchial Arches
thraa
Pronephric Ducts
thrb
Jaw Cartilage
thrb
Pituitary
dio2
BDE-47
96 hpf larvae
thrb
dio1
dio2
dio3b
pax8
ugt1ab
ttr
Pronephric Ducts
thraa
thrb
dio1
dio3b
Liver
thraa
dio1
thrb
Intestinal Bulb
thrb
Brain
thraa
thrb
Branchial Arches
thraa

970

971 Figure 11. Overview of the key changes in the transcript level (measured via qRT-PCR) and tissue expression pattern (measured
972 via WISH) of HPT axis target genes in zebrafish embryo-larvae exposed to TBBPA and BDE-47. Dark blue arrow blocks indicate
973 transcript levels were up-regulated and light blue arrow blocks indicate transcript levels were down-regulated.

974 ## Tables

975 Table 1: LC_x and EC_x values (µM) with corresponding 95% confidence intervals for
976 zebrafish embryo-larvae following 96-h exposures to TBBPA and BDE-47.

	TBBPA	BDE-47
LC_{10}	0.6 (0.5-0.7)	NC
LC_{50}	0.9 (0.8-1)	NC
LC_{90}	1.3 (1.2-1.5)	NC
EC_{10}	0.4 (0.3-0.5)	0.03 (0-0.23)
EC_{50}	0.7 (0.6-0.9)	38.2 (10.4-475.9)
EC_{90}	1.25 (0.9-2.2)	NC

977