Expression dynamics of genes in the hypothalamic-pituitary-thyroid (HPT) cascade and their responses to 3,3′,5-triiodo-L-thyronine (T3) highlights potential vulnerability to thyroid-disrupting chemicals in zebrafish (*Danio rerio*) embryo-larvae.

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Abstract

Some chemicals in the environment disrupt thyroid hormone (TH) systems leading to alterations in organism development, but their effect mechanisms are poorly understood. In fish, this has been limited by a lack of fundamental knowledge on thyroid gene ontogeny and tissue expression in early life stages. Here we established detailed expression profiles for a suite of genes in the hypothalamic-pituitary-thyroid (HPT) axis of zebrafish (*Danio rerio*) between 24-120 hours post fertilisation (hpf) and quantified their responses following exposure to 3,3',5-triiodo-L-thyronine (T3) using whole mount *in situ* hybridisation (WISH) and qRT-PCR (of whole-body extracts). All of the selected genes in the HPT axis demonstrated dynamic transcript expression profiles across the developmental stages examined. The expression of thyroid receptor alpha (*thraa)* was observed in the brain, gastrointestinal tract, craniofacial tissues and pectoral fins, while thyroid receptor beta (*thrb)* expression occurred in the brain, otic vesicles, liver and lower jaw. The TH deiodinases (*dio1, dio2* and *dio3b*) were expressed in the liver, pronephric ducts and brain and the patterns differed depending on life stage. Both *dio1* and *dio2* were also expressed in the intestinal bulb

(96-120 hpf), and *dio2* expression occurred also in the pituitary (48-120 hpf). Exposure of zebrafish embryo-larvae to T3 (30 and 100 μg L^{-1}) for periods of 48, 96 or 120 hpf resulted in the up-regulation of *thraa*, *thrb*, *dio3b*, thyroid follicle synthesis proteins (*pax8*) and corticotropin-releasing hormone (*crhb)* and down-regulation of *dio1*, *dio2*, glucuronidation enzymes (*ugt1ab*) and thyroid stimulating hormone (*tshb)* (assessed via qRT-PCR) and responses differed across life stage and tissues. T3 induced *thraa* expression in the pineal gland, pectoral fins, brain, somites, gastrointestinal tract, craniofacial tissues, liver and pronephric ducts. T3 enhanced *thrb* expression in the brain, jaw cartilage and intestine, while *thrb* expression was suppressed in the liver. T3 exposure suppressed the transcript levels of *dio1* and *dio2* in the liver, brain, gastrointestinal tract and craniofacial tissues, while *dio2* signalling was also suppressed in the pituitary gland. *Dio3b* expression was induced by T3 exposure in the jaw cartilage, pectoral fins and brain. The involvement of THs in the development of numerous body tissues and the responsiveness of these tissues to T3 in zebrafish highlights their potential vulnerability to exposure to environmental thyroid-disrupting chemicals.

Keywords: thyroid hormone, zebrafish, development, gene expression, endocrine disruption

1. Introduction

The ability of xenobiotic compounds to alter endocrine function has been reported widely over the last two decades, with attention largely focused on chemicals that disrupt the reproductive system of humans and wildlife (Tyler et al., 1998). Growing awareness of the role of thyroid hormones (TH) during development has led to

increasing concern over environmental contaminants which act as thyroid-disrupting chemicals (TDCs), such as polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), perchlorates, phthalates and brominated flame retardants (BFR) (Boas et al., 2006). These TDCs act via a wide variety of mechanisms to disrupt TH homeostasis in vertebrates. For example, some compounds can alter the function of the thyroid gland itself, by inhibiting the uptake of iodide and/or inhibiting the activity of thyroid peroxidase and subsequently decreasing TH synthesis. Other compounds act by altering the biliary elimination of TH via the induction of the metabolising enzymes, altering the activity of blood and cellular TH transporters, interfering with hepatic, serum and target tissue deiodinase activity and/or altering TH-responsive genomic signalling in target tissue (reviewed in (Crofton, 2008)). Aquatic and semi-aquatic species, including fish and amphibians, are especially vulnerable to TDCs, with uptake occuring via the skin and gills, via the diet and they can even be transferred to the offspring of exposed adults (Brown et al., 2002; Kim et al., 2011; Wu et al., 2009; Yu et al., 2011).

TH dynamics are primarily under the control of the hypothalamic-pituitary-thyroid (HPT) axis, a complex regulatory network which coordinates TH synthesis, secretion, transport and metabolism (Zoeller et al., 2007). L-3,5,3',5'-Tetraiodothyronine (Thyroxine; T4) is the main TH secreted by the thyroid follicles of teleost fish, but 3,3′,5-triiodo-L-thyronine (T3) is the biologically active form that is under the control of peripheral tissues (Power et al., 2001). The genomic actions of THs depend on the binding of T3 with nuclear thyroid hormone receptors (TRs) and the subsequent interaction with specific thyroid response elements (TREs) in the promoters of target genes, which either enhance or repress their transcription (Power et al., 2001). The

iodothyronine deiodinase enzymes type I, II and III (D1, D2 and D3) regulate the activity of TH by removing iodine moieties from T3 or T4. D2 generates T3 via deiodination of T4, while D3 produces the inactive metabolites, 3,5',3'-triiodothyronine (rT3) and 3,3'-diiodothyronine (3,3′-T2) by deiodination of T4 and T3, respectively. D1 is kinetically inefficient and can deiodinate both the inner and outer rings of T4, and therefore has activating and inactivating abilities (Power et al., 2001).

THs are involved in a variety of physiological and developmental processes in vertebrates. In teleost fish, developmental roles include mediating the metamorphic transition from larval to adult stages and influencing the maturation of tissues including bone, gonads, intestine and the central nervous system (Campinho et al., 2014; Matta et al., 2002; Power et al., 2001). In adults, they modulate growth, energy homeostasis, cardiac rhythm, the smolting process, osmoregulation and the behaviours/physiology associated with rheotaxis and migration (Boeuf et al., 1989; Eric et al., 2004; Godin et al., 1974). Consequently, even minor alterations in TH levels, particularly during sensitive developmental windows, can have significant acute and potentially long-term health effects. In recent years, the expression profiles of several genes in the HPT axis of teleost fish have been used as indicators for thyroid disruption by different environmental pollutants (Parsons et al., 2019; Shi et al., 2009). However, the spatial and temporal expression of many of these genes during zebrafish early life stages and their regulation by THs have not been thoroughly evaluated. A greater understanding of the transcriptional dynamics of TH-related genes in teleost fish would greatly facilitate the identification of target genes, tissues and developmental stages which may be particularly sensitive to TDCS.

In this study, we studied the ontogeny of expression of several genes in the HPT axis of zebrafish *(Danio rerio),* and both identified their tissue localisations and measured their regulatory responses to THs. We first characterised the expression dynamics of genes of the HPT axis from 24 to 120 hours post fertilisation (hpf), and subsequently examined the responses of these genes to T3 at key developmental stages. The overall objective of this work was to establish whether T3 differentially regulated the gene ontogenic expression of target genes and by doing so identify some of the potential mechanisms and thyroid targets related to TDCs. We used a combination of both whole-mount *in situ* hybridisation (WISH) assays to identify tissue-specific gene expression patterns in whole zebrafish embryo-larvae, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays to quantify changes in gene transcript levels in whole body extracts.

2. Materials and Methods

2.1 Materials and reagents

3,3',5-Triiodo-L-thyronine (T3; CAS 6893-02-3) (purity ≥ 95%) was purchased from Sigma-Aldrich (Gillingham, UK). T3 stock solutions were prepared in dimethylsulfoxide (DMSO).

2.2 Maintenance of zebrafish and embryo collection

Zebrafish [*casper* (*mitfa; roy*) mutant strain] embryos were collected from breeding adults at the University of Exeter, as described by Parsons *et al*., (2019). (Parsons et al., 2019). All experiments were carried out according to the UK Home Office regulations and approved protocols.

2.3 HPT axis gene transcript levels during early development

One hundred fertilised embryos were randomly placed into three glass tanks with 100 ml of embryo culture water. At the desired developmental stage (24, 48, 72, 96 and 120 hpf), 80 individuals from each tank were sampled for WISH experiments and the remaining 20 embryo-larvae were pooled and sampled for qRT-PCR analyses, as described by Parsons *et al*., 2019 (Parsons et al., 2019). Each experiment was repeated three times.

2.4 The effect of T3 treatment on gene transcripts in the HPT axis

An initial range finding study was carried out to determine the toxicity of T3 to zebrafish embryo-larvae. T3 exposures were carried out at 10, 30, 100 and 300 µg L^{-1} (in 0.01% DMSO). These doses were chosen based on a study by Liu and Chan (2002) showing that increased TR transcript levels occurred in zebrafish embryos exposed to T3 at 50 nM (approx. 30 µg L^{-1})(Liu and Chan, 2002). Embryo-larvae in control groups were incubated in DMSO at 0.01%. Twenty fertilised embryos were selected for each treatment group. Exposures were conducted for 96 h from fertilisation and half of the exposure solutions were replaced every 24 h. The number of dead embryo-larvae, hatching success, phenotypic deformities and swim bladder inflation success were recorded every 24 h. Experiments were carried out in triplicate and each experiment was repeated three times. In this range finding study, T3 at 30 and 100 µg L^{-1} induced morphological deformities but no significant mortalities in zebrafish embryo-larvae and therefore these concentrations were selected for subsequent gene transcript analysis experiments.

To assess the effect of T3 on gene transcripts in the HPT axis, zebrafish embryos were exposed to exogenous T3 at concentrations of 0, 30 and 100 µg L^{-1} (in 0.01% DMSO), for 48, 96 and 120 h from fertilisation. Control groups were incubated in embryo culture water with 0.01% DMSO. Fifty fertilised embryos were allocated to each treatment group and each treatment group was performed in triplicate. At the desired developmental stage, 40 individuals from each group were sampled for WISH and 10 individuals from each group were pooled and sampled for qRT-PCR analyses. The experiment was repeated three times.

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

The following target genes in the HPT axis of zebrafish were selected for qRT-PCR analyses: *corticotropin-releasing hormone* (*crhb*), *deiodinases type I, II and III* (*dio1*, *dio2*, *dio3b*), *paired box 8* (*pax8*), *thyroid receptors* (*thraa* and *thrb*), *transthyretin* (*ttr*), *thyroid-stimulating hormone* (*tshb*) and *uridine diphosphate-glucuronosyltransferase* (*ugt1ab*). qRT-PCR assays were optimised and carried out according to the protocol described previously (Parsons et al., 2019) and the sequences of primers used to quantify target gene expressions are provided in the supplementary material (Table S1). Briefly, total RNA was extracted using Tri reagent (Sigma-Aldrich, UK) according to the manufacturer's protocol. RNA was treated with RQ1 RNase-Free DNase (Promega, Southampton, UK) and subsequently reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega) and random hexamers (Eurofins Genomics), following manufacturer's instructions. The expression of target mRNA was subsequently determined by qPCR using target-specific SybrGreen assays on a

CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Ltd., Hercules, CA, US). For this, there was an initial activation step of 95˚C for 15 min followed by 40 cycles of denaturation (95˚C, 10 s) and annealing (appropriate annealing temperature, 30s) and a final melt curve analysis (95˚C for 1 min, 55 ˚C for 1 min, followed by 100 cycles each for 5 sec beginning at 55°C and increasing 0.5°C per cycle). Ribosomal protein l8 (*rpl8*) was used as a reference gene as this has been shown to be expressed at a consistent level across tissue types and experimental conditions (Filby and Tyler, 2005) after exposure to a wide range of chemicals and stressors in fish. This includes for (zebrafish exposed to the organophosphorus flame retardant tris(1,3-dichloro-2-propyl) phosphate (Wang et al., 2015), the thyroid disruptor Di-(2 ethylhexyl) phthalate (DEHP) (Jia et al., 2016) and silver (van Aerle et al., 2013), as well as to EE2 in adult mangrove killifish (*Mangrove rivulus*) (Farmer and Orlando, 2012) and to hypoxia in fathead minnow (*Pimephales promelas*) (Hala et al., 2012)). A consistent level of expression of *rpl8* during the different life stages studied was furthermore shown in our pilot work (see: Fig. S1 and S2). Ontogeny results are expressed as fold changes ± standard error (SE), relative to the earliest time point (24 hpf). T3 exposure results are expressed as fold-changes relative to the control ± SE.

2.6 Whole-mount *in situ* hybridisation (WISH)

Tissue-specific changes in the expression of selected genes of interest in the HPT axis were examined via WISH assays. Selected genes included thyroid receptors (*thraa and thrb)*, deiodinases *(dio1, dio2 and dio3b)* and transthyretin (*ttr*). The WISH protocol used in this study was modified from Thisse and Thisse (Thisse and Thisse, 2008) and detailed methodologies are provided in Parsons *et al.,* 2019 (Parsons et al.,

2019). Briefly, digoxigenin (DIG) antisense RNA probes were synthesised using purified zebrafish target gene DNA, that were subsequently treated with DNAse, purified by lithium chloride precipitation and diluted with hybridisation buffer. For WISH assays, fixed embryos were rehydrated through a series of PBS washes, treated with proteinase K and hybridised with an antisense probe (100 µL of hybridisation buffer containing approximately 15 ng of antisense DIG-labelled RNA probe) overnight at 65˚C. Embryos were subsequently incubated in blocking solution and then incubated with an anti-DIG antibody conjugated with alkaline phosphatase (Roche; x5000, diluted 1/100 with blocking solution). Embryos were then stained in BM-Purple AP Substrate (Roche) until signal or background staining became visible. Staining times varied depending on the probe (Table S2 and S3). Embryos were observed and photographed using Nikon SMZ1500 microscope equipped with a digital camera.

2.7 Statistical analyses

Statistical analyses were performed in R (R Studio, 1.1.423) (RStudio Team, 2016). Using the *lme4* package, general linear mixed models (GLMM) with Gaussian error structures were used to assess the differences in gene transcript levels between zebrafish developmental stages (Bates et al., 2015). Maximum likelihood tests of the full model against a reduced model were performed to obtain p values. Developmental stage was incorporated as a fixed effect into the model and each experiment was incorporated as a random effect. When a significant effect was identified, pair-wise comparisons were conducted to determine within group differences using the *multcomp* package within R (Hothorn et al., 2008). A similar approach using GLMMs was used to examine the differences in transcript levels between the thyroid receptor

and deiodinase encoding genes at each of the developmental stages. Again, GLMMs were used to examine the effect of T3 treatment on gene transcripts levels, with T3 treatment incorporated as a fixed effect into the model. Prior to the analysis of all gene transcript data, outliers in the gene expression data were identified according to Chauvenet's criterion and subsequently removed (Chauvenet, 1863). Shapiro–Wilk tests were used to test for equal variance and normality and non-normal data were either log transformed or inverse transformed. Generalised linear mixed models (GLzMM) with binomial error structures were used to asses the effects of T3 treatment on measured toxicological endpoints (mortality, hatching success, deformities and swim bladder inflation), with T3 treatment incorporated as a fixed effect into the model and random intercepts incorporated for each experiment. When a significant effect was identified, pair-wise comparisons were conducted to determine within group differences as outlined above. All data was considered statistically significant when $P < 0.05$. Data was plotted using the *ggplot2* R package (Wickham, 2009).

3. Results

3.1 HPT axis gene transcript levels during early development

3.1.1 Whole-body transcript levels (qRT-PCR)

The transcript profiles of the 10 genes studied followed a similar pattern of expression during embryogenesis, with relatively low levels at 24 hpf increasing significantly at either 48 hpf or 72 hpf (Fig. 1A-J, Table S4-5). The transcript levels of *thraa*, *tshb* and *pax8* peaked between 48-72 hpf and subsequently declined thereafter. In contrast, transcript levels of *thrb*, *ttr*, *dio1*, *dio2*, *dio3*, *crhb* and *ugt1ab* continued to increase further at 96 hpf and/or 120 hpf. Levels of *thrb* transcripts were significantly higher

than *thraa* transcripts throughout all of the developmental stages tested ($p<0.001$; Fig. S3A, Table S6A). Similarly, *dio3b* mRNA levels were significantly higher than for *dio1* and *dio2* at all stages from 24-120 hpf ($p<0.001$; Fig. S3B, Table S6B), while *dio1* transcript levels were significantly higher than *dio2* levels at 24, 96 and 120 hpf ($p<0.001$; Fig. S3B, Table S6B)

3.1.2 Tissue transcript expression patterns (WISH)

At 24 hpf, *thraa* signalling was detected in the brain and head region (Fig. 2A). Subsequently, at 48 hpf, *thraa* expression signals were observed in the branchial arches of the head, pronephric ducts, pectoral fins and throughout the brain (Fig. 2B). Expression in these tissues persisted between 72-120 hpf, with additional and/or enhanced signalling detected in the cartilage of the lower jaw, pericardia, liver, intestinal tract and brain (Fig. 2C-E).

At 24 hpf, *thrb* expression was observed in the brain, in a small group of cells in the region of the otic vesicle and was highly localised in the prospective fin buds (Fig. 2F). *Thrb* expression in the brain and otic vesicles was observed at all other stages examined (48-120 hpf). *Thrb* expression was also observed in the pectoral fins and/or pronephric ducts at 48 and 72 hpf, and in the liver and cartilage of the lower jaw at 96 and 120 hpf (Fig. 2G-J).

Ttr signalling was detected throughout the brain and head region at 24 hpf (Fig. 2K). No specific *ttr* signalling was observed at 48 hpf, though subsequently, at 72 hpf, a

strong *ttr* expression signal was seen in the left lobe of the liver (Fig. 2L-M), with greater and more expansive expression in the liver at 96 and 120 hpf (Fig. 2N-O).

During the embryonic stages of development (24-72 hpf), expression of *dio1* was not detected (Fig. 3A-C). At 96 hpf, *dio1* was expressed in the brain, liver, intestinal bulb and pronephric ducts and this expression pattern persisted at 120 hpf, with additional expression observed in the intestine (Fig. 3D-E). *Dio2* signalling was first detected at 48 hpf in the pituitary gland (Fig. 3F-G). Between 72-120 hpf, *dio2* signalling persisted in the pituitary gland and occurred in the brain, liver, pronephric ducts, intestinal bulb and intestine (Fig. 3H-J). A strong *dio3b* signal was detected in 24 hpf embryos initially in the pronephric ducts (Fig. 3K) and then subsequently in the brain (48 hpf) and prospective liver (72 hpf). At these times the *dio3b* signal in the pronephric ducts appeared to be greatly reduced (Fig. 3L-M). A strong *dio3b* expression signal was detected in the liver and yolk syncytial layer in the embryo-larvae at 96 and 120 hpf (Fig. 3N-O).

3.3 Effects of T3 on gene transcripts in the HPT axis

3.3.1 Toxicity of T3

T3 had no significant effect on mortality and hatching success across the whole concentration range tested (10-300 µg L^{-1}) compared with the controls (Fig. 4A-B, Table S7-8). The number of surviving zebrafish larvae showing morphological deformities increased significantly in all treatment groups, with 16 ± 6%, 62 ± 9%, 74 ± 8%, 93 ± 3% mean deformities observed in the 10, 30, 100 and 300 µg T3 L^{-1} exposure groups, respectively ($p<0.001$, Fig. 4C, Table S7-8). At 72 hpf and 96 hpf,

larvae exposed to T3 had deformities such as small eyes, oedema, curved spine, swollen yolk sac, reduced pigmentation and craniofacial deformities (Fig. 4E-F). At 96 hpf, the number of surviving larvae with inflated swim bladders significantly decreased in all T3 exposure groups, with mean swim bladder inflation at 97 ± 2%, 85 ± 6%, 44 ± 10%, 36 ± 10% and 11 ± 3% in groups exposed to 10, 30,100 and 300 µg T3 L^{-1}, respectively ($p<0.001$, Fig. 4D, Table S7-8).

3.3.2 Whole-body transcript levels (qRT-PCR)

All ten target genes examined in the HPT axis were significantly altered in whole-body zebrafish samples following exposures to exogenous T3, and changes were dependent on the developmental stage and T3 exposure concentration (Fig. 5A-J, Table S9-10). At all time points, transcript levels of *thraa*, *thrb* and *dio3b* were significantly higher in T3 treated groups compared with controls, while *pax8* and *crhb* transcript levels were elevated only in T3 treated embryos at 48 and 96 hpf, respectively. *Dio1* (96 and 120 hpf), *dio2* (96 hpf), *ttr* (96 hpf) and *ugt1ab* (120 hpf) levels were significantly reduced in T3 groups compared to controls, although this effect was most pronounced for *dio1.* Transcript levels of *tshb* were significantly reduced in T3 exposed embryos compared with the control at 48 hpf, while in 120 hpf exposed larvae, *tshb* levels were significantly elevated.

3.3.3 Tissue transcript expression (WISH)

At 48 hpf, *thraa* mRNA expression was observed in the pineal gland, pectoral fins, branchial arches, pronephric ducts, pronephros, brain and the somites of T3 exposed individuals (both 30 and 100 µg L^{-1} groups), whereas limited signal was detected in

controls embryos (Fig. 6A-C). At 120 hpf, a strong (and concentration-related) *thraa* signal was observed in the pineal gland, lower jaw cartilage, liver, pectoral fins, brain and intestine of T3 exposed larvae, whereas a weak signal in the brain was the only *thraa* expression observed in control larvae (Fig. 6D-F).

A strong *thrb* expression signal was observed in the brain of 48 hpf embryos exposed to T3 (30 and 100 µg L^{-1}), whereas expression in control embryos appeared weak (Fig. 6G-I). The *thrb* expression pattern was similar in control and T3 exposed larvae at 120 hpf, however the signal intensity across the tissues appeared to differ between groups. For instance, *thrb* signal appeared to be enhanced in the lower jaw, intestine and brain (with a pronounced effect on the hindbrain) compared to the controls, whereas expression appeared to be reduced in the liver and otic vesicles of larvae exposed to 100 µg T3 L^{-1} (Fig. 6J-L). At 96 hpf, the expression of *ttr* appeared to be reduced in the liver of larvae exposed to both T3 concentrations (30 and 100 µg L^{-1}) compared with controls (Fig. 6M-O).

T3 treatments had no apparent effect on the tissue expression patterns of *dio1* at 48 hpf (Fig. S4A-C), but subsequently, at 96 hpf, the *dio1* expression signal in the liver, brain, swim bladder, intestinal bulb and jaw cartilage appeared to be reduced in T3-treated (100 µg L^{-1}) larvae compared to controls (Fig. 7A-C).

At 48 hpf, there was a strong *dio2* expression signal in the pituitary gland of control embryos, however the signal appeared weak or was not detected in a number of T3-exposed embryos (Fig. 7D-F). In the control groups, *dio2* expression was detected in the pituitary of 96% of embryos, but only in 65% and 48% of embryos treated with 30

and 100 µg T3 L^{-1}, respectively. At 96 hpf, the overall expression pattern of *dio2* was similar between control and T3-treated larvae, however the signalling intensity in the liver, intestinal bulb, intestine, and brain appeared to be weaker in T3-exposed (100 µg L^{-1}) individuals compared with controls (Fig. 7G-I). In addition, the proportion of individuals in which *dio2* expression was detected in the pituitary gland was greatly reduced in T3-exposed lavae compared to controls. For example, *dio2* signalling was detected in the pituitary gland of 54% of control individuals compared with only 4% and 0% of individuals treated with 30 and 100 µg T3 L^{-1}, respectively. When *dio2* signalling was detected in T3-treated (30 µg T3 L^{-1}) larvae, the intensity appeared greatly reduced compared with control larvae (Fig. 7Gi-Ii).

A clear *dio3b* signal was observed in the pectoral fins and pronephric ducts of 48 hpf control embryos, and this signal appeared to be enhanced in embryos exposed to both 30 and 100 µg T3 L^{-1}(Fig. 7J-L). At 96 hpf, *dio3b* expression was observed in the liver and yolk syncytial layer of control larvae, however, *dio3b* expression was additionally observed in the brain, lower jaw cartilage, caudal fin and pectoral fins of the T3-treated individuals (Fig. 7M-O). Furthermore, the *dio3b* signal in the yolk syncytial layer appeared to be stronger, while the signal in the liver appeared weaker, in the T3-treated larvae compared with controls (Fig. 7M-O).

4. Discussion

Here we report novel data regarding TH signalling in the developing zebrafish, including ontogenic transcript profiles of key genes in the HPT axis (including those involved in TH transport, metabolism, synthesis and signalling), their tissue specific

expression patterns during early life stages and the effects of T3 exposure on these transcript profiles and expression patterns. Our findings indicate that: (1) genes encoding key TH-related molecules are expressed in the developing zebrafish in a highly spatio-temporal manner and, (2) T3 regulates the expression of several key genes in the HPT axis pathway which varies across life stages and tissues. These findings increase our understanding of the role and regulation of THs during fish early life stages and identify potential mechanistic pathways and target tissues for TDCs.

4.1. Transcript profiling of TH-related genes.

Here, based on PCR data, we have shown that the transcript levels of a number of the examined TH-related genes (*ttr, dio1, dio2, dio3b, crhb, ugt1ab)* were relatively low in 24 hpf zebrafish embryos, increasing considerably thereafter (either at 48 hpf or 72 hpf) and remaining at relatively high levels until 120 hpf. In contrast, we found that while transcript levels of *thraa*, *thrb, pax8* and *tshb* increased at the 48 hpf life stage, their levels were subsequently reduced at either the 96 hpf and/or 120 hpf life stages. This increased expression of the examined transcripts after the 24 hpf coincides with a number of key TH-dependent processes including the development of fully functioning thyroid follicles and the initial zygotic synthesis of THs, hatching, and the early stages of the embryonic to larval transitory phase, which marks the switch from yolk sac dependency to exogenous feeding larvae (Liu and Chan, 2002; Opitz et al., 2012; Porazzi et al., 2009; Wendl et al., 2002). The ontogenic profiles established in this study confirm that transcript levels of these genes are expressed differentially and they align for the most part with previous reports in developing zebrafish (Liu and Chan, 2002; Vergauwen et al., 2018; Walter et al., 2018). Some small differences in

the transcript profiles of the TR and deiodinase encoding genes where evident in comparison to the above studies. For example, while we observed that both *thraa*, *thrb* and *dio1* mRNA levels increased significantly from 48 hpf, Walter *et al.,* (2018) reported that *thraa* and *dio1* transcript levels in zebrafish did not increase significantly until the 72 hpf life stage, and *thrb* levels did not significantly change between 12-120 hpf (Walter et al., 2018). Liu and Chan (2002) reported a similar *thrb* transcript profile to that observed here, however, in their study *thraa* levels only increased after hatching (Liu and Chan, 2002). As far as we are aware no studies to date have characterised the ontogenic expression of *pax8*, *crhb* and *ugt1ab* in zebrafish early life stages.

In our comparisons of the TR- and deiodinase-encoding gene transcript profiles during zebrafish embryo-larval development, we found that *thrb* levels were consistently higher than *thraa* at all stages examined. This suggests an important role of *thrb* during these early developmental stages and this is consistent with the study by Liu and Chan (2002) which examined TR transcript levels in zebrafish from 1-168 hpf (Liu and Chan, 2002). Interestingly and in contrast to our results, Marelli *et al.,* (2016) more recently observed that zebrafish *thraa* expression was predominant over *thrb* expression between 1-120 hpf (Marelli et al., 2016). We observed that *dio3b* levels were higher than *dio1* and *dio2* levels at all the developmental stages examined, consistent with D3's role in deactivating THs and the largely accepted view that D3 protects tissues against high levels of THs (both maternal and zygotic) during vertebrate development (Heijlen et al., 2014). Here we found also that *dio1* transcript levels were often higher than *dio2* transcript levels in zebrafish embryo-larvae (24, 96 and 120 hpf). D2 proteins are considered to be the major TH-activating enzymes, catalysing the conversion of

T4 to T3 and thereby maintaining TH homeostasis systemically and locally (Bianco et al., 2002). Although D1 proteins are capable of activating and deactivating THs, only recently has it been proposed that the primary role of D1 in fish is to activate THs (Van der Geyten et al., 2001). D1, nevertheless, is considered to be less important than D2 in intracellular TH activation during normal zebrafish embryonic development and may only be significant when the activity of D2 is reduced and TH levels are depleted (Schneider et al., 2001; Walpita et al., 2010). Our results suggest that D1 proteins may have a significant role in maintaining TH homeostasis.

4.2 Tissue-specific expression of TH-related genes

WISH analyses demonstrated that *thraa* and *thrb* were expressed in a tissue-specific manner. For example, *thraa* was widely expressed in a number of tissues including the brain, pectoral fin, heart, intestine, pronephric ducts and jaw cartilage, in line with earlier reports on zebrafish early life stages (Bertrand et al., 2007; Marelli et al., 2016). Interestingly, the *thrb* expression pattern observed here was somewhat different to expression patterns previously described. For example, we observed the expression of *thrb* in the liver, heart and pectoral fin which has not previous been observed in WISH observations (Marelli et al., 2016). In addition, we did not observe *thrb* expression in the eye or pituitary gland at any stages examined as previously observed (Bertrand et al., 2007; Marelli et al., 2016).

In terms of developing a better understanding the role of TR encoding genes during early development, through the WISH analyses we show the co-expression of *thraa* and *thrb* in several zebrafish tissues at specific developmental stages (both genes were co-expressed in the brain between 24-120 hpf, pronephric ducts between 48-72

hpf, liver between 72-120 hpf, pericardium at 72 hpf, craniofacial tissue at 120 hpf and pectoral fins at 72 hpf) which may indicate co-operative roles in mediating the actions of THs. Previous studies have also detected the simultaneous expression of both *thraa* and *thrb* in the brain (Bertrand et al., 2007; Marelli et al., 2016), liver (Lema et al., 2009; Nelson and Habibi, 2006), skeletal tissues (Yamano and Miwa, 1998) and heart (Yamano and Miwa, 1998) of several fish species. The tissue localisations of these TR-encoding genes align with their well-known roles in mediating the actions of THs during brain (Rovet, 2014), fin (pectoral, pelvic and dorsal), cranial skeletal tissues and heart development in several fish species (Brown, 1997; Okada et al., 2003; Shkil et al., 2012). While TRs appear to be co-expressed in several zebrafish tissues, studies into the distribution pattern of these TRs at a cellular level and their target genes in these tissues are required to dissect their functional differences in zebrafish.

There were also distinct expression domains seen for *thraa* and *thrb*. For example, *thrb* expression (but not *thraa*) occurred in the otic vesicles of zebrafish at all the developmental stages examined. These tissues are responsible for hearing, balance and sensing acceleration in vertebrates (Nicolson, 2005) and while TRβ2 has been shown to mediate the effect of THs on auditory functions in mammals, influencing cellular differentiation in the cochlea (inner ear) (Rusch et al., 2001), as far as we are aware no such information is available for fish. The expression of *thrb* in the otic vesicles, as observed here, strongly suggests a role for THs, mediated principally by TRβ receptors, in the development of sensory functions in fish. We detected a domain of *thraa* expression (but not *thrb*) in the intestinal bulb of zebrafish larvae (96-120 hpf) which is similar to the expression pattern observed in the developing stomach of Japanese flounder (Yamano and Miwa, 1998). THs have long been suggested to be

a regulator of intestinal development (Miwa et al., 1992; Plateroti et al., 2001; Shi et al., 1996), and TH-dependent intestinal remodelling has been well characterised during amphibian metamorphosis (Tata, 1993). The expression of *thraa* in the intestine of developing zebrafish we observed here is consistent with the role of TRα isoforms in meditating T3-dependent functions in intestinal epithelial cells of rodents (Plateroti et al., 2001). Given the wide range of tissues in which TR-encoding genes are expressed in the zebrafish early life stages, this indicates that some TDC (depending on their mode of action) could have wide pervasive effects through alterations to the development and/or functions of these TR-dependent tissues in wild fish.

While the abundance of *dio3b* mRNAs during early life stages of fish has been examined previously (using pooled embryo-larval samples) (Heijlen et al., 2014; Vergauwen et al., 2018), relatively little is known about the tissue-specific *dio3b* expression patterns. *dio3b* expression has been observed previously in the pronephric ducts and brain of zebrafish embryos from the 6 somite stage to 24 hpf (Dong et al., 2013), but here we also observed *dio3b* expression in the pronephric ducts (24-72 hpf), liver (72-120 hpf) and brain (48-72 hpf) of zebrafish embryo-larvae. *Dio3b* expression in the liver and pronephric ducts are likely involved in controlling systemic levels of TH whereas expression in the brain suggests a localised D3 regulation of T3 during distinct neurodevelopmental windows.

We observed that the tissue expression domains for *dio1* and *dio2* were overlapping in zebrafish larvae (in the liver, pronephric ducts, intestinal bulb, intestine and brain at 96 and 120 hpf) consistent with previous findings (Dong et al., 2013; Thisse et al., 2003), and suggesting that D1 and D2 proteins co-operate to control systemic T3

levels and/or local T3 availability during development (Walpita et al., 2010). It should be noted however that the distinct *dio2* expression pattern observed in the pituitary gland (48-120 hpf), is suggestive of D2 alone regulating the availability of T3 in the pituitary and thereby influencing the negative feedback loop of the central HPT axis.

4.3 T3 regulation of TH-related gene transcript levels

The transcript levels of all of the selected TH-related genes examined in our study were altered in zebrafish embryo-larvae exposed to exogenous T3, with several of these genes affected for multiple time points. For example, *thraa*, *thrb* and *dio3b* RNA levels were significantly elevated at 48 hpf, 96 hpf and 120 hpf stages, while *dio1* levels were significantly reduced at 96 hpf and 120 hpf. It is interesting that several of the genes examined however were only affected at a very specific developmental stages, including *pax8* (48 hpf), *ugt1ab* (120 hpf), *crhb* (120 hpf) and *dio2* (96 hpf). These results suggest that different TH pathways may be vulnerable to perturbations in TH levels by TDCs at very specific developmental windows, with thyroid synthesis (via *pax8* signalling) potentially vulnerable for a short period of time while thyroid target gene signalling (via TRs) potentially vulnerable for much longer periods.

Our results show that the effect of T3 on *thrb* transcript levels was more pronounced than on *thraa* transcript levels, in line with previous studies that exposed zebrafish to T3, at concentration similar to those tested in the present study (3-60 µg L^{-1}), from 6-72 hpf, 6-120 hpf and 72-144 hpf (Liu et al., 2000; Walter et al., 2018). In contrast, however, a study by Walpita *et al.,* (2007) found that *thrb* mRNA levels were not affected in zebrafish embryos exposed to T3 (3 µg L^{-1}) up to 75 hpf, while *thraa* mRNA

levels was similarly unaffected between 8-36 hpf and at 75 hpf (but mRNA levels increased at 48 hpf) (Walpita et al., 2007). Taken together, these results suggest that the transcriptional regulation of TRs by T3 may be stage-dependent in zebrafish early life stages and differ depending on the T3 concentration to which they are exposed. Indeed, although T3 exposure resulted in increased *thrb* transcript levels across all developmental stages, the effect was more pronounced at 96 hpf compared with 48 and 120 hpf (6-fold increase compared to 2- and 4-fold increases, respectively). At 96 hpf, zebrafish larvae are entering the embryonic-larval transition phase, which is associated with increased endogenous TH levels, and this may explain the observed greater *thrb* responsiveness to T3 at this stage. It also raises the possibility that TH signalling, mediated by TRβ receptors, at 96 hpf may be a particularly vulnerable developmental window in terms of altered TH status (Campinho et al., 2010).

T3 differentially regulated the transcript levels of the three deiodinase encoding genes examined here in zebrafish embryo-larvae and these observed mRNA changes align well with their documented roles in regulating both systemic and local TH levels. For example, the suppression of *dio1* (96 and 120 hpf) and *dio2* (96 hpf) transcript levels is consistent with their functional role in converting T4 into T3 and supports the idea that changes in their transcript levels may help to regulate T3 status in peripheral tissues. The T3-induced suppression of *dio1* and *dio2* observed here is consistent with several studies that have reported reduced mRNA levels/protein activity in the liver, brain, gonads or whole-body samples in various fish species (zebrafish [56] sea bream (*Sparus aurata)* (Campinho et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Bres et al., 2006; Fines et al., 1999), striped parrotfish (*Scarus iseri*) (Johnson and Lema, 2011), and killifish (*Fundulus heteroclitus*) (García-G et al., 2004)) exposed to T3. The

strong induction of *dio3b (*in contrast to the suppression of *dio1* and *dio2)* in all of the zebrafish life stages examined here is consistent with its proposed function of protecting tissues from high levels of TH, as discussed above, and is in line with previous findings in other fish species including striped parrotfish (Johnson and Lema, 2011), rainbow trout (Bres et al., 2006) and sea bream (Campinho et al., 2010). We did not observe a significant change in *dio1* or *dio2* transcript levels in 48 hpf embryos and although it has previously been reported that these deiodinase encoding genes are unaffected by T3 exposures in zebrafish early life stage (8-48 hpf) (Walpita et al., 2007), the underlying reason(s) for is unknown. We can, however, hypothesise based on our findings that the significant induction of *dio3b* at the 48 hpf stage may lead to sufficiently high levels of D3 proteins that would be capable of regulating TH levels at these earlier stages (prior to hatching) without the need for D1 and D2.

An interesting finding from our study was the differential regulation of *tshb* transcript levels by T3, with a dose-dependent reduction in *tshb* levels observed in 48 hpf embryos and a significant increase in *tshb* levels in larvae post hatching. Thyroid stimulating hormone (TSH), encoded by the *tshb* gene, controls expression of thyroglobulin mRNA, thyroid peroxidase, TSH receptors and the sodium-dependent iodide ions uptake in thyrocytes and therefore plays a crucial role in TH synthesis and release from the thyroid follicles (Pierce and Parsons, 1981). While the reduced expression of *tshb* at 48 hpf observed our study, is consistent with the negative feedback mechanisms that controls TSH and therefore TH levels, the increased *tshb* levels at 96 hpf is difficult to interpret, particularly without a measurement of whole body TH content, which we did not do in this study.

4.4 T3 regulation of TH-related tissue specific gene expression patterns

A novel element of our study was the examination of tissue-specific expression changes for a number of zebrafish thyroid-related genes (via WISH) after exposure to T3 for varying lengths of time. We observed that exposure to exogenous T3 induced distinct changes in the tissue expression patterns of *thraa* and *thrb* in zebrafish embryo-larvae. At both developmental stages examined (48 and 120 hpf), the expression of *thraa* and *thrb* appeared to be substantially higher in the brain of T3-exposed embryo-larvae compared to control individuals. In addition, we found that T3 appeared to induced *thraa* and *thrb* expression in the intestine, liver, craniofacial tissues and pectoral fins (*thraa* only). As far as we are aware, no previous studies have assessed the tissue-specific regulation of TR transcripts by T3 during early fish life stages. Previous studies have, however, studied TH-regulation of TR transcripts in a few tissues in adult fish. In adult fathead minnows, exposure to exogenous T3 has been shown to result in elevated *thraa* and *thrb* expression in the liver and brain and elevated *thrb* transcripts in the gonads (Lema et al., 2009). Interestingly, in adult goldfish (*Carassius auratus*), T3 exposure was shown to down-regulate *thra*a and *thrb* expression in the gonads (Nelson et al., 2011). In the striped parrotfish, T3 exposure increased *thra*a and *thrb* transcripts in the liver, gonad and brain in males but only in the brain in females (Johnson and Lema, 2011). Taken together these results suggest that changes to TR transcript level within specific tissues in response to T3 exposure can differ between species, sexes and life stages.

A novel finding in our study was the induction of *thraa* by T3 in the pineal gland of zebrafish embryo-larvae. The pineal gland is the site of melatonin production and

influences the light-dark rhythm (circadian rhythms) in most vertebrates, including teleost fish (FalcÓn et al., 2006). Melatonin has also been shown to be involved in the regulation of smoltification, reproduction and the immune system in fish (Ángeles Esteban et al., 2006; Ekstrzm and Meissl, 1997). Interestingly, early studies on walking catfish (*Clarias batrachus)* indicated a potential role of the pineal gland/melatonin in controlling thyroidal uptake of iodine and circulating levels of THs (Nayak and Singh, 1987a, b). The expression of *thraa* in the pineal gland here may indicate a role of melatonin in regulating TH synthesis as a compensatory response to exogenous T3 exposure. Future studies should consider whether altered pineal gland signalling is a mechanistic pathway of TDCs and if so fully define adverse outcome pathways for the potential effects of TDCs on pineal gland signalling and function.

Exposure to exogenous T3 altered the tissue specific expression patterns of all of the deiodinase-encoding genes and the observed changes varied depending on the zebrafish life stage examined. As far as we are aware, no other study to date has examined the tissue-specific changes to deiodinase-encoding gene expression by T3 in the early life stages of teleost fish. Here we observed that *dio1* and *dio2* expression appeared to be suppressed in a wide variety of tissues in T3-exposed larvae including craniofacial tissues, brain, liver, gastrointestinal tract and pituitary gland (*dio2* only). The reduced *dio2* expression in the pituitary may be a compensatory mechanism to elevated T3 levels, as reducing the conversion of T4 to T3 (as a result of reduced D*2* activity/ levels) in the pituitary may increase local T4 levels and therefore inhibit TH release by the thyroid follicles. Here, we demonstrate for the first time that *dio3b* expression appeared to be induced in the jaw cartilage, pectoral fins and brain (in 96 h larvae) of T3-exposed embryo-larvae, suggesting these tissues are particularly

sensitive to elevated levels of THs at these earlier stages of development. It is, therefore, important to consider that TH-signalling in these tissues may be vulnerable to some TDCs.

In summary, the data presented illustrate widespread and highly dynamic tissue expression of key genes in the HPT axis in zebrafish embryo-larvae, supporting their roles in multiple developmental processes. Furthermore, the responsiveness of these genes to T3 highlights the high vulnerability of TH-dependant tissues and physiological processes during early developmental windows to altered TH signalling and captures some of the potential mechanisms of action of TDCs. Future work would be well placed to further investigate the downstream adverse outcomes of altered TH signalling in some of the key target tissues identified within this study, such as the pineal gland, the brain, pectoral fins and intestine, in order to better assess the potential environmental risks associated with exposure to TDCs.

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Author contributions: The project was conceived and designed by AP, CRT and TK. AP carried out the exposure studies, WISH and PCRs, and data analysis. AL supported in conducting the molecular work. AP and CRT wrote the manuscript, with input from AL, TH and TK.

Conflicts of interest: The contributing authors have no conflicts of interest.

Figures

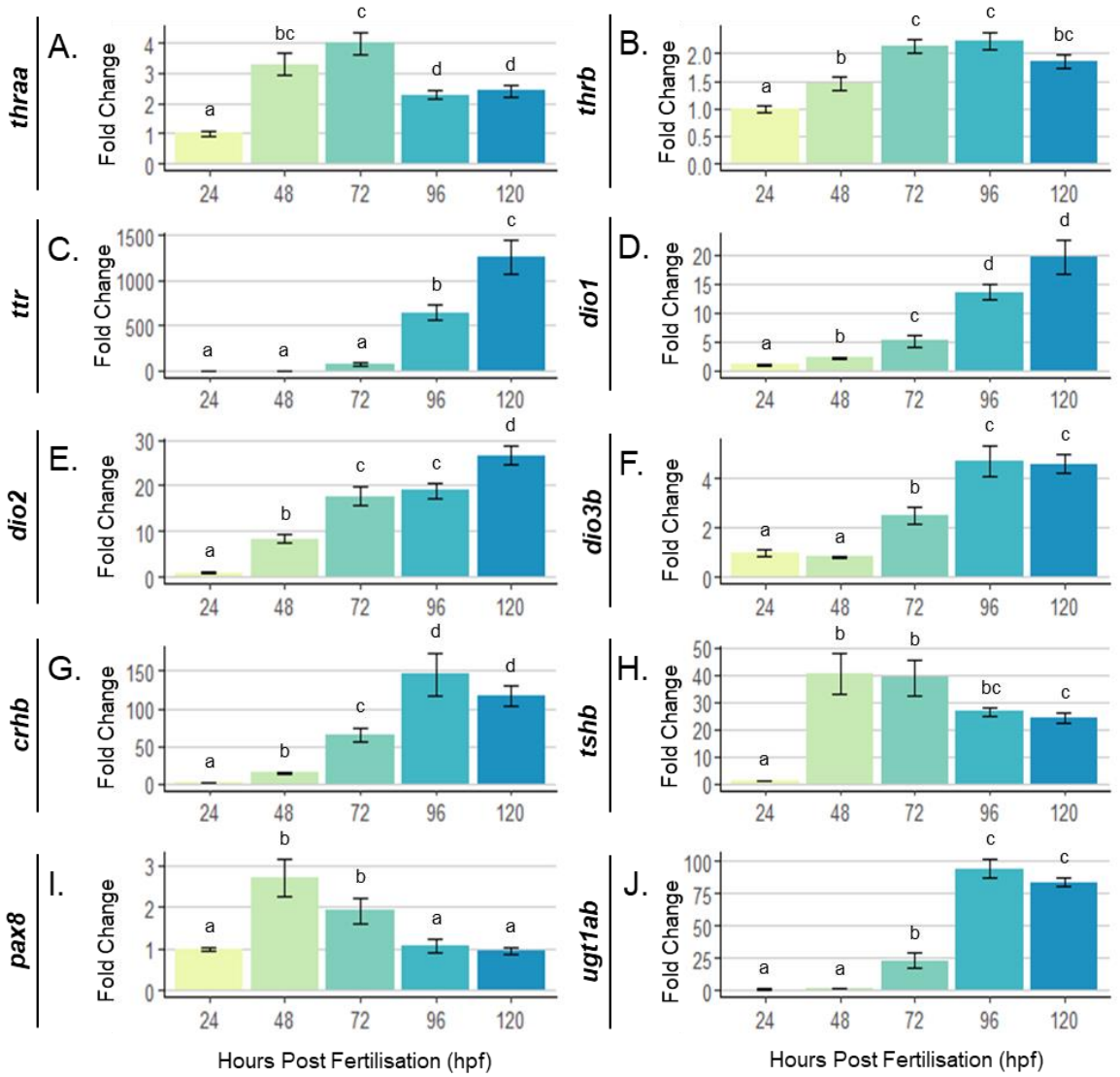


Figure 1. Transcript profile of genes in the HPT axis of zebrafish during embryo-larvae development. Relative expression levels of (A) thyroid receptor alpha (*thraa)*, (B) thyroid receptor beta (*thrb)*, (C) transthyretin (*ttr*), (D) deiodinase type I (*dio1*), (E) deiodinase type II (*dio2*), (F) deiodinase type III (*dio3b*), (G) corticotropin-releasing hormone (*crhb),* (H) thyroid-stimulating hormone beta (*tshb),* (I) paired box 8 (*pax8)* and (J) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR and differences between developmental stages were assessed using

general linear mixed models. Plotted data are presented as fold changes (normalised against the expression of the reference gene, *rpl8*) ± SEM, relative to the earliest developmental stage (24 hpf). Outliers, as identified in the text, were excluded from the analysis, resulting in a replication of n=7-9 samples per developmental stage. Different letters indicate significant differences between developmental stages ($p<0.05$).

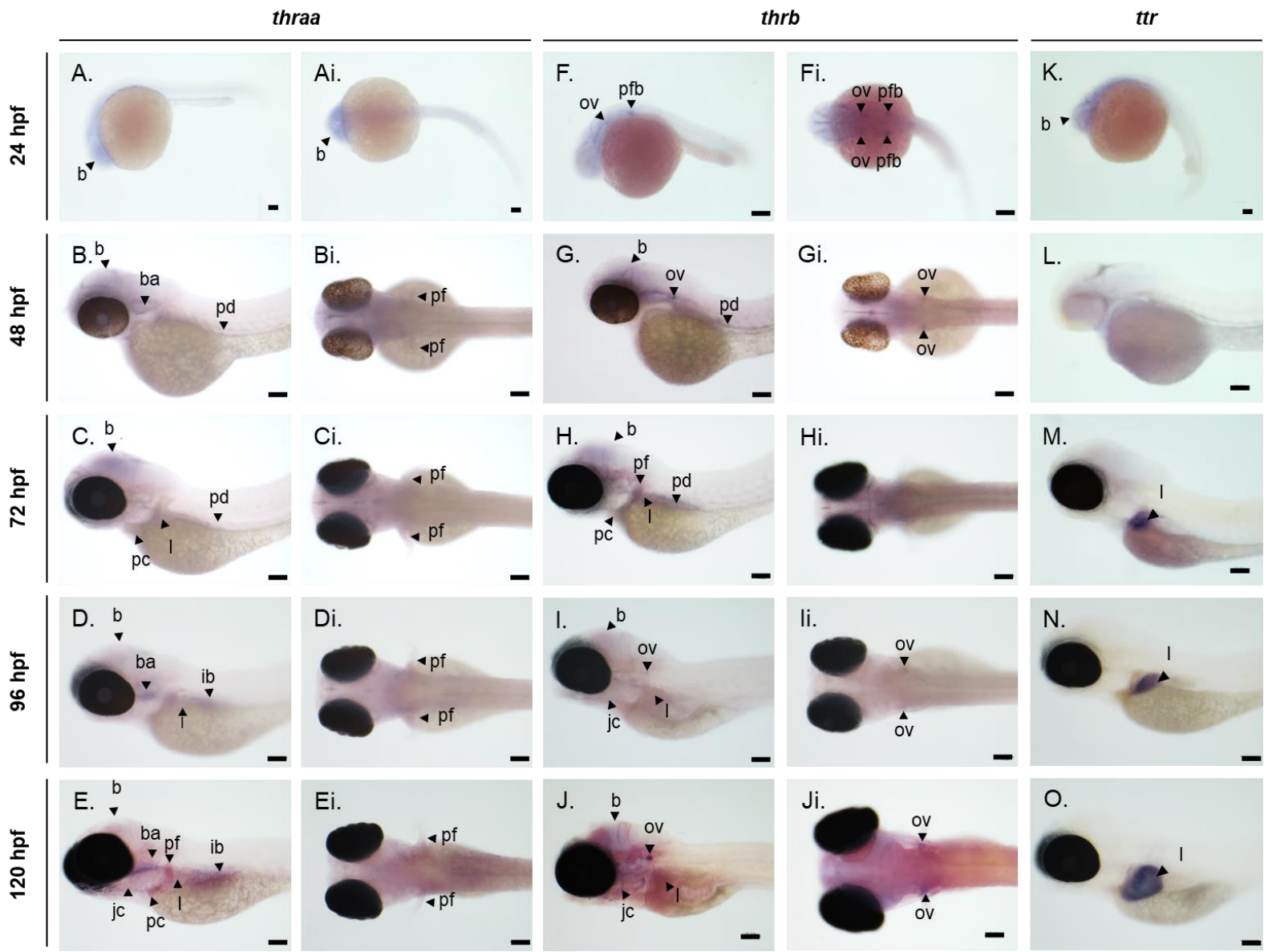

thraa
thrb
ttr
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
A.
Ai.
F.
Fi.
K.
B.
Bi.
G.
Gi.
L.
C.
Ci.
H.
Hi.
M.
D.
Di.
I.
Ii.
N.
E.
Ei.
J.
Ji.
O.
b
ba
pd
pf
ov
pfb
ib
jc
pc
l

Figure 2. WISH images of TR and transthyretin mRNA expression in zebrafish embryo-larvae. Tissue expression pattern of (A-E) thyroid receptor alpha (*thraa*), (F-J) thyroid receptor beta (*thrb*) and (K-O) transthyretin *(ttr)* zebrafish embryo-larvae between 24-120 hpf. Lateral (A-O), and dorsal (Ai-Ji) views of whole embryo-larvae are shown with anterior orientated to the left and the focal areas of expression indicated by black arrowheads. b=brain, ba=branchial arches, ib=intestinal bulb, jc=jaw cartilage, l=liver, ov=otic vesicle, pc=pericardia, pd=pronephric ducts, pf=pectoral fins, pfb=prospective fin bud. Scale bar=100 μm.

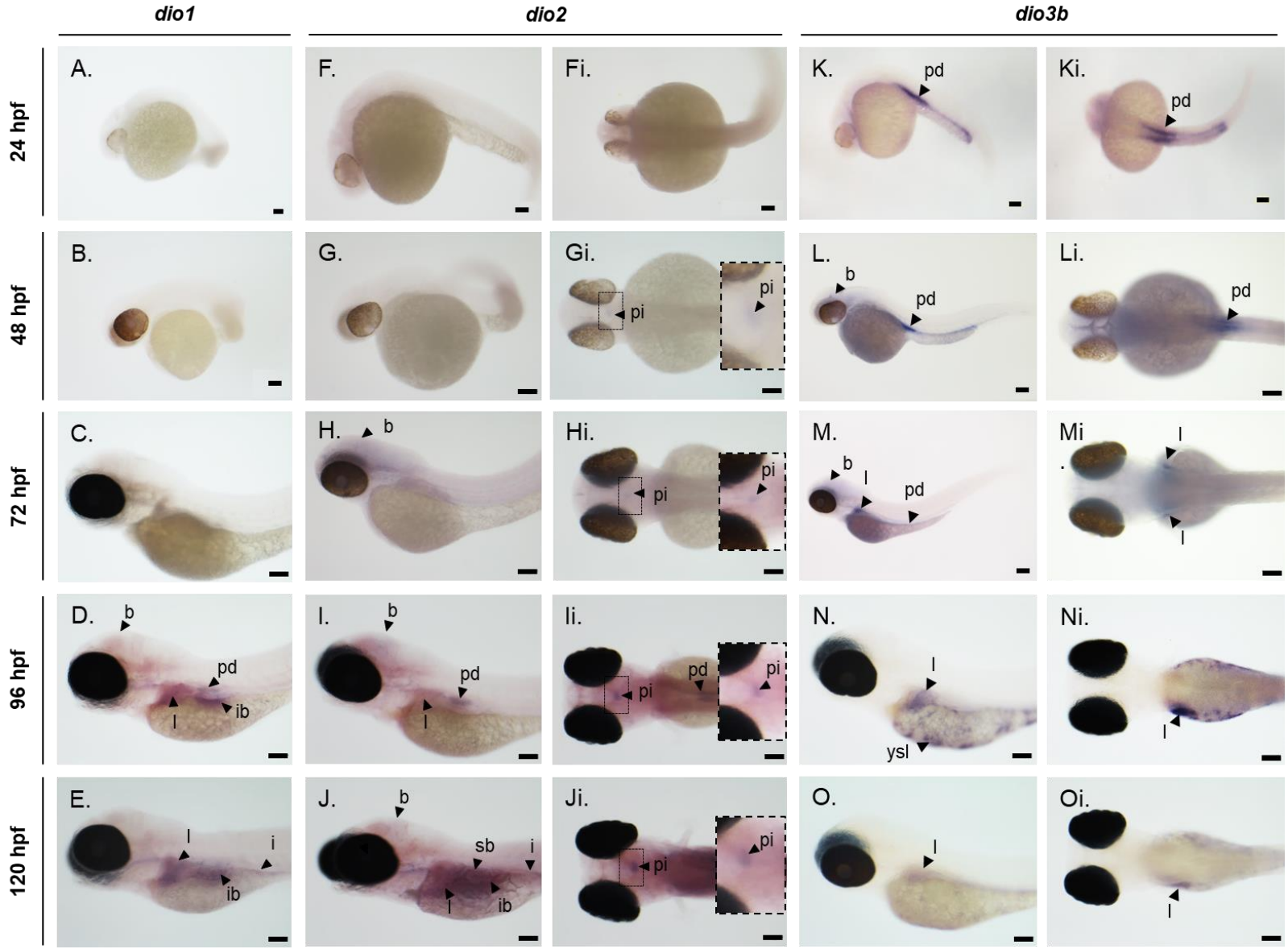
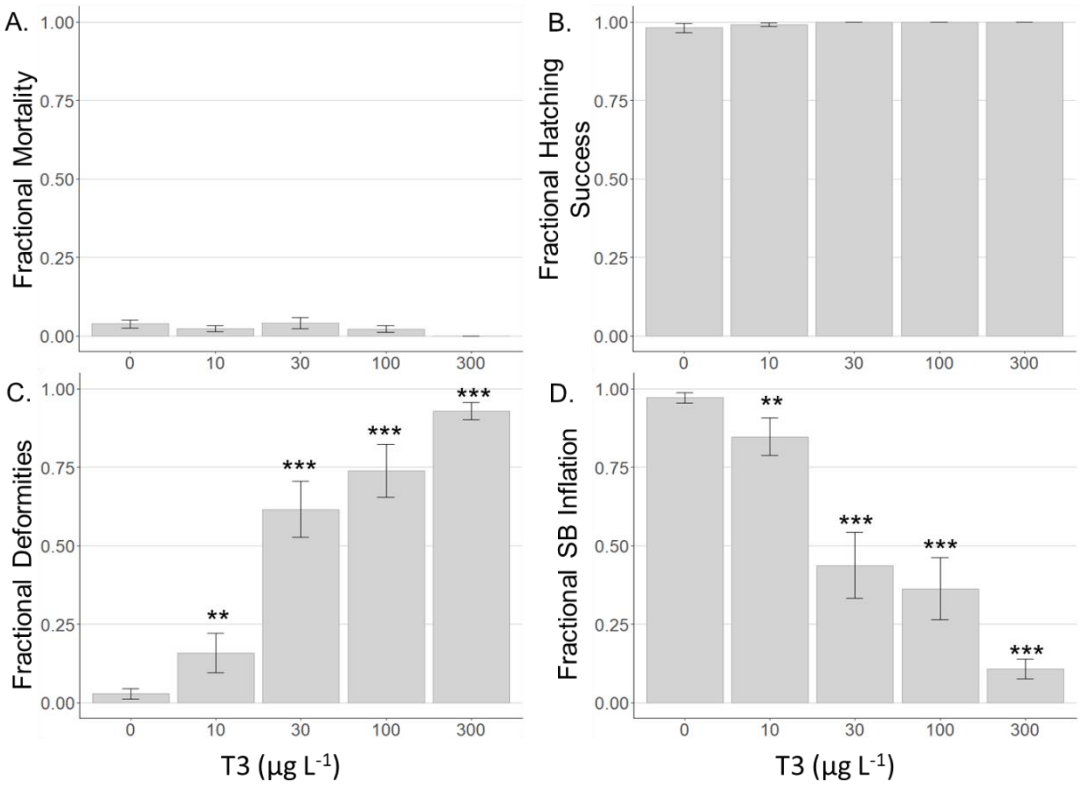

dio1
dio2
dio3b
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
A.
F.
Fi.
K.
pd
Ki.
pd
B.
G.
Gi.
pi
pi
L.
b
pd
Li.
pd
C.
H.
b
Hi.
pi
pi
M.
b
l
pd
Mi
l
l
D.
b
pd
l
ib
I.
b
pd
l
Ii.
pd
pi
pi
N.
l
ysl
Ni.
l
E.
l
i
ib
J.
b
sb
i
l
ib
Ji.
pi
pi
O.
l
Oi.
l

Figure 3. WISH images of deiodinase type I (*dio1*), type II (*dio2*) and type III (*dio3b*) mRNA expression patterns in zebrafish embryo-larvae. Tissue expression pattern of (A-E) *dio1, (*F-J) *dio2* and (K-O) *dio3b* mRNA in zebrafish embryo-larvae between 24-120 hpf. Lateral (A-O) and dorsal (Fi-Oi) views of whole embryo-larvae are shown with anterior orientated to the left and the focal areas of expression are indicated by black arrowheads. b=brain, i=intestine, ib=intestinal bulb, l=liver, pd=pronephric ducts, pi=pituitary, ysl=yolk syncytial layer. Scale bar=100 μm.


A.
Fractional Mortality
1.00
0.75
0.50
0.25
0.00
0
10
30
100
300
B.
Fractional Hatching Success
C.
Fractional Deformities
**
***
D.
Fractional SB Inflation
T3 (µg L-1)


Ei.
72 hpf
Control
Eii.
5
1
2
30 µg L-1
Eiii.
3
100 µg L-1
Eiv.
300 µg L-1
Fi.
96 hpf
Fii.
10 µg L-1
Fiii.
6
4
Fiv.

Figure 4. Mean (A) mortality, (B) hatching success, (C) morphological deformities and (D) swim bladder (SB) inflation success in zebrafish larvae following T3 exposure for 96 hours post fertilisation (hpf). The relationship between toxicity endpoints and T3 concentration was assessed using generalised linear mixed models Significance codes compared to control: **p<0.01, ***p<0.001. (E) Images of zebrafish embryo-larvae at 72 hpf exposed to (i) Control, (ii) 30 µg T3 L^{-1}, (iii) 100 µg T3 L^{-1} and (iv) 300 µg T3 L^{-1}. (F) Images of zebrafish larvae at 96 hpf exposed to (i) Control, (ii) 10 µg T3 L^{-1} (iii) 100 µg T3 L^{-1} and (iv) 300 µg T3 L^{-1} showing—1. oedema, 2. swollen yolk sac, 3. bent spine, 4. lower jaw deformity, 5. reduced pigmentation and 6. small eyes. Scale bar=100 µm.

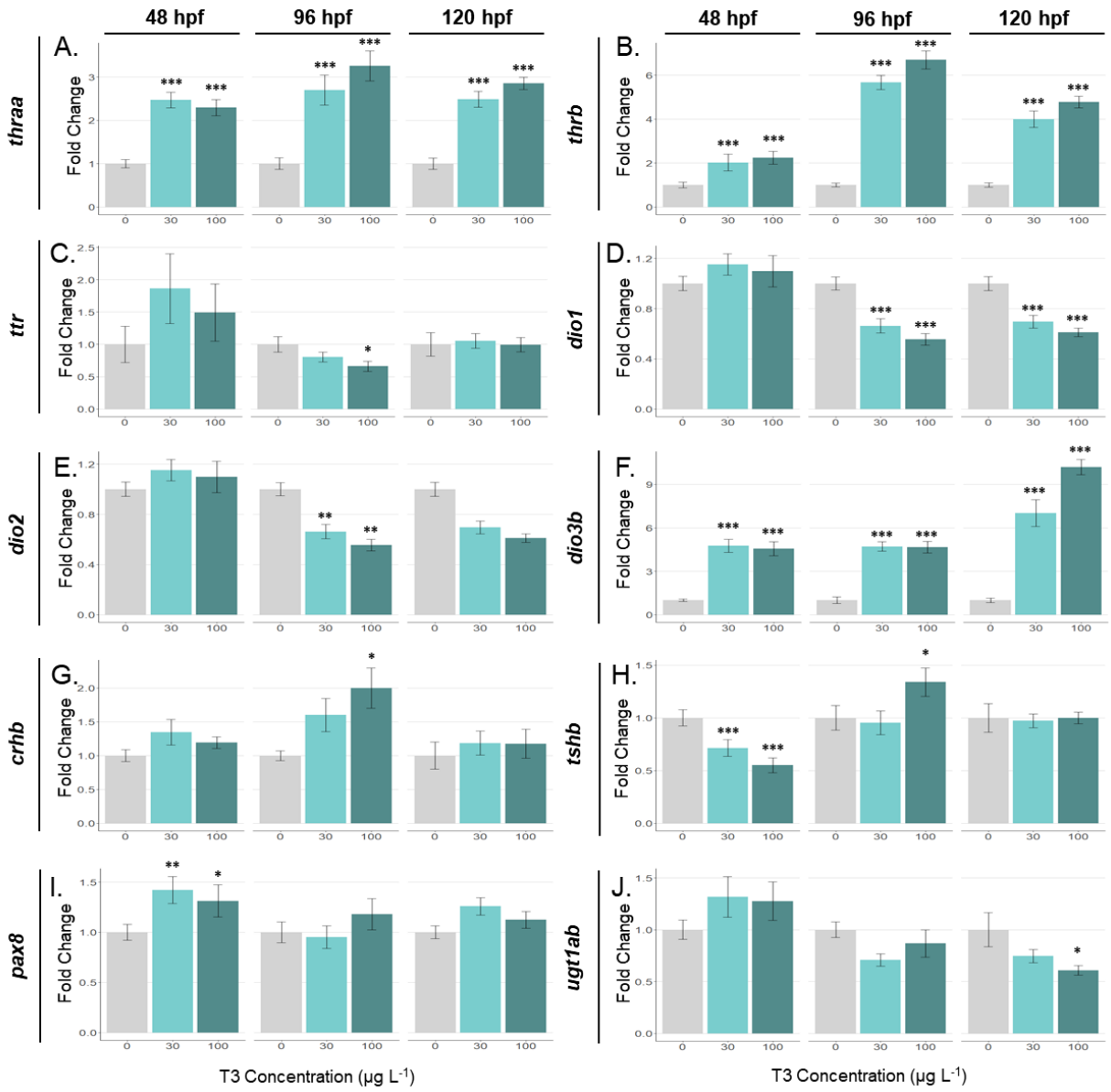

48 hpf
96 hpf
120 hpf
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
thraa
thrb
ttr
dio1
dio2
dio3b
crhb
tshb
pax8
ugt1ab
Fold Change
0
30
100
T3 Concentration (µg L-1)

Figure 5. Transcript profile of genes in the HPT axis of zebrafish embryo-larvae following exposure to T3. Changes in (A) thyroid receptor alpha (*thraa)*, (B) thyroid receptor beta (*thrb)*, (C) transthyretin (*ttr*), (D) deiodinase type I (*dio1*), (E) deiodinase type II (*dio2*), (F) deiodinase type III (*dio3b*), (G) corticotropin-releasing hormone (*crhb),* (H) thyroid-stimulating hormone beta (*tshb),* (I) paired box 8 (*pax8)* and (J) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* in zebrafish embryo-larvae following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR and the relationship between transcript expression and T3 concentration was assessed using general linear mixed models. Plotted data are presented as 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=8-9 homogenised sample per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

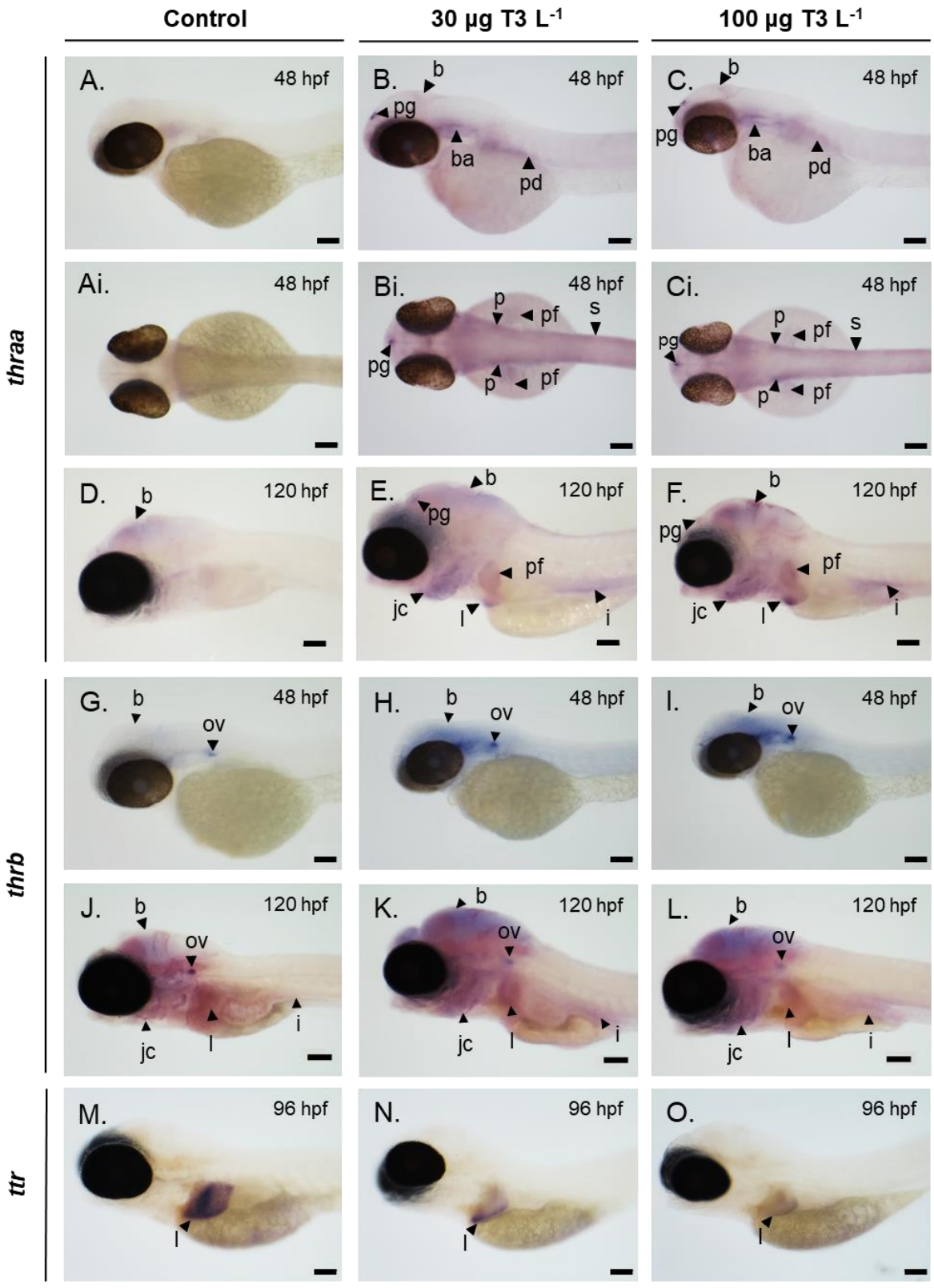


Figure 6. WISH images of thyroid receptor alpha (*thraa*), thyroid receptor beta (*thrb*) and transthyretin (*ttr*) mRNA expression patterns in zebrafish embryo-larvae following

exposure to T3. Representative images of (A-F) *thraa*, (G-L) *thrb* and (M-O) *ttr* expression patterns in 48 hpf and 120 hpf zebrafish embryo-larvae treated with T3 (0, 30 and 100 µg L^{-1}). Lateral (A-O) and dorsal (Ai-Ci) views of whole embryo-larvae are shown with anterior orientated to the left and focal areas of expression are indicated by black arrowheads. b=brain, ba=branchial arch, i=intestine, jc=jaw cartilage, l=liver, ov=otic vesicle, p=pronephros, pd=pronephric ducts, pf=pectoral fins, pg=pineal gland, s=somites. Scale bar=100 µm.

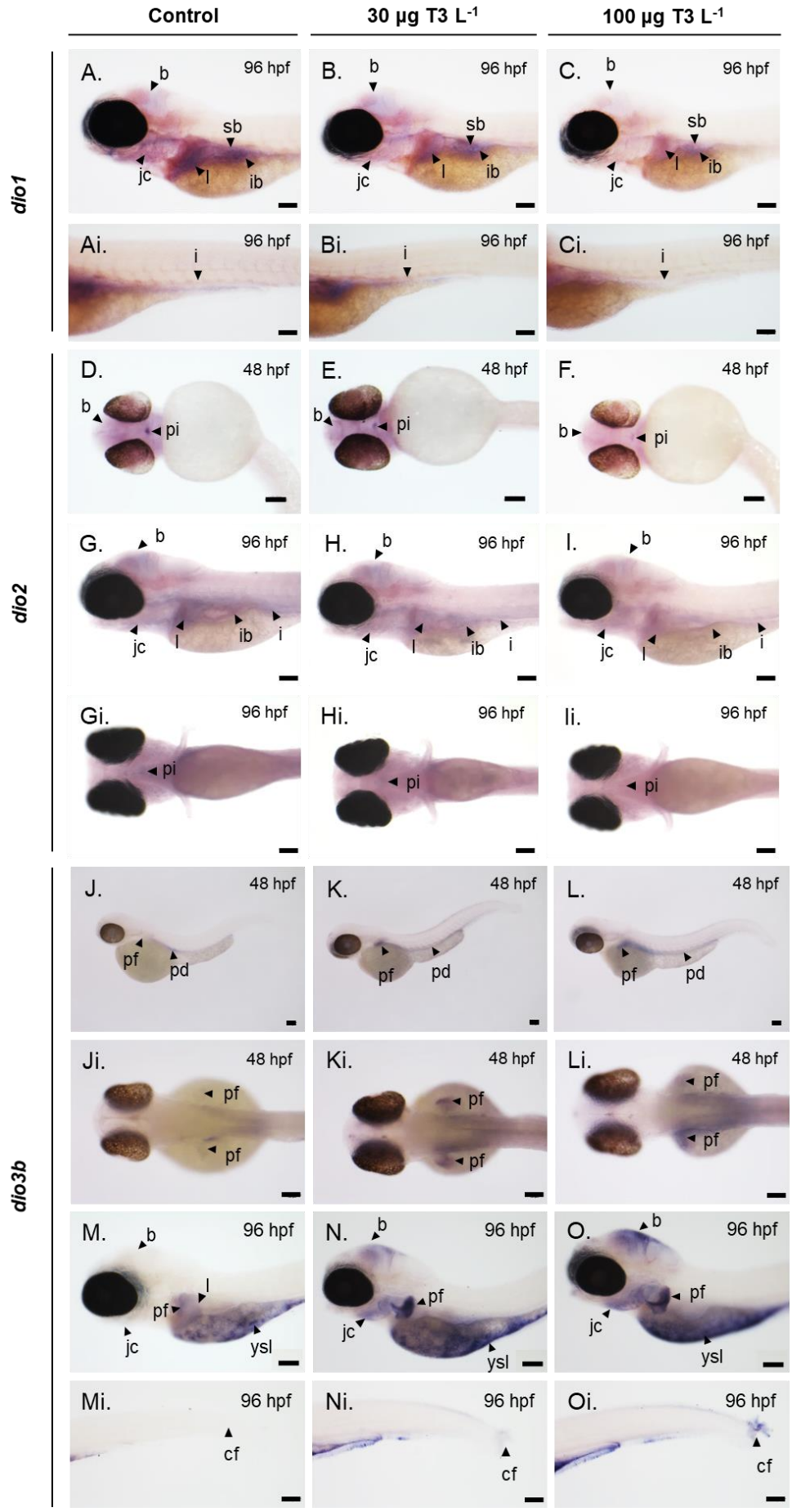

Control
30 µg T3 L-1
100 µg T3 L-1
dio1
dio2
dio3b
A.
B.
C.
Ai.
Bi.
Ci.
D.
E.
F.
G.
H.
I.
Gi.
Hi.
Ii.
J.
K.
L.
Ji.
Ki.
Li.
M.
N.
O.
Mi.
Ni.
Oi.
96 hpf
48 hpf
b
sb
jc
l
ib
i
pi
pf
pd
ysl
cf

Figure 7. WISH images of deiodinase type I (*dio1*), type II (*dio2*) and type III (*dio3b*) mRNA expression patterns in zebrafish embryo-larvae following exposure to T3. Representative images of (A-C) *dio1,* (D-I) *dio2* and (J-O) *dio3b* expression patterns in 48 hpf and/or 96 hpf zebrafish embryo-larvae treated with T3 (0, 30 and 100 µg L^{-1}). Lateral (A-C; G-O; Ai-Ci; Mi-Oi), ventral (Di-Fi; Gi-Ii) and dorsal (Ji-Li) views of whole embryo-larvae are shown with anterior orientated to the left and focal areas of expression indicated by black arrowheads. b=brain, cf=caudal fin, i=intestine, ib=intestinal bulb, jc=jaw cartilage, l=liver, pd=pronephric ducts, pf=pectoral fins, pi=pituitary gland, sb=swim bladder, ysl= yolk syncytial layer. Scale bar=100 µm.

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Supplementary Material

Expression dynamics of genes in the hypothalamic-pituitary-thyroid (HPT) cascade and their responses to 3,3′,5-triiodo-L-thyronine (T3) highlights potential vulnerability to thyroid-disrupting chemicals in zebrafish (*Danio rerio*) embryo-larvae.

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Supplemental Tables and Figures

Table S1. Forward and reverse primer sequences for target genes in the HPT axis of zebrafish used in qRT-PCR analysis, with annealing temperatures (Ta), product size (base pairs), efficiency values and accession numbers.

Gene Name	Gene Symbol	Primer Sequence (5' – 3') (F: forward; R: reverse)	Product Size (bp)	Ta (°C)	PCR Efficiency (%)	Accession Number	Reference
thyroid receptor alpha	*thraa*	F: GGC GTC CTG TAA CTG CTG R: GGT TGT GCT CCT GCT CTG	142	61	101	NM_131396.1	[1]
thyroid receptor beta	*thrb*	F: TGG GTG TCT CGC TGT CCTC R: ACA ACG CTC TAT CCG CTC AAC	119	60	93	NM_131340.1	[1]
transthyretin	*ttr*	F: CGC ACA CCT TTC CAC CAG R: TTG ACG ACC ACA GCA GTT G	122	60.5	109	NM_001005598.2	[1]
deiodinase enzymes type I	*dio1*	F: GTA ATC GTC CAC TGG TTC TGA G R: TGA GGA AAT CTG CGA CAT TGC	114	60.5	107	NM_001007283.1	[1]
deiodinase enzymes type II	*dio2*	F: TCT GGA GGA GAG GAT GTT TGC R: CTC GTA GGA CAC ACC GTA GG	124	59.5	105	NM_212789.3	[1]
deiodinase enzymes type III	*dio3b*	F: AGG GCT CCG CAG GTG TG R: AGG AAG TCC AGC AGG CAG AG	106	63	98	NM_001177935.2	[1]
corticotropin-releasing hormone	*crhb*	F: TTC GGG AAG TAA CCA CAA GC R: CTG CAC TCT ATT CGC CTT CC	163	59.5	110	NM_001007379.1	[1]
thyroid-stimulating hormone	*tshb*	F: CAG GGA CAG TAA CAT AAA GGA G R: CTG GGT AGG TGA AGT GAG G	137	60.5	112	NM_181494.2	[1]
paired box 8	*pax8*	F: CCG TCA CTC CTC CTG AAT CTC R: GCT CTC CTG GTC ACT GTC ATC	128	62.5	106	AF072549.1	[1]
uridine diphosphate-glucuronosyltransferase	*ugt1ab*	F: CCA CCA AGT CTT TCC GTG TT R: GCA GTC CTT CAC AGG CTT TC	168	62.5	105	NM_213422.2	[1]
ribosomal protein l8	*rpl8*	F: CCG AGA CCA AGA AAT CCA GAG R: CCA GCA ACA ACA CCA ACA AC	91	59.5	102	NM_200713.1	[2]

Table S2: Whole mount *in situ* hybridisation staining times (h) for the target genes in zebrafish embryo-larvae studied at various stages of development; 24, 48, 72, 96 and 120 hpf. Staining times are shown for the following genes: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*) and transthyretin (*ttr*).

Gene	***hpf***				
	24	***48***	***72***	***96***	***120***
thraa	*8.5*	*7.5*	*7.5*	*7.5*	*7.5*
thrb	*8.5*	*8.5*	*8.5*	*8.5*	*8.5*
ttr	*6.5*	*6.5*	*5.0*	*5.0*	*5.0*
dio1	*8.5*	*8.5*	*8.5*	*7.5*	*7.5*
dio2	*8.5*	*5.0*	*7.0*	*8.5*	*8.5*
dio3b	*5.0*	*5.0*	*6.5*	*5.0*	*3.5*

Table S3: Whole mount *in situ* hybridisation staining times (h) for target genes in zebrafish embryo-larvae following exposures to T3. Embryo-larvae were sampled at various developmental stages; 48, 96 and/or 120 hpf. Staining times are shown for the following genes: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*) and transthyretin (*ttr*).

Gene	**hpf**		
	48	**96**	**120**
thraa	2.0	-	3.0
thrb	3.5	-	3.5
ttr	4.5	3.5	-
dio1	4.5	10.0	-
dio2	3.5	10.0	-
dio3b	2.0	2.0	-

Table S4. Output of maximum likelihood tests performed to assess differences in gene transcript levels between zebrafish developmental stages. Developmental stage was incorporated as a fixed effect into the GLMM model and each experiment were incorporated as a random effect.

Gene	**df**	**X^2**	**p value**
thraa	4	68.9	<0.001
thrb	4	50.0	<0.001
ttr	4	125.1	<0.001
dio1	4	119.5	<0.001
dio2	4	77.6	<0.001
dio3b	4	80.7	<0.001
crhb	4	74.7	<0.001
tshb	4	40.1	<0.001
pax8	4	40.2	<0.001
ugt1ab	4	110.6	<0.001

Table S5. Output of pairwise comparison analyses used to compare differences in the transcript levels of each gene between the zebrafish developmental stages examined (24-120 hpf).

Gene	Stage Comparison	Estimate	SE	z value	p value	Gene	Stage Comparison	Estimate	SE	z value	p value
thraa	24-48	0.02	0.00	9.56	<0.001	*dio3b*	24-48	-0.06	0.07	-0.94	0.880
	24-72	0.02	0.00	12.37	<0.001		24-72	0.40	0.07	5.87	<0.001
	24-96	0.01	0.00	5.48	<0.001		24-96	0.67	0.07	9.65	<0.001
	24-120	0.01	0.00	5.41	<0.001		24-120	0.68	0.07	9.74	<0.001
	48-72	0.00	0.00	2.41	0.111		48-72	0.46	0.07	6.82	<0.001
	48-96	-0.01	0.00	-3.98	<0.001		48-96	0.74	0.07	10.57	<0.001
	48-120	-0.01	0.00	-3.64	0.003		48-120	0.75	0.07	10.66	<0.001
	72-96	-0.01	0.00	-6.50	<0.001		72-96	0.28	0.07	3.95	0.001
	72-120	-0.01	0.00	-6.07	<0.001		72-120	0.28	0.07	4.05	0.001
	96-120	0.00	0.00	0.16	0.999		96-120	0.01	0.07	0.09	0.999
thrb	24-48	0.01	0.01	2.97	0.025	*crhb*	24-48	0.02	0.01	3.02	0.022
	24-72	0.04	0.01	7.48	<0.001		24-72	0.04	0.01	7.51	<0.001
	24-96	0.04	0.01	8.11	<0.001		24-96	0.07	0.01	11.39	<0.001
	24-120	0.03	0.01	4.89	<0.001		24-120	0.06	0.01	10.19	<0.001
	48-72	0.02	0.01	4.51	<0.001		48-72	0.03	0.01	4.49	<0.001
	48-96	0.03	0.01	5.14	<0.001		48-96	0.05	0.01	8.38	<0.001
	48-120	0.01	0.01	2.14	0.204		48-120	0.04	0.01	7.27	<0.001
	72-96	0.00	0.01	0.63	0.970		72-96	0.02	0.01	3.89	<0.001
	72-120	-0.01	0.01	-2.06	0.239		72-120	0.02	0.01	2.92	0.029
	96-120	-0.01	0.01	-2.64	0.063		96-120	-0.01	0.01	-0.85	0.914
ttr	24-48	-0.49	0.16	-3.09	0.017	*tshb*	24-48	0.00	0.00	6.97	<0.001
	24-72	1.40	0.16	8.76	<0.001		24-72	0.00	0.00	6.74	<0.001
	24-96	2.85	0.17	17.23	<0.001		24-96	0.00	0.00	4.51	<0.001
	24-120	3.14	0.17	18.98	<0.001		24-120	0.00	0.00	3.71	0.002
	48-72	1.90	0.16	11.85	<0.001		48-72	0.00	0.00	-0.05	1
	48-96	3.34	0.17	20.22	<0.001		48-96	0.00	0.00	-2.34	0.132

	48-120	3.63	0.17	21.98	<0.001		48-120	0.00	0.00	-3.14	0.015
	72-96	1.44	0.17	8.74	<0.001		72-96	0.00	0.00	-2.23	0.168
	72-120	1.73	0.17	10.50	<0.001		72-120	0.00	0.00	-3.00	0.023
	96-120	0.29	0.17	1.71	0.429		96-120	0.00	0.00	-0.77	0.938
dio1	24-48	0.36	0.06	6.29	<0.001	*pax8*	24-48	0.37	0.07	5.51	<0.001
	24-72	0.66	0.06	11.71	<0.001		24-72	0.23	0.07	3.47	0.005
	24-96	1.12	0.06	20.01	<0.001		24-96	-0.02	0.07	-0.28	0.999
	24-120	1.27	0.06	21.85	<0.001		24-120	-0.05	0.07	-0.67	0.962
	48-72	0.29	0.06	5.05	<0.001		48-72	-0.14	0.06	-2.11	0.218
	48-96	0.76	0.06	13.10	<0.001		48-96	-0.39	0.06	-5.98	<0.001
	48-120	0.90	0.06	15.08	<0.001		48-120	-0.41	0.07	-6.20	<0.001
	72-96	0.47	0.06	8.30	<0.001		72-96	-0.25	0.06	-3.87	0.001
	72-120	0.61	0.06	10.51	<0.001		72-120	-0.28	0.07	-4.16	<0.001
	96-120	0.14	0.06	2.47	0.098		96-120	-0.03	0.07	-0.41	0.994
dio2	24-48	0.00	0.00	3.97	<0.001	*ugt1ab*	24-48	0.31	0.12	2.57	0.075
	24-72	0.00	0.00	8.85	<0.001		24-72	1.37	0.12	11.47	<0.001
	24-96	0.00	0.00	9.48	<0.001		24-96	2.12	0.12	17.14	<0.001
	24-120	0.00	0.00	13.10	<0.001		24-120	2.07	0.12	16.79	<0.001
	48-72	0.00	0.00	4.88	<0.001		48-72	1.07	0.12	8.90	<0.001
	48-96	0.00	0.00	5.64	<0.001		48-96	1.81	0.12	14.65	<0.001
	48-120	0.00	0.00	9.25	<0.001		48-120	1.77	0.12	14.29	<0.001
	72-96	0.00	0.00	0.91	0.895		72-96	0.75	0.12	6.03	<0.001
	72-120	0.00	0.00	4.52	<0.001		72-120	0.70	0.12	5.68	<0.001
	96-120	0.00	0.00	3.50	0.004		96-120	-0.04	0.13	-0.35	0.997

Table S6. Output of pairwise comparison analyses used to compare differences in the transcript levels of (A) thyroid receptors *thraa* and *thrb* and (B) deiodinases *dio1*, *dio2* and *dio3* at each of the zebrafish developmental stages examined (24-120 hpf).

A) Comparison of thyroid receptor transcript levels					
Gene comparison	**HPF**	**Estimate**	**SE**	**z value**	**p value**
thraa-thrb	24	0.04	0.003	10.8	<0.001
	48	0.02	0.004	4.7	<0.001
	72	0.04	0.004	10.1	<0.001
	96	0.06	0.005	11.2	<0.001
	120	0.04	0.005	8.7	<0.001
B) Comparison of deiodinase transcript levels					
Gene comparison	**HPF**	**Estimate**	**SE**	**z value**	**p value**
dio1-dio2	*24*	-0.62	0.07	-8.49	<0.001
dio1-dio3b	*24*	1.64	0.07	22.56	<0.001
dio2-dio3b	*24*	2.26	0.07	31.04	<0.001
dio1-dio2	48	70.98	65.1	1.09	0.52
dio1-dio3b	48	-581.65	65.10	-8.94	<0.001
dio2-dio3b	48	-652.62	63.15	-10.33	<0.001
dio1-dio2	72	-10.28	54.13	-0.19	0.98
dio1-dio3b	72	-330.92	54.13	-6.11	<0.001
dio2-dio3b	72	-320.64	54.13	-5.92	<0.001
dio1-dio2	96	186.88	20.81	8.98	<0.001
dio1-dio3b	96	-100.95	20.81	-4.851	<0.001
dio2-dio3b	96	-287.83	21.40	-13.45	<0.001
dio1-dio2	120	128.46	18.18	7.07	<0.001

dio1-dio3b	120	-75.01	18.18	-4.13	<0.001
dio2-dio3b	120	-203.47	18.18	-11.19	<0.001

Table S7. Mean fractional mortality, hatching success, deformities and swim bladder (SB) inflation success (± SEM) following exposure of zebrafish to a range of T3 concentrations over the period to 96 h post fertilisation.

T3 (µg L^{-1})	N	Mean Mortality	Mean Hatching Success	Mean Deformities	Mean SB Inflation Success
0	9	0.04 (± 0.01)	0.98 (± 0.02)	0.03 (± 0.02)	0.97 (± 0.02)
10	9	0.02 (± 0.01)	0.99 (± 0.01)	0.16 (± 0.06)	0.85 (± 0.06)
30	9	0.04 (± 0.02)	1.00 (± 0.00)	0.62 (± 0.09)	0.44 (± 0.10)
100	9	0.02 (± 0.01)	1.00 (± 0.00)	0.74 (± 0.08)	0.36 (± 0.10)
300	9	0.00 (± 0.00)	1.00 (± 0.00)	0.93 (± 0.03)	0.11 (± 0.03)

Table S8. Output of (A) maximum likelihood tests and the subsequent (B) pairwise comparison analyses performed to assess the effect of T3 treatment on mortality, hatching success, deformities and swim bladder inflation in zebrafish larvae following a 96-h exposure. NS=Not significant.

(A) Output of maximum likelihood tests

Measured Endpoints	X^2	df	p value
Mortality	10.1	4	0.05
Hatching Success	5.9	4	0.21
Deformities	513.4	4	<0.001
Swim Bladder Inflation	469.8	4	<0.001

(B) Output of pairwise comparisons

Measured Endpoints	Treatment Comparison	Estimate	SE	z value	p value
Mortality	0-10	NS	NS	NS	NS
	0-30	NS	NS	NS	NS
	0-100	NS	NS	NS	NS
	0-300	NS	NS	NS	NS
Hatching Success	0-10	NS	NS	NS	NS
	0-30	NS	NS	NS	NS
	0-100	NS	NS	NS	NS
	0-300	NS	NS	NS	NS
Deformities	0-10	2.01	0.52	3.84	0.001
	0-30	5.10	0.57	9.02	<0.001
	0-100	5.77	0.58	10.00	<0.001

	0-300	7.55	0.64	11.85	<0.001
Swim Bladder Inflation	0-10	-1.99	0.53	-3.77	0.002
	0-30	-5.10	0.58	-8.84	<0.001
	0-100	-5.52	0.58	-9.46	<0.001
	0-300	-7.38	0.63	-11.80	<0.001

Table S9. Output of maximum likelihood tests performed to assess differences in gene transcript levels and T3 exposure conditions in 48, 96 and 120 hpf zebrafish larvae. T3 concentration was incorporated as a fixed effect into the GLMM model and each experiment were incorporated as a random effect.

Gene	hpf	X^2	df	p value	Gene	hpf	X^2	df	p value
thraa	48	31.6	2	<0.001	***dio3b***	48	41.6	2	<0.001
	96	30.0	2	<0.001		96	38.6	2	<0.001
	120	40.3	2	<0.001		120	44.1	2	<0.001
thrb	48	14.2	2	<0.001	***crhb***	48	3.9	2	0.144
	96	46.5	2	<0.001		96	6.6	2	0.037
	120	52.2	2	<0.001		120	0.6	2	0.748
ttr	48	2.2	2	0.355	***tshb***	48	29.5	2	<0.001
	96	8.5	2	0.014		96	8.1	2	0.018
	120	0.2	2	0.920		120	0.1	2	0.965
dio1	48	1.4	2	0.491	***pax8***	48	9.8	2	0.007
	96	23.7	2	<0.001		96	2.0	2	0.368
	120	23.7	2	<0.001		120	4.5	2	0.104
dio2	48	1.1	2	0.588	***ugt1ab***	48	3.6	2	0.163
	96	13.1	2	0.001		96	4.3	2	0.117
	120	0.8	2	0.668		120	6.7	2	0.035

Table S10. Output of pairwise comparison analyses used to compare differences in gene transcript levels between T3 treatment groups (30 and 100 µg L^{-1} versus control) at different zebrafish developmental stages (48, 96 and 120 hpf). NS=Not significant.

Gene	hpf	Treatment Comparison	Estimate	SE	z value	p value	Gene	hpf	Treatment Comparison	Estimate	SE	z value	p value
thraa	48	0-30	0.03	0.00	7.20	<0.001	*dio3b*	48	0-30	0.08	0.01	9.28	<0.001
		0-100	0.03	0.01	6.17	<0.001			0-100	0.07	0.01	8.77	<0.001
	96	0-30	0.04	0.01	5.52	<0.001		96	0-30	0.88	0.11	7.79	<0.001
		0-100	0.05	0.01	7.31	<0.001			0-100	0.87	0.12	7.49	<0.001
	120	0-30	0.03	0.00	7.13	<0.001		120	0-30	0.58	0.08	7.52	<0.001
		0-100	0.04	0.00	8.88	<0.001			0-100	0.90	0.08	11.22	<0.001
thrb	48	0-30	0.05	0.02	3.20	0.004	*crhb*	48	0-30	NS	NS	NS	NS
		0-100	0.06	0.00	4.02	<0.001			0-100	NS	NS	NS	NS
	96	0-30	0.20	0.02	8.84	<0.001		96	0-30	0.11	0.07	1.51	0.286
		0-100	0.24	0.02	11.05	<0.001			0-100	0.18	0.07	2.56	0.028
	120	0-30	0.20	0.02	9.80	<0.001		120	0-30	NS	NS	NS	NS

		0-100	0.25	0.02	12.35	<0.001			0-100	NS	NS	NS	NS
ttr	48	0-30	NS	NS	NS	NS	*tshb*	48	0-30	-4.65E-04	9.22E-05	-5.04	<0.001
		0-100	NS	NS	NS	NS			0-100	-6.77E-04	9.53E-05	-7.11	<0.001
	96	0-30	-0.02	0.01	-1.86	0.150		96	0-30	-6.41E-05	1.95E-04	-0.33	0.942
		0-100	-0.03	0.01	-3.10	0.006			0-100	4.96E-04	2.02E-04	2.46	0.037
	120	0-30	NS	NS	NS	NS		120	0-30	NS	NS	NS	NS
		0-100	NS	NS	NS	NS			0-100	NS	NS	NS	NS
dio1	48	0-30	NS	NS	NS	NS	*pax8*	48	0-30	0.003	0.00	3.22	0.004
		0-100	NS	NS	NS	NS			0-100	0.002	0.00	2.38	0.046
	96	0-30	-0.003	0.001	-4.40	<0.001		96	0-30	NS	NS	NS	NS
		0-100	-0.003	0.001	-5.64	<0.001			0-100	NS	NS	NS	NS
	120	0-30	-0.002	0.001	-4.43	<0.001		120	0-30	NS	NS	NS	NS
		0-100	-0.003	0.001	-5.68	<0.001			0-100	NS	NS	NS	NS
dio2	48	0-30	NS	NS	NS	NS	*ugt1ab*	48	0-30	NS	NS	NS	NS
		0-100	NS	NS	NS	NS			0-100	NS	NS	NS	NS

	96	0-30	-0.001	0.000	-3.53	0.001**		96	0-30	NS	NS	NS	NS
		0-100	-0.001	0.000	-3.21	0.004**			0-100	NS	NS	NS	NS
	120	0-30	NS	NS	NS	NS		120	0-30	-0.11	0.07	-1.66	0.223
		0-100	NS	NS	NS	NS			0-100	-0.18	0.07	-2.66	0.021*

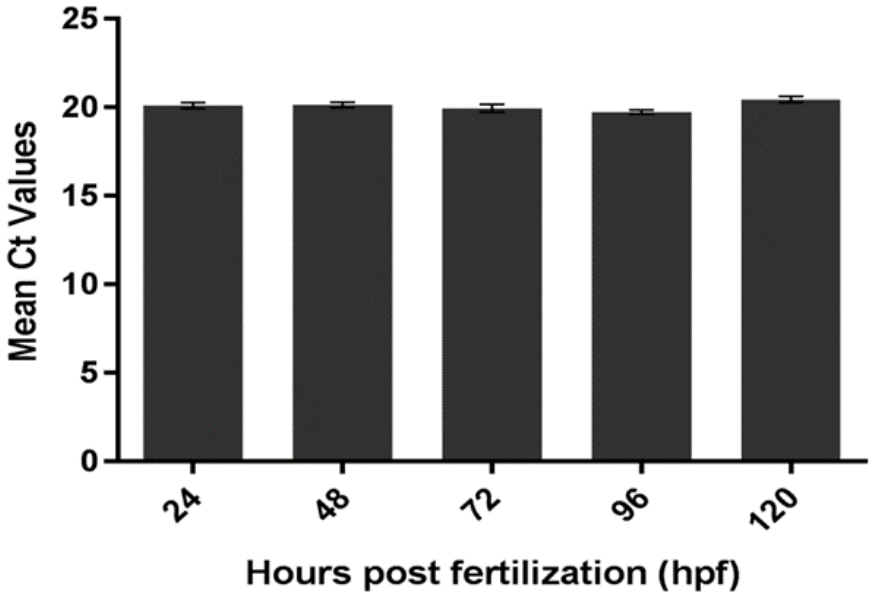

Figure S1: Mean *rpl8* mRNA levels expressed as Ct values (threshold cycle) in zebrafish embryo-larvae at 24, 48, 72, 96 and 120 hpf (n=9). There were no significant differences in Ct values between developmental stages (GLM). Error bars represent standard error.

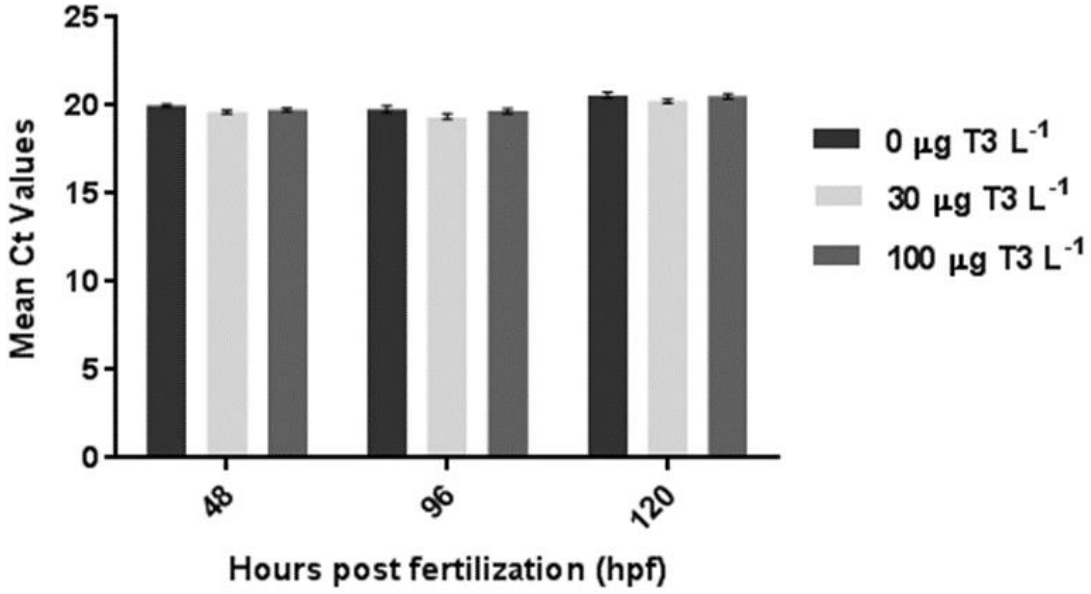

Figure S2: Mean levels of *rpl8* mRNA expressed as Ct values (threshold cycle) in zebrafish embryo-larvae exposed to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hpf (n=9). There were no significant differences in Ct values between treatment groups (GLM). Error bars represent standard error.

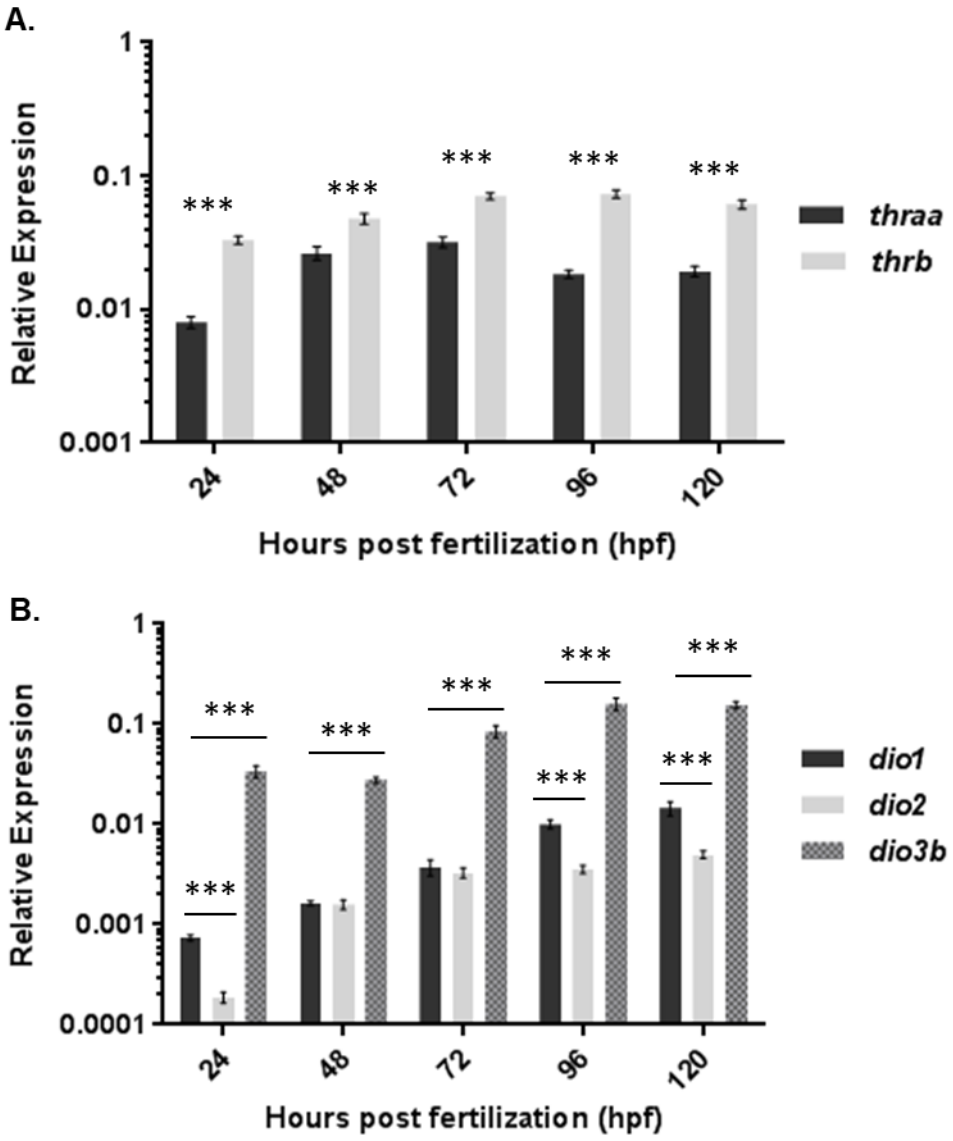

Figure S3. Comparative transcript profiles of (A) *thraa* and *thrb* and (B) *dio1, dio2* and *dio3b* in zebrafish embryo-larvae at 24, 48, 72, 96 and 120 h post fertilisation (hpf). Transcript profiles were determined using qRT-PCR and differences in transcript levels were assessed using general linear mixed models. Plotted data are presented as mean relative expression (normalised against the expression of the reference gene, *rpl8*) ± SEM. Outliers, as identified in the text, were excluded from the analysis, resulting in a replication of n=7-9 samples per developmental stage. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

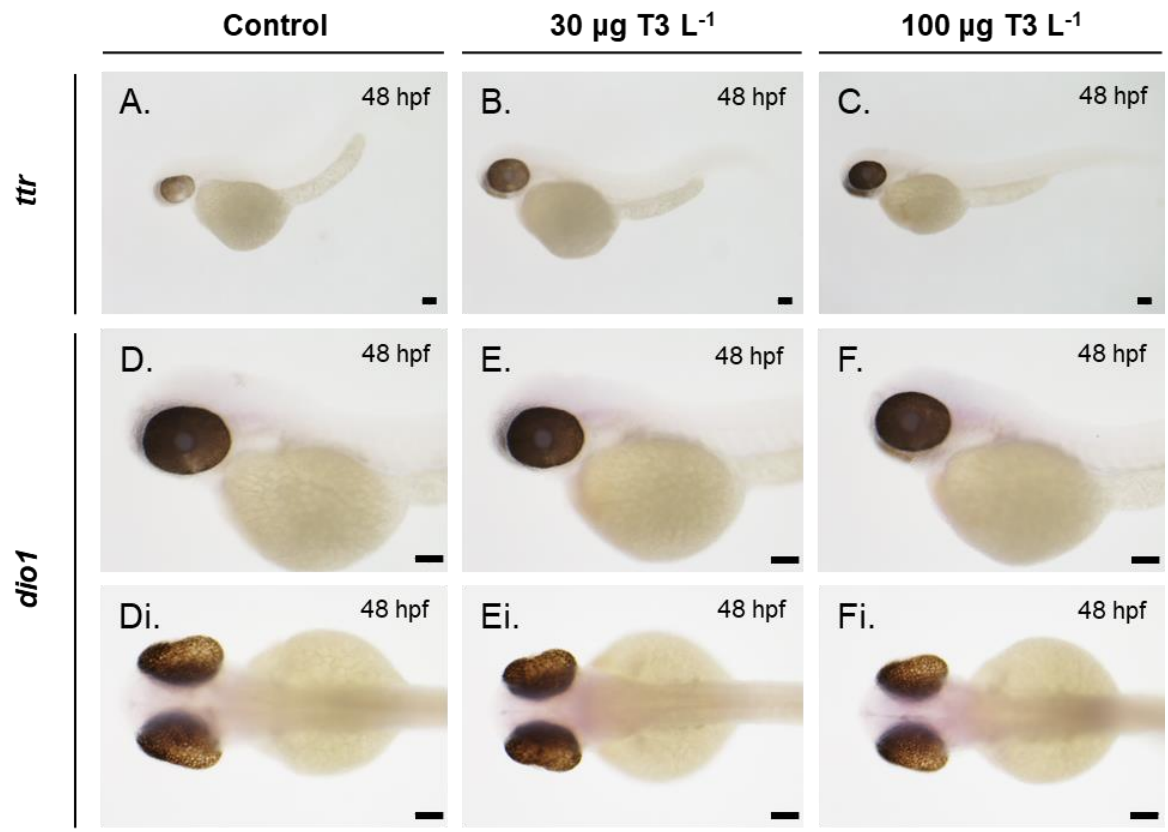

Figure S4. Representative images of the mRNA expression patterns for (A-C) transthyretin (*ttr*) and (D-F) deiodinase type I (*dio1*) in zebrafish embryos treated with T3 (0, 30 and 100 µg L^{-1}) for 48 h post fertilisation. Lateral (A-F) and dorsal (Di-Fi) views of whole embryos are shown with anterior to the left. Scale bar=100 µm.

References

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2. Filby, A.L., et al., *Unravelling the neurophysiological basis of aggression in a fish model.* BMC Genomics, 2010. **11**(1): p. 498.