



Effects of thyroid hormone disruption on the ontogenetic expression of thyroid hormone signaling genes in developing zebrafish (*Danio rerio*)

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ABSTRACT

Thyroid hormones (THs) regulate neurodevelopment, thus TH disruption is widely posited as a mechanism of developmental neurotoxicity for diverse environmental chemicals. Zebrafish have been proposed as an alternative model for studying the role of TH in developmental neurotoxicity. To realize this goal, it is critical to characterize the normal ontogenetic expression profile of TH signaling molecules in the developing zebrafish and determine the sensitivity of these molecules to perturbations in TH levels. To address these gaps in the existing database, we characterized the transcriptional profiles of TH transporters, deiodinases (DIOs), receptors (TRs), nuclear coactivators (NCOAs), nuclear corepressors (NCORs), and retinoid X receptors (RXRs) in parallel with measurements of endogenous TH concentrations and *tshb* mRNA expression throughout the first five days of zebrafish development. Transcripts encoding these TH signaling components were identified and observed to be upregulated around 48–72 h post fertilization (hpf) concurrent with the onset of larval production of T4. Exposure to exogenous T4 and T3 upregulated *mct8*, *dio3-b*, *tra-a*, *trβ*, and *mbp-a* levels, and downregulated expression of *oatp1c1*. Morpholino knockdown of TH transporter *mct8* and treatment with 6-propyl-2-thiouracil (PTU) was used to reduce cellular uptake and production of TH, an effect that was associated with downregulation of *dio3-b* at 120 hpf. Collectively, these data confirm that larval zebrafish express orthologs of TH signaling molecules important in mammalian development and suggest that there may be species differences with respect to impacts of TH disruption on gene transcription.

1. Introduction

Thyroid hormones (THs) are critical for normal brain and somatic development (de Escobar et al., 2004; DeLong et al., 1985; Gore et al., 2015; Horn and Heuer, 2010). Environmental chemicals can interfere with the function of the thyroid gland or cellular action of thyroid hormones by disrupting TH production, transport in plasma and into cells, metabolism in the liver and target cells, and receptor-mediated signaling (Boas et al., 2012; Zoeller, 2010). Concerns about human health effects of chemical-induced TH disruption have promoted research efforts to develop higher throughput tools to screen chemicals for their potential to disrupt TH function and/or signaling, and to generate mechanistic data linking TH disruption to specific health

outcomes, including adverse neurodevelopmental outcomes. Existing cell-based *in vitro* models for identifying TH disrupting chemicals (Murdock et al., 2013) do not fully recapitulate the complex physiological regulation of the hypothalamic-pituitary-thyroid (HPT) axis, the changing spatiotemporal expression patterns of TH signaling components throughout development, and cell-specific action of THs. Thus, simple systems-based models have been proposed as alternative models for chemical screening and mechanistic studies of TH disruption. Zebrafish (*Danio rerio*) are particularly promising in this regard. Zebrafish retain the physiologic complexity of traditional *in vivo* models, yet they are more tractable to gene editing, and their rapid external development and small size make them amenable to medium-throughput chemical toxicity screens (Levin and Tanguay, 2011; Mandrell et al., 2012;

Abbreviations: DIO, Deiodinase; EM, embryo medium; FW, fish water; hpf, hours post-fertilization; HPT, Hypothalamic-pituitary-thyroid; NCOA, Nuclear coactivator; NCOR, Nuclear corepressor; PTU, 6-propyl-2-thiouracil; RXR, Retinoid X Receptor; T3, Triiodothyronine; T4, Thyroxine; TH, Thyroid hormone; TR, Thyroid hormone receptor; TSH, Thyroid stimulating hormone

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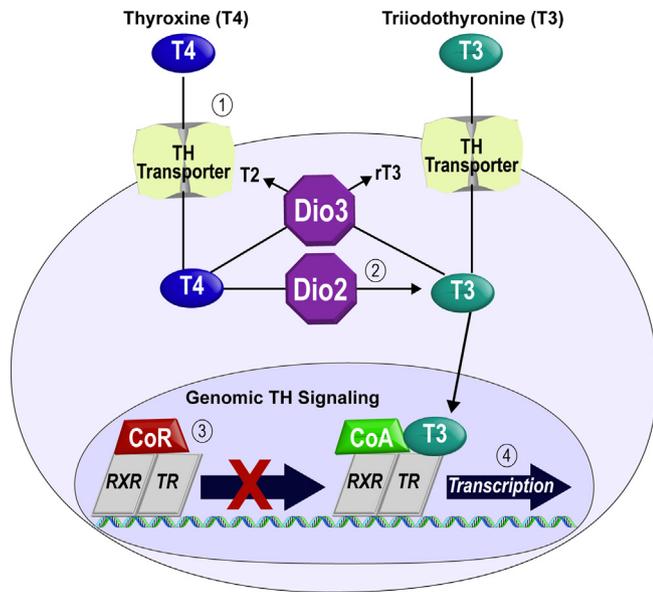


Fig. 1. Canonical thyroid hormone (TH) signaling. (1) Circulating levels of T4, the predominant form of TH in the blood, and T3 are transported into target cells by TH transporters. (2) T4 is converted to the more active T3 by deiodinase-2 (Dio2). T4 and T3 can also be converted to rT3 and T2, respectively, by Dio3. (3) In the nucleus, the thyroid hormone receptor (TR) localizes to DNA, usually as a heterodimer with the retinoid-X-receptor (RXR). In the absence of T3 ligand, a corepressor (CoR) prevents transcription. (4) When T3 translocates into the nucleus and binds to the TR-RXR heterodimer, the CoR dissociates and a coactivator (CoA) is recruited, inducing transcription of TH-responsive genes.

Truong et al., 2011).

THs are produced in the thyroid gland, typically in the form of thyroxine (T4) and to a lesser extent triiodothyronine (T3). Circulating levels of T4 and T3 are tightly regulated by negative feedback through the HPT axis, predominantly by altering the release of thyroid stimulating hormone (TSH) from the pituitary. The genomic action of T4 and T3 is mediated through interaction with nuclear TH receptors (TRs), which act as ligand-modulated transcription factors. Canonical genomic signaling, which is summarized in Fig. 1, has been previously reviewed (Cheng et al., 2010). Thyroid gland development and function, and the HPT axis, are highly conserved between zebrafish and humans (Blanton and Specker, 2007; Fagman and Nilsson, 2010; Porazzi et al., 2009). Zebrafish orthologs to mammalian TH signaling components include TH transporters (*lat1*, *lat2*, *mct8*, and *oatp1c1*), TH deiodinases (*dios1/2/3*) with *dio3* having two paralogs (*dio3-a* and *dio3-b*), TH receptors (*tra-a*, *tra-b*, and *trβ*), nuclear co-repressors (*ncor1* and *ncor2*), nuclear co-activators (*ncoa1/2/3*), and retinoid X receptors (*rxra-a*, *rxra-b* and *rxrβ-b*) (Campinho et al., 2014; Dong et al., 2013; Guo et al., 2014; Heijlen et al., 2013; Linney et al., 2011; Liu et al., 2000; Vatine et al., 2013).

While genes encoding core TH signaling molecules have been identified in zebrafish, their temporal expression patterns throughout early zebrafish development, particularly in the context of changing levels of endogenous TH levels, have not been systematically evaluated. It is also not known whether disruption of endogenous TH levels shifts the normal ontogenetic expression of these genes. The identification of TH-sensitive genes that could be leveraged as reliable readouts of TH disruption in developing zebrafish remains an outstanding question in the field. Several genes previously identified as being sensitive to TH-signaling in mammalian systems, including myelin basic protein (*mbp*), myelin associated glycoprotein (*mag*), rc3/neurogranin (Dowling and Zoeller, 2000), sonic hedgehog (*shh*) (Desouza et al., 2011), kruppel-like factor 9 (*klf9*) (Denver and Williamson, 2009; Gilbert et al., 2016), and brain derived neurotrophic factor (*bdnf*), are known to have zebrafish orthologs, but it has yet to be determined whether their

expression in zebrafish is modulated by changes in TH levels. Earlier reports have documented the effects of environmental chemicals on expression of genes in the HPT axis of developing zebrafish (Chan and Chan, 2012; Chen et al., 2012; Liang et al., 2015; Shi et al., 2009; Tu et al., 2016; Wang et al., 2013; Yu et al., 2010), and a recent microarray study identified genes responsive to TR agonists (Haggard et al., 2018). While the latter study is an important advance, it did not address the effects of hypothyroidism on gene expression, an important data gap given that many TH disrupting chemicals, including polychlorinated biphenyls, polybrominated flame retardants, pesticides, perfluorinated chemicals, perchlorate, phthalates, and bisphenol-A, have been shown to decrease serum TH levels in humans (Boas et al., 2012). In addition, experimental models, including developing zebrafish, have demonstrated both increases and decreases in T4 and T3 following chemical exposures (Chen et al., 2012; Huang et al., 2016; Liang et al., 2015; Liu et al., 2011; Shi et al., 2009; Tang et al., 2015; Tu et al., 2016; Wang et al., 2013; Yang et al., 2016; Yu et al., 2010).

To address these data gaps, we investigated the ontogenetic expression of transcripts for a core subset of TH signaling components and mammalian TH-regulated genes in parallel with quantification of endogenous concentrations of T4, T3, reverse T3 (rT3), and 3,3'-T2 (T2). In addition, we experimentally increased or decreased TH levels to determine the impact of TH disruption on expression of these genes in larval zebrafish.

2. Materials and methods

2.1. Zebrafish husbandry

All zebrafish work was approved and performed in accordance with the University of California Davis Institutional Animal Care and Use Committee (IACUC) protocols 17645 and 19391. Adult wildtype zebrafish (5D) were obtained from the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University (Corvallis, OR) and subsequent generations were raised at UC Davis. Adult zebrafish were maintained under standard laboratory controlled conditions consisting of a 14:10 h light (~850 lx):dark photoperiod (Harper and Lawrence, 2016), water temperature of 28.5 ± 0.5 °C, pH of 7.2 ± 0.4 , and conductivity of 700 ± 100 μ S. Adult fish were fed twice daily with live *Artemia nauplii* (INVE Aquaculture, Inc., Salt Lake City, UT, USA) and a mixture of the following commercial flake foods: Zeigler Zebrafish Granule (Ziegler Bros, Inc. Gardners, PA, USA), Spirulina flake (Zeigler Bros, Inc.), Cyclopeeze (Argent Aquaculture, Redmond, WA, USA), and Golden Pearl (Brine Shrimp Direct, Ogden, UT, USA). Adult zebrafish were spawned naturally in groups of 8–10 fish; embryos were collected and staged following fertilization as previously described (Kimmel et al., 1995). Embryos were kept in an incubator at 28.5 °C until plated and/or used for chemical exposures (~6 hpf).

2.2. Chemicals

Thyroid hormones L-thyroxine (T4 > 98%; Sigma T2376) and triiodothyronine (T3 > 95%; Sigma 2877), and 6-propyl-2-thiouracil (PTU > 98%; Sigma 82460) used for chemical treatment of zebrafish were purchased from Sigma-Aldrich (St. Louis, MO, USA). T4 and T3 were reconstituted by solubilizing 1 mg in 1 mL NaOH (1 N) and then adding this basic solution to 49 mL deionized water. Aliquots of T4 and T3 were stored as stock solutions of 25.7 and 30.7 μ M, respectively, at -80 °C. PTU was reconstituted by solubilizing 50 mg in 1 mL NaOH (1 N) and then adding to 4 mL deionized water to make a 72.5 mM stock (1.0%) and stored at -20 °C. Stock aliquots of T4, T3, and PTU were diluted to yield final concentrations at the time of exposure. Analytical standards used for LC/MS/MS determination of thyroid hormones in zebrafish larvae were described previously (Chen et al., 2018).

2.3. LC/MS quantitation of thyroid hormones in embryonic and larval zebrafish

The concentrations of four thyroid hormones, T4, T3, rT3, and T2 were measured in zebrafish embryos and larvae raised in control embryo medium (EM; 15 mM NaCl, 0.5 mM KCl, 1.0 mM MgSO₄, 150 μM KH₂PO₄, 50 μM Na₂HPO₄, 1.0 mM CaCl₂, 0.7 mM NaHCO₃) (Westerfield, 2000) in the absence or presence of 30 nM T4, 30 nM T3, or 0.01% PTU at 24, 72, and 120 h post fertilization (hpf) using the LC-MS/MS method previously described and validated in zebrafish larvae (Chen et al., 2018). Zebrafish embryos were placed in 6-well plates (BD Falcon, Corning, Lowell, MA, USA) containing 2 mL EM at a density of 30 embryos per well. At 6 hpf, 1 mL of a 3x concentration of T4, T3, or PTU or an additional 1 mL of EM was added to wells for final concentrations of 30 nM T4, 30 nM T3, or 0.01% PTU. Embryos were collected at 24, 72, and 120 hpf, washed 6 times in PBS, and pooled into samples of 150 embryos each. Excess water was removed, and samples were stored at −20 °C. Samples were later thawed and homogenized using a Virtis Virsonic 100 ultrasonic cell disrupter (SP Industries, Warminster, PA, USA) in 400 μL Ultrapure distilled water (Invitrogen, Carlsbad, CA, USA). Samples were re-frozen at −20 °C until TH extraction. Extractions and LC-MS/MS quantitation of THs in zebrafish tissues was performed as previously described (Chen et al., 2018). The lower limit of detection (LOD) and quantification (LOQ) were established based on the lowest calibrator with a signal-to-noise ratio of 3:1 and 10:1, respectively. In order to facilitate the statistical analysis, a non-detected congener was assigned a value of the corresponding LOD divided by ½. Data are presented as the ng TH per embryo. Statistically significant differences in the measured TH concentrations between untreated EM controls and T4- or T3-treated embryos were determined by student's *t*-test with significance set at *p* < 0.05.

2.4. Zebrafish sample collection for developmental gene expression analysis

Embryos were collected from spawning tanks, staged, and raised in 6-well plates (BD Falcon, Corning) with 30 embryos per well in 3 mL EM. Plates were covered with Parafilm M (Bemis NA, Neenah, WI) to minimize evaporation and kept in an incubator at 28.5 °C with 14 h light (~300 lx)/10 h dark cycles. Zebrafish embryos and larvae were collected at 12, 24, 48, 72, 96, and 120 hpf to assess the developmental expression of TH signaling genes and suspected TH-responsive genes using quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR). At the time of sample collection, embryos were removed from EM, washed in fish water (FW) from the adult zebrafish husbandry racks (pH of 7.2 ± 0.4, and conductivity of 700 ± 100 μS) and placed in 2 mL Eppendorf tubes with 1 mL RNAlater stabilization solution (Thermo Fisher Scientific, Waltham, MA, USA). Embryos were left at 4 °C for 24 h and then stored at −20 °C until RNA was extracted. Three biological replicates were collected at each time point with embryos spawned from different adult pairs.

2.5. Thyroid hormone treatment in zebrafish embryos

To identify genes with expression regulated by thyroid hormones, zebrafish were treated with exogenous T4 and T3 via static waterborne exposure beginning at 6 hpf and continuing until tissues were collected. T4 and T3 have been shown to be stable in serum and plasma at room temperature or 4 °C for at least 72 h (Oddo et al., 2012; Reimers et al., 1983); however, some degradation may occur by 120 h at 37 °C (Diver et al., 1994). Embryos were placed in 6-well plates (BD Falcon, Corning) with 20 embryos per well in 2 mL of EM. At 6 hpf, 1 mL of a 3X concentration of the selected compound in EM was added to each well. Control wells were treated with 1 mL of EM. Final concentrations in the well were 10, 30, and 100 nM T4 or T3. Plates were covered with Parafilm M to minimize evaporation and kept in an incubator at 28.5 °C with 14 h light (~300 lx)/10 h dark cycles. At 24, 72, or 120 hpf,

zebrafish embryos/larvae were removed from treatment solution, washed in FW, and placed in 2 mL Eppendorf tubes with 1 mL RNAlater stabilization solution (Thermo Fisher Scientific). Embryos were left at 4 °C for 24 h and then stored at −20 °C until RNA extraction. Three biological replicates were collected for each time point and TH treatment combination with embryos spawned from different adult pairs.

2.6. *Mct8* morpholino knockdown and PTU treatment

To identify genes influenced by decreased TH levels, the TH transporter *mct8* was knocked-down using the fluorescein-tagged splice blocking morpholino-modified antisense oligonucleotide *mct8*(E2I2) MO (5'-ataaaatcatgtatttcactggtgcca-3'); the control morpholino for these experiments was the fluorescein-tagged Gene Tools standard control MO (5'-ctcttacctcagttacaattata-3') (Gene Tools, Philomath, OR). The *mct8*(E2I2) MO interferes with the splicing of the second exon/intron, introducing a premature stop codon, as described and validated previously (Vatine et al., 2013). Wildtype 5D zebrafish embryos were injected at the 1–2 cell stage with approximately 0.5 pmol of 0.15 mM morpholino. At 6 hpf, injected embryos were placed in polystyrene 96-well plates with EM or PTU. At 24 hpf, consistent incorporation of the morpholino was confirmed by visualizing the fluorescein tag in embryos using an automated imaging system (ImageXpress, Molecular Devices, Sunnyvale, CA, USA). Embryos without MO incorporation were not included in gene expression analysis. At 120 hpf, zebrafish larvae were removed from treatment solution, washed in FW, and placed in 2 mL Eppendorf tubes with 1 mL RNAlater stabilization solution (Thermo Fisher Scientific). Embryos were left at 4 °C for 24 h and then stored at −20 °C until RNA extraction. Three biological replicates were collected with embryos spawned from different adult pairs.

2.7. RNA extraction, cDNA synthesis, and quantitative real time RT-PCR (qRT-PCR)

To extract RNA, zebrafish were removed from RNAlater and homogenized in 350 μL of RLT buffer (Qiagen RNeasy kit, Valencia, CA) with 1 mm glass beads using the Bullet Blender (Next Advance, Troy, NY). Homogenates were transferred to 1.5 mL Eppendorf tubes and spun in a tabletop centrifuge at maximum speed (21,000 × *g*) for 3 min. The supernatant was transferred to 2 mL Eppendorf tubes and RNA was extracted using a Qiagen RNeasy kit using an automated QIAcube robotic workstation (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Nucleic acid purity was determined by the ratio of absorbance at 260 nm to 280 nm (A₂₆₀/A₂₈₀ of 1.8–2.2 were accepted) and the absence of genomic DNA contamination was determined by visualization on an agarose gel stained with SYBR Green nucleic acid gel stain (Thermo Fisher Scientific). RNA was reverse transcribed to complementary DNA (cDNA) using of 1 μg of total RNA per 20 μL reaction with Superscript VILO Mastermix cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. This kit uses a master mix with random primers for cDNA synthesis. Reactions were incubated for 10 min at 25 °C, 60 min at 42 °C followed by a 5 min denaturation step at 85 °C. Quantitative real-time PCR (qRT-PCR) was performed on a laser 7900 HT FAST platform (Applied Biosystems, Foster City, CA, USA). Samples were run in a 384-well format with 12 μL of PCR mix per well. PCR mix consisted of 6 μL 2X SYBR Green Power Master Mix (Life Technologies, Foster City, CA, USA), 0.25 μL of 10 mM stock forward primer (200 nM final concentration), 0.25 μL of 10 mM stock reverse primer (200 nM final concentration), 1 μL cDNA (~50 ng/reaction), and 4.5 μL UltraPure water. The PCR amplification protocol was as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The amplification protocol was followed by a standard melting curve to confirm the production of a single amplicon and absence of primer dimers. Each

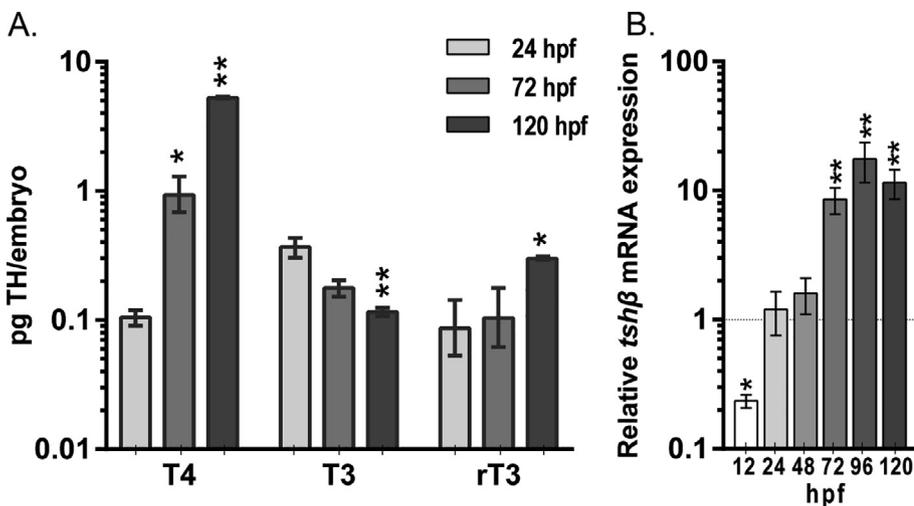


Fig. 2. T4, T3, and rT3 concentrations and *tshβ* mRNA expression during zebrafish development. (A) Concentrations of T4, T3, and rT3 were determined by LC-MS/MS at 24, 72, and 120 hpf. Data are presented as the mean TH concentration normalized per embryo (pg TH/embryo) \pm SE (n = 3 replicates, each with 150 pooled embryos). (B) Expression of *tshβ* at 12, 48, 72, 96, and 120 hpf is presented as the mean \pm SE of the fold change in mRNA levels relative to expression at 24 hpf ($2^{-\Delta\Delta Ct}$). * p < 0.05; ** p < 0.01 as determined by ANOVA (TH measurements) or REST relative expression software (qRT-PCR data).

cDNA sample was run as three technical replicates and averaged for expression analysis. Primers were designed using NCBI Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and obtained from Integrated DNA Technology (Integrated DNA Technologies, Inc., Coralville, IA, USA). Supplemental Table 1 provides primer sequences, accession numbers, and amplicon size for each gene included in analysis.

2.8. Expression analysis of TH-signaling genes and suspected TH-responsive genes

Expression of TH transporters, TH deiodinases, TH receptors, nuclear coactivators and corepressors, retinoid X receptors, and suspected TH-responsive genes were analyzed by qRT-PCR. Amplification results were analyzed using the 7500 Fast System SDS software (Applied Biosystems) to obtain Ct values (Pfaffl et al., 2002). Amplification efficiencies (AE) for each primer pair were determined using seven-point standard dilution curves with three replicates using the following equation: $AE = 10^{[-1/\text{slope}]}$ as previously described (Pfaffl, 2001). Amplification efficiencies were all between 90 and 110%. The Ct values for each target gene were normalized to the geometric mean of the Ct values for two reference genes, *β-actin* and *elf1a*, (McCurley and Callard, 2008) within the same sample. To compare the relative expression between signaling genes in the whole fish, delta Ct (ΔCt) values were calculated as the AE adjusted difference between the Ct value of the target gene and the geometric mean of the Ct values of *β-actin* and *elf1a* for the same sample using the following equation: $\Delta Ct = [Ct_{tgt} \times AE_{tgt}] - [Ct_{ref} \times AE_{ref}]$ where tgt is the target gene and ref is the reference gene. Data are presented as the mean fold expression difference between the target gene and the reference genes ($2^{-\Delta Ct}$) to compare relative expression levels between TH signaling genes during embryonic development (24–48 hpf) and larval development (72–120 hpf). To evaluate changes in expression through development and the impact of experimental treatments on gene expression, the ΔCt values for each sample were normalized to the ΔCt of the same gene in the control sample, calculating AE adjusted delta-delta Ct values ($\Delta\Delta Ct$) using the following equation as previously described (Yuan et al., 2008):

$\Delta\Delta Ct = [(Ct_{tgt, trt} \times AE_{tgt, trt}) - (Ct_{ref, trt} \times AE_{ref, trt})] - [(Ct_{tgt, ctrl} \times AE_{tgt, ctrl}) - (Ct_{ref, ctrl} \times AE_{ref, ctrl})]$ where tgt is the target gene, ref is the reference gene, trt is the treatment, and ctrl is the control. For evaluating expression changes across developmental time points, 24 hpf was used as the reference point and other time points were considered as the treatment in the calculation, thus, gene expression was normalized to the expression level at 24 hpf. Data are presented as the fold expression change at each time point relative to 24 hpf controls ($2^{-\Delta\Delta Ct}$). To

compare the expression changes resulting from experimental treatments (T4 or T3 exposure or *mct8MO* + 0.01% PTU), the $\Delta\Delta Ct$ values were calculated by normalizing treated samples to their respective controls (EM or ctrlMO). For the analysis of gene expression changes following TH treatment, data is presented as the fold expression change of each sample at each time point relative to 24 hpf EM controls ($2^{-\Delta\Delta Ct}$) but also shows the fold expression change of each TH-treated sample relative to controls at the same time point. This approach allowed us to show TH-induced changes in gene expression within the context of normal developmental expression changes.

Statistical analysis was performed to determine significant changes in expression between developmental time points or between experimental treatments using REST 2009 relative expression software (Qiagen). The REST 2009 software assesses the statistical significance of differences in AE adjusted expression between control and experimental groups using the applied Pair Wise Fixed Reallocation Randomization Test© for each sample (random pairing of controls and samples from the gene of interest and the reference gene, and calculation of their expression ratios). Randomization tests have the advantage of making no distributional assumptions about the data, thus they are a useful alternative to parametric tests. A full description of the development of the REST software and validation of the statistical model have been previously described (Pfaffl et al., 2002).

3. Results

3.1. Thyroid hormone concentrations and *tshβ* mRNA expression during development

Concentrations of T4, T3, rT3, and T2 were measured in pooled samples of 150 zebrafish at 24, 72, and 120 hpf (Fig. 2A). At 24 hpf, the concentration of T3 in whole embryos (0.37 ± 0.05 pg/embryo) was the highest of the THs measured, reflecting the high maternal contribution of T3 in eggs. The concentration of T3 decreased over the first five days of development to 0.18 ± 0.02 pg/embryo at 72 hpf and 0.12 ± 0.007 pg/embryo at 120 hpf. The T4 concentration in embryos at 24 hpf (0.1 ± 0.01 pg/embryo) increased approximately 10-fold by 72 hpf and 50-fold by 120 hpf. These findings are consistent with previous research demonstrating that in developing zebrafish, thyroid follicles develop and begin producing T4 around 48–72 hpf (Porazzi et al., 2009). At 24 hpf, the concentration of rT3 was measured at 0.09 ± 0.05 pg/embryos and increased slightly by 72 hpf to 0.1 ± 0.06 pg/embryo and further to 0.3 ± 0.01 pg/embryos by 120 hpf (p < 0.05), indicating that an increasing amount of T4 was being deiodinated to rT3. The concentration of T2 in zebrafish embryos and larvae was below the limit of detection (0.2 pg/g or 0.003 pg/

embryo) at all time points evaluated (data not shown).

Thyroid-stimulating hormone (TSH) is a glycoprotein hormone synthesized and secreted from thyrotrope cells in the anterior pituitary to stimulate T4 production and secretion from thyroid follicles. We measured mRNA expression of the TSH beta subunit (*tshβ*) throughout zebrafish development to evaluate activity of the hypothalamic-pituitary-thyroid (HPT) axis. As illustrated in Fig. 2B, *tshβ* mRNA was detectable at 24 hpf, and expression increased approximately 8-fold at 72 hpf, and remained significantly elevated at 96 and 120 hpf, indicating increased functionality of the HPT axis by 72 hpf, which coincides with the simultaneous increase in T4 measured in zebrafish tissue.

3.2. Ontogenetic profile of mRNA encoding TH signaling genes and transcriptional regulators

The ontogenetic profile of transcripts encoding core TH signaling genes was determined over the first 120 hpf using quantitative real time RT-PCR (qRT-PCR). Data are presented as: (1) the fold change in mRNA levels relative to reference genes ($2^{-\Delta C_t}$) during embryonic development (defined as the developmental period between 24 and 48 hpf) and larval development (defined as the developmental period between 72 and 120 hpf) in order to compare expression levels between genes; and (2) as the fold change in transcript levels of each gene at each time point relative to expression of the gene at 24 hpf ($2^{-\Delta\Delta C_t}$) in order to evaluate the developmental expression patterns of each gene.

3.2.1. TH transporters

The mRNA levels were quantified for four TH transporters, including two non-specific TH transporters, L-type amino acid transporters 1 and 2 (*lat1* and *lat2*), and two TH-specific transporters, monocarboxylate anion transporter 8 (*mct8*) and organic anion transporter 1C1 (*oatp1c1*) (Fig. 3A–B). Transcripts for all four transporters were present as early as 12 hpf and exhibited moderate increases in expression from early embryonic development to larval developmental time points (Fig. 3A). *Lat1* and *lat2* were expressed in similar amounts during embryonic and larval development, and mRNA levels of both were higher than either *mct8* or *oatp1c1*. Three transporters, *lat2*, *mct8*, and *oatp1c1*, exhibited similar developmental expression profiles, with no significant change in expression between 12 hpf and 48 hpf, followed by significantly increased expression at 72 hpf, which peaked at 96 hpf, evident as a 4-fold increase in *lat2* and *mct8*, and an 8-fold increase in *oatp1c1* relative to levels at 24 hpf (Fig. 3B). In contrast, *lat1* mRNA expression was approximately 2.5-fold lower at 12 hpf than at 24 hpf ($p < 0.05$), increased significantly from 24 hpf to 48 hpf by approximately 3-fold, and remained elevated at 72, 96, and 120 hpf, peaking at 96 hpf with a 7-fold higher expression than at 24 hpf (Fig. 3B).

3.2.2. TH deiodinases

The mRNA levels were quantified for four TH deiodinases, including deiodinase 1 (*dio1*), which catalyzes deiodination of T4 to T3 or rT3, deiodinase 2 (*dio2*), which catalyzes the deiodination of T4 to T3, and two paralogs of deiodinase 3 (*dio3-a* and *dio3-b*), which deiodinate T4 and T3 to the inactive metabolite rT3 and T2, respectively (Fig. 3A & C). Of these, *dio2* exhibited the lowest expression during both embryonic and larval development, with *dio1*, *dio3-a*, and *dio3-b* showing more similar levels of expression during both developmental stages (Fig. 3A). All four deiodinases increased over the first five days of development (Fig. 3C). Expression of *dio1* did not change between 12 and 48 hpf, then increased significantly by 7-fold at 72 hpf, 31-fold at 96 hpf, and 47-fold at 120 hpf, relative to expression at 24 hpf. At 12 hpf, *dio2* mRNA levels were 4.3-fold lower than those measured at 24 hpf. By 48 hpf, *dio2* expression increased by 7.5-fold relative to 24 hpf, peaked at 72 and 96 hpf with levels 18-fold higher than at 24 hpf, and then decreased slightly by 120 hpf. Developmental patterns of *dio3-a* and *dio3-b* expression were very similar over the first five days

of development. At 12 hpf, *dio3-a* and *dio3-b* mRNA levels were 12.5 and 10-fold lower than at 24 hpf, respectively. Transcript levels for either gene did not change between 24 and 48 hpf, then increased by approximately 5-fold at 72 hpf and remained elevated at both 96 hpf and 120 hpf at levels between 6 and 9-fold above the 24 hpf levels.

3.2.3. TH receptors

The mRNA levels were quantified for three TH receptors, including two paralogs of *tra*, *tra-a* and *tra-b*, and *trβ*, (Fig. 3A & D). Each receptor gene exhibited unique developmental expression profiles. Of the three, *tra-a* expression was the highest during both embryonic and larval development. Levels of *tra-b* were somewhat lower but exhibited a larger increase during development. Expression of *trβ* was similar to that of *tra-b* during embryonic development; however, *trβ* expression was consistent across developmental time points (Fig. 3A). As illustrated in Fig. 3D, *Tra-a* expression was 2-fold lower at 12 hpf than at 24 hpf, unchanged between 24 and 48 hpf, increased by 4-fold at 72 hpf, and remained elevated at 96 and 120 hpf at levels that were 5.5-fold and 4-fold higher than 24 hpf levels, respectively. mRNA levels of *tra-b* were 1.4-fold lower at 12 hpf relative to 24 hpf, and by 48 hpf, a small but significant 2-fold increase was observed. Expression of *tra-b* continued to increase by 9-fold at 72 hpf, peaking at a 26-fold elevation at 96 hpf, and returning by 120 hpf to levels that were 9-fold higher than 24 hpf levels. While *trβ* mRNA was significantly lower at 12 hpf than at 24 hpf, levels did not change significantly beyond 24 hpf (Fig. 3D).

3.2.4. Retinoid X receptors (RXRs)

Transcripts were quantified for four RXRs, the TR heterodimerization partner, including two paralogs of *rxra* (*rxra-a* and *rxra-b*) and two paralogs of *rxrβ* (*rxrβ-a* and *rxrβ-b*) (Fig. 4A–B). While *rxra-a* and *rxrβ-b* exhibited similar levels of expression during embryonic and larval development, *rxra-b* was measured in much lower amounts at all time points assessed (Fig. 4A). *Rxrβ-a* mRNA was not detected during zebrafish development (data not shown). Of the detected RXRs, *rxra-a*, *rxra-b*, *rxrβ-b* each exhibited a slightly different pattern of expression, yet for all, the peak expression level was observed at 96 hpf (Fig. 4B). The expression of *rxra-a* increased somewhat consistently from 12 hpf to 96 hpf, reaching a statistically significant 3-fold increase relative to 24 hpf at 96 hpf and 2-fold at 120 hpf. Levels of *rxra-b* mRNA were negligible at 12 hpf, gradually increased after 24 hpf, peaking at 96 hpf with expression 4.3-fold higher than 24 hpf levels. Levels of *rxrβ-b* mRNA exhibited a u-shaped pattern over the first five days of development: expression at 12 hpf was 2-fold higher than at 24 hpf, remained unchanged between 24 and 48 hpf, and then increased from 72 to 120 hpf. Expression at 72, 96, and 120 hpf was increased by 2.2, 4.0, and 2.4-fold relative to expression at 24 hpf (Fig. 4B).

3.2.5. Nuclear co-activators

The mRNA levels were measured for three nuclear coactivators, including *ncoa1* (also known as *src-1*), *ncoa2* (also known as *scr-2/tif2/grip1*), and *ncoa3* (Fig. 4A & C). These coactivators are recruited to TRs, as well as other nuclear receptors, upon ligand interaction and enhance transcriptional activation (Leo and Chen, 2000). Expression levels of *ncoa1/2/3* were similar during embryonic and larval development, with *ncoa3* having a slightly higher expression during the embryonic period than *ncoa1* or *ncoa2* (Fig. 4A). As shown in Fig. 4C, at 12 hpf, expression of *ncoa1*, *ncoa2*, and *ncoa3* was 1.6, 1.7, and 1.8-fold higher than at 24 hpf, respectively; however, this pattern was only statistically significant for *ncoa1* and *ncoa2*. For all three coactivators, expression levels did not change significantly between 24 and 48 hpf, then increased at 72 hpf approximately 1.4–2.0-fold, peaking at 96 hpf with a 3–4-fold increase relative to 24 hpf. Only the changes in *ncoa1* and *ncoa2* mRNA levels were statistically significant at 72, 96, and 120 hpf (Fig. 4C).

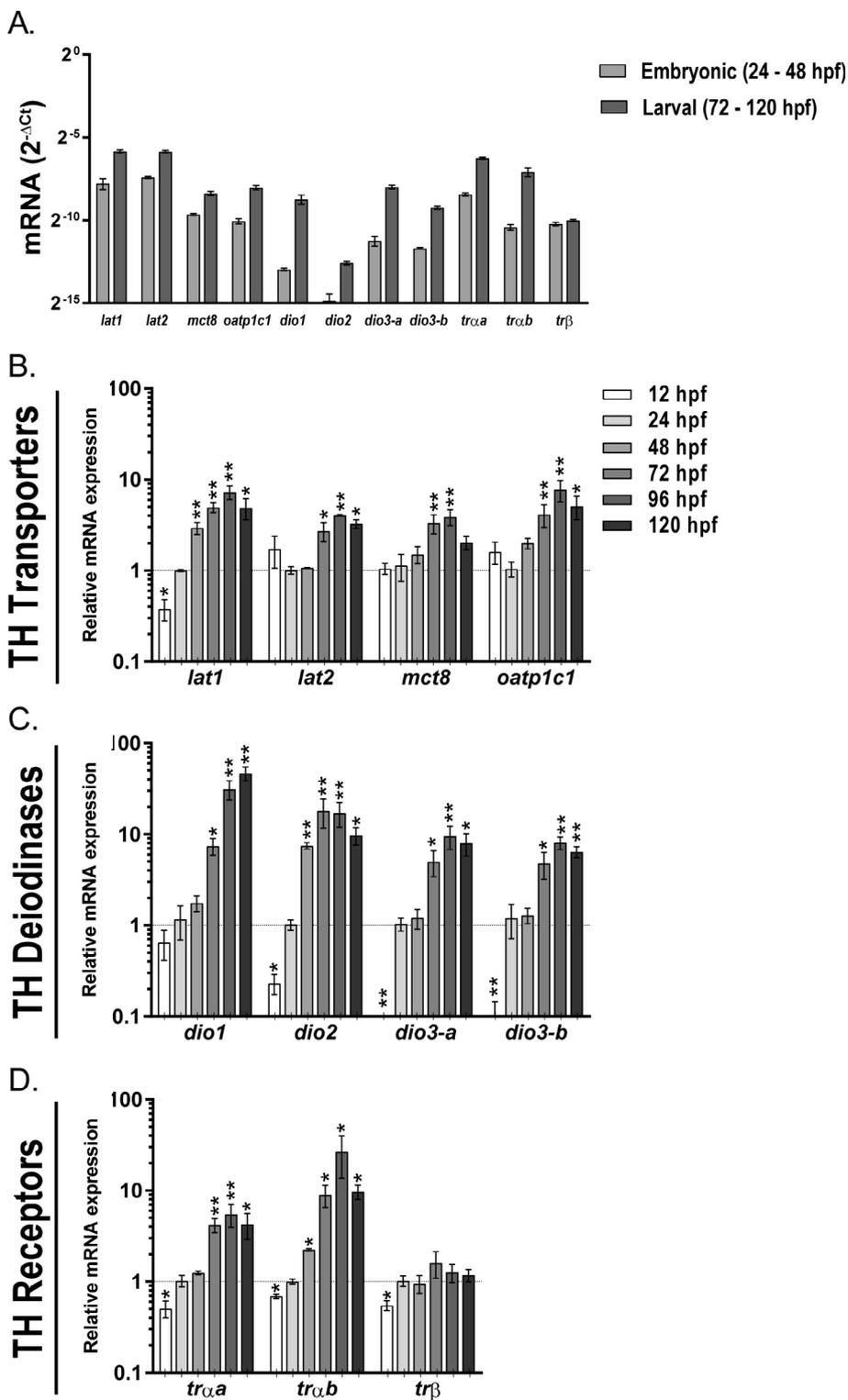


Fig. 3. Ontogenetic transcription of TH transporters, deiodinases, and receptors. Pooled samples were collected at 12, 24, 48, 72, 96, and 120 hpf to quantify changes in gene expression as determined by qRT-PCR. (A) To compare the relative expression between genes, data are presented as the average fold expression change relative to the geometric mean of two reference genes, *β-actin* and *elf1a*, ($2^{-\Delta Ct}$) during embryonic development (24–48 hpf) and larval development (72–120 hpf). (B–D) Expression of each gene is also presented as the relative expression measured at each time point relative to expression at 24 hpf ($2^{-\Delta\Delta Ct}$), as determined using the Pfaffl delta-delta Ct method. All samples were normalized to the reference genes *β-actin* and *elf1a*. Error bars represent the SE (n = 3 replicates of 30 pooled embryos per group). *Significantly different from 24 hpf at $p < 0.05$, ** $p < 0.01$ as determined using REST relative expression software.

3.2.6. Nuclear co-repressors

The developmental mRNA expression was determined for two nuclear corepressors, *ncor1* and *ncor2* (also known as *smrt*) (Fig. 4A & D). In the absence of ligand, NCORs localize to DNA-bound TRs, and other nuclear receptors, and repress transcription (Watson et al., 2012). Transcript levels of *ncor1* and *ncor2* were similar during embryonic and larval development (Fig. 4A), and the two genes exhibited similar developmental expression profiles (Fig. 4D). Levels of mRNA did not change between 12 and 48 hpf. However, from 72 to 120 hpf, small increases in expression were observed with transcript levels of both

genes peaking at 96 hpf, with 3-fold increases relative to expression at 24 hpf (Fig. 4D).

3.3. Developmental expression of candidate TH-responsive genes

Rodent studies have identified many genetic targets of TH with known roles in neurodevelopment, which have been used to assess the cellular impact of thyroid hormone disrupting chemicals. These include myelin basic protein (*mbp*) and myelin associated glycoprotein (*mag*) (Dong et al., 2009), *rc3/neurogranin* (Dowling and Zoeller, 2000), sonic

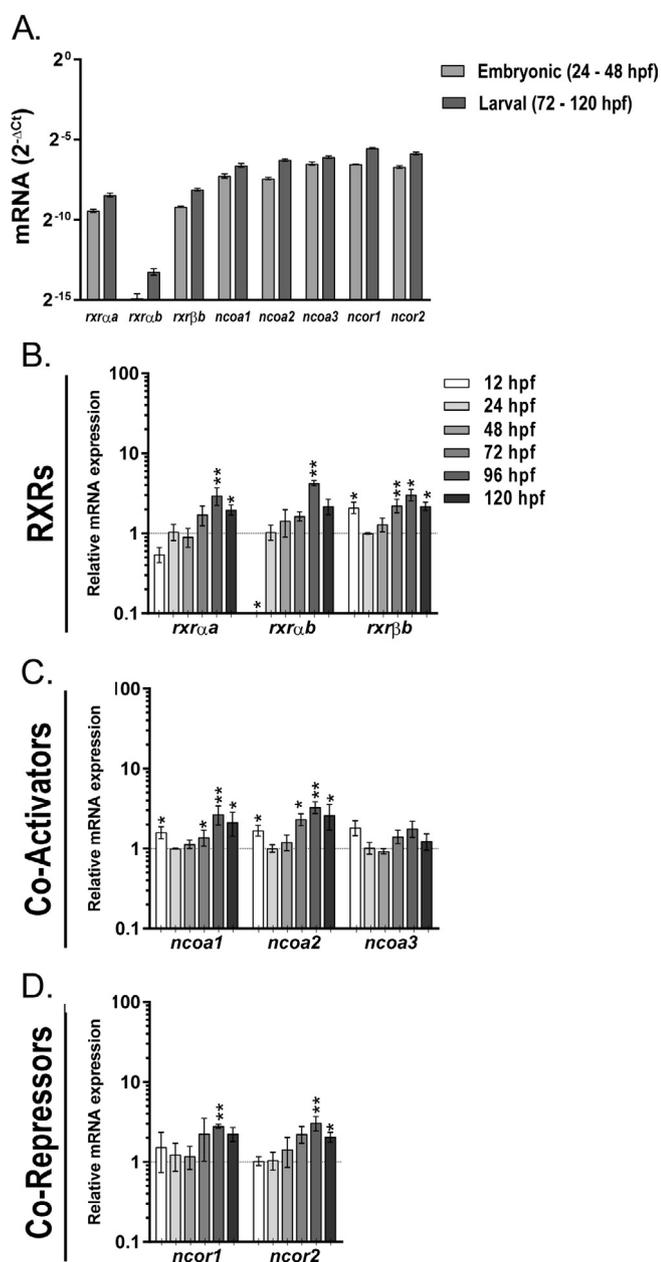


Fig. 4. Ontogenetic transcription of RXRs, nuclear co-activators, and nuclear co-repressors. Pooled samples were collected at 12, 24, 48, 72, 96, and 120 hpf to quantify changes in gene expression as determined by qRT-PCR. (A) To compare the relative expression between genes, data are presented as the average fold expression change relative to the geometric mean of two reference genes, β -actin and $elf1a$, ($2^{-\Delta\Delta Ct}$) during embryonic development (24–48 hpf) and larval development (72–120 hpf). (B–D) Expression of each gene is also presented as the relative expression measured at each time point relative to expression at 24 hpf ($2^{-\Delta\Delta Ct}$), as determined using the Pfaffl delta-delta Ct method. All samples were normalized to the reference genes β -actin and $elf1a$. Error bars represent the SE ($n = 3$ replicates of 30 pooled embryos per group). *Significantly different from 24 hpf at $p < 0.05$, ** $p < 0.01$ as determined using REST relative expression software.

hedgehog (*shh*) (Desouza et al., 2011), kruppel-like factor 9 (*klf9*) (Denver and Williamson, 2009; Gilbert et al., 2016), and brain derived neurotrophic factor (*bdnf*). The impact of varying TH levels on expression of these known TH-target genes has not yet been evaluated in the zebrafish model. Developmental transcriptomic profiles of these six genes were assessed at 24, 72, and 120 hpf (Fig. 5). With the exception of *shha*, mRNA levels of these genes increased significantly with

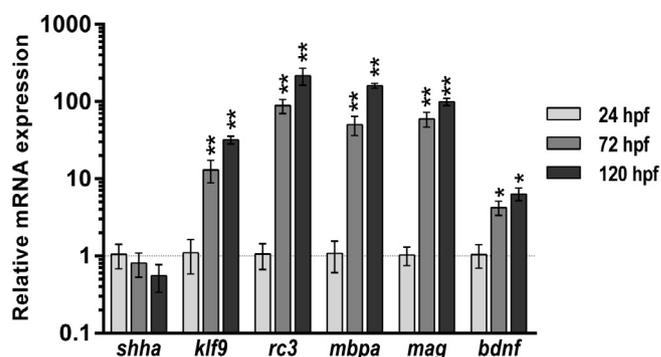


Fig. 5. Ontogenetic transcription of candidate TH-responsive genes. Pooled samples were collected at 24, 72, and 120 hpf to quantify changes in gene expression as determined by qRT-PCR using the Pfaffl delta-delta Ct method. Bars represent the fold expression change relative to 24 hpf samples. All samples were normalized to the reference genes β -actin and $elf1a$. Error bars represent the SE ($n = 3$ replicates of 30 pooled embryos per group). *Significantly different from 24 hpf at $p < 0.05$, ** $p < 0.01$ as determined using REST relative expression software.

increasing developmental times. From 24 hpf to 72 hpf, expression of *klf9*, *rc3*, *mbpa*, *mag*, and *bdnf* increased by 13, 90, 50, 60, and 4-fold, respectively, while expression from 24 hpf to 120 hpf increased by 32, 216, 160, 100, and 6-fold, respectively (Fig. 5).

3.4. Uptake and biotransformation of T4 and T3 in zebrafish embryos/larvae from static waterborne exposure to TH

The concentrations of T4, T3, rT3, and T2 were measured at 24, 72, and 120 hpf in zebrafish embryos/larvae treated with 30 nM T4 or T3 to evaluate the extent of TH uptake and impact on the TH profile across development (Fig. 6). Treatment with exogenous T4 increased the tissue concentration of T4 at all time points measured, with the greatest relative increase occurring at 24 hpf (Fig. 6A). At 24 hpf, T4 in embryos was increased 100-fold relative to age-matched EM controls. At 24 hpf, levels of T3, rT3, and T2 were not altered by T4 treatment. At 72 (Fig. 6B) and 120 hpf (Fig. 6C), T4 concentration in tissue was elevated by 2.6-fold and 1.7-fold, respectively, relative to age-matched EM controls. T4 treatment also increased the larval concentrations of rT3, by 9-fold at 72 hpf and 2.5-fold at 120 hpf, and T2, by 8-fold at 72 hpf and 40-fold at 120 hpf (Fig. 6B & C), indicating that the excess T4 taken up was metabolized to these two inactive products by tissue deiodinases. No statistically significant changes in T3 were measured at any time points following treatment with exogenous T4 (Fig. 6A–C). Treatment with exogenous T3 increased the T3 in zebrafish tissue at 24 (Fig. 6A), 72 (Fig. 6B), and 120 hpf (Fig. 6C), by approximately 8-fold, 4-fold, and 6-fold, respectively. T3 treatment also increased concentrations of T2 at 24, 72, and 120 hpf by approximately 3-fold, 8-fold, and 13-fold, respectively, relative to age-matched EM controls (Fig. 6A–C), indicating that T3 was deiodinated in tissue to form T2. No significant changes in tissue T4 or rT3 were measured at any time points following treatment with exogenous T3; however, a small increase in T4 in T3-treated zebrafish at 24 hpf neared statistical significance ($p = 0.06$) (Fig. 6A–C).

3.5. Effects of varying TH levels on gene expression

To determine whether mRNA expression of TH signaling genes and suspected TH-responsive genes are regulated by THs, zebrafish embryos were exposed to T4 or T3 to simulate developmental hyperthyroidism or TR agonism. Zebrafish were continuously exposed to T4 and T3 at concentrations of 10, 30, or 100 nM, beginning at 6 hpf and samples were collected at 24, 72, or 120 hpf for analysis of gene expression by qRT-PCR. Of the genes evaluated (TH transporters *lat1*, *lat2*, *mct8*, and

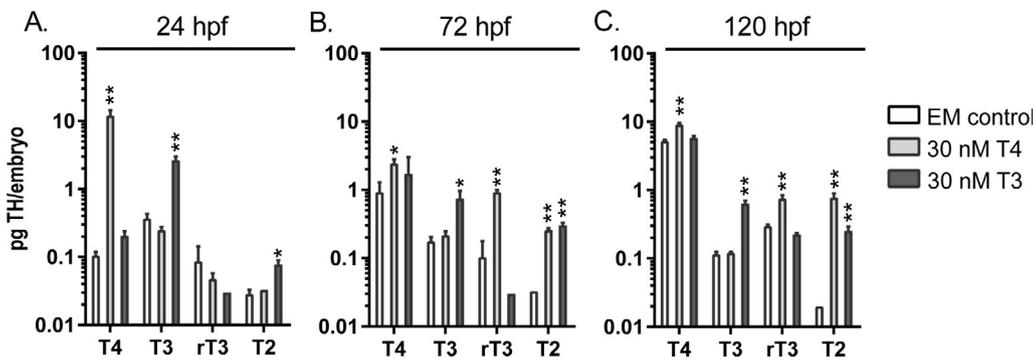


Fig. 6. Concentrations of the thyroid hormones T4, T3, rT3, and T2 in zebrafish larvae exposed to exogenous T4 or T3. Zebrafish were raised in control embryo medium (EM) in the absence or presence of 30 nM T4 or 30 nM T3. T4 or T3 treatment began at 6 hpf and samples were collected and measured at 24 hpf, 3 dpf, and 5 dpf. Concentrations of T4, T3, rT3, and 3,3'-T2 are presented as the mean pg TH/embryo \pm SE measured from three pooled samples of 150 embryos each ($n = 3$ per group). Significantly different from EM controls at * $p < 0.05$, ** $p < 0.01$, as determined using unpaired student's t -test.

oatp1c1, TH deiodinases *dio2*, *dio3a*, and *dio3b*, TH receptors *tra-a*, *tra-b*, and *trb*, and suspected TH responsive genes (*mbp-a*, *bdnf*, *shh-a*, *rc3*, *klf9*, and *mag*), the expression of six was observed to be significantly altered by addition of exogenous TH (Fig. 7). Data for the remaining 10 genes whose expression was not significantly changed by increased levels of T4 or T3 are presented in Supplemental Fig. 1. While a few of these 10 genes did exhibit statistically significant changes in gene expression in some individual samples, the changes were less consistently observed over multiple doses, time points, or TH treatments, thus these genes were deemed less likely to be useful as biomarkers of developmental TH disruption.

Two TH transporters, *mct8* and *oatp1c1*, exhibited statistically significant changes in gene expression following treatment with either T4 or T3 (Fig. 7A–D). Expression of *mct8* was increased at 72 hpf by all concentrations of both T4 and T3 relative to untreated controls (Fig. 7A–B). However, at 120 hpf, *mct8* expression was significantly increased only by the highest concentration of T4 (100 nM), and by T3 at 10 and 100 nM. In contrast, expression of *oatp1c1* was significantly decreased by T4 at 30 and 100 nM at both 72 and 120 hpf, and by T3 exposure at 100 nM at both 72 and 120 hpf (Fig. 7C–D). No changes were observed in expression of *lat1* or *lat2* following T4 or T3 exposure (Fig. S1A–D).

Of the TH deiodinases, *dio2*, *dio3-a*, and *dio3-b*, only *dio3-b* mRNA levels were significantly altered by exposure to exogenous THs (Fig. 7E–F). However, of all the mRNAs measured, *dio3-b* transcripts exhibited the highest sensitivity to either T4 or T3, and the largest increases. *Dio3-b* was the only gene whose expression was altered by TH exposure at 24 hpf, although significantly increased *dio3-b* mRNA levels at 24 hpf were only observed in fish exposed to T4 (Fig. 7E), not T3 (Fig. 7F). At 72 hpf, T4 at 10, 30, and 100 nM increased *dio3-b* expression by 2.2, 3.0, and 5.6-fold, respectively (Fig. 7E), while T3 at 10, 30, and 100 nM increased *dio3-b* expression by 1.5, 2.2, and 3.6-fold, respectively (Fig. 7F). At 120 hpf, *dio3-b* expression was increased by 2.1, 3.6, and 8.2-fold in response to T4 at 10, 30 and 100 nM, respectively (Fig. 7E), and by 1.8, 3.0, and 5.2-fold in response to T3 at 10, 30, and 100 nM, respectively (Fig. 7F). Interestingly, expression of *dio2*, which deiodinates T4 to form T3, was decreased by exposure to T4; however, only at 72 hpf (Fig. S1E). In contrast, *dio3-a* expression was upregulated by 100 nM T3 at 72 hpf, but only by 1.6-fold (Fig. S1H).

Of the three nuclear TH receptor genes, *tra-a*, *tra-b*, and *trb*, mRNA levels of two, *tra-a* and *trb*, were significantly increased by exogenous TH at 72 and 120 hpf (Fig. 7G–J). At 72 hpf, all concentrations of T4 and T3 significantly upregulated expression of *tra-a*, with fold increases of 1.4, 1.7, and 2.1 for 10, 30, and 100 nM T4, respectively (Fig. 7G), and slightly higher fold increases of 1.6, 1.9, and 2.5 following exposure to T3 at 10, 30, or 100 nM, respectively (Fig. 7H). At 120 hpf, only the higher concentrations of T4 and T3 increased expression of *tra-a*. Exposure to T4 at 30 or 100 nM increased *tra-a* expression by 1.7 and 2.1-fold, respectively (Fig. 7G), while exposure to T3 at 100 nM increased

tra-a expression by 1.8-fold (Fig. 7H). Exposure to all concentrations of T4 and T3 increased expression of *trb* at 72 hpf and 120 hpf. At 72 hpf, T4 concentrations of 10, 30, and 100 nM increased *trb* expression by 1.6, 2.1, and 2.6-fold, respectively (Fig. 7I), and T3 concentrations of 10, 30, and 100 nM increased *trb* expression by 2.1, 2.3, and 3.2-fold, respectively (Fig. 7J). At 120 hpf, *trb* expression was increased by T4 and T3 at of 10, 30, and 100 nM by 1.7, 2.7, and 4.5-fold and 3.0, 3.4, and 3.9-fold, respectively (Fig. 7I–J). In contrast to *tra-a* and *trb*, expression of *tra-b*, was only minimally influenced by exposure to exogenous THs. Minimal (< 1.5-fold) increases in expression were observed at 72 hpf following exposure to T3 (Fig. S1J).

Of six genes previously identified as TH-responsive genes in rodent models, including *mbp-a*, *bdnf*, *shh-a*, *rc3*, *klf9*, and *mag*, *mbp-a* exhibited the greatest number of statistically significant changes in gene expression in response to exogenous THs (Fig. 7K–L). Expression of *mbp-a* was upregulated only at 120 hpf; however, significant increases were measured in response to all concentrations of T4 and T3 (Fig. 7K–L). Among the remaining TH-responsive genes, each exhibited only minimal changes in expression that were statistically significant, and some were contradictory to what has previously been observed in other models (Fig. S1K–T). *Shh-a* expression was upregulated at 120 hpf by 30 and 100 nM T4 only, showing fold increases of 1.3 and 1.5 (Fig. S1M–N). Expression of *rc3* was significantly decreased at 120 hpf following exposure to T4 and T3 at 30 and 100 nM (Fig. S1O–P). At 30 and 100 nM, T4 decreased *rc3* expression by 1.25 and 1.43-fold, respectively, and T3 at 30 and 100 nM decreased *rc3* expression by 1.43 and 1.67-fold, respectively. Expression of *klf9* was reduced at 72 hpf in zebrafish exposed to T3, exhibiting 1.67, 1.43, and 2.0-fold decreased expression in response to T3 at 10, 30, and 100 nM, respectively (Fig. S1R). Expression of *mag* was 1.25-fold lower in 120 hpf zebrafish exposed to T3 at 30 or 100 nM T3 (Fig. S1T).

3.6. Effects of decreased TH signaling on gene expression

Injection with the *mct8*MO decreased mRNA expression of *mct8* by 6.25-fold at 24 hpf and 1.43-fold at 120 hpf, relative to *mct8* mRNA levels in fish injected with ctrlMO (Fig. S2). To counteract the diminishing downregulation of *mct8* mRNA with increasing time post-injection, a subset of *mct8*MO embryos were also treated with 0.01% 6-propyl-2-thiouracil (PTU), a thyroperoxidase inhibitor which decreased production of T4. This concentration of PTU was previously shown to decrease T4 immunoreactivity without causing gross morphological abnormalities in zebrafish (Elsalini and Rohr, 2003). Similarly, we observed that 0.01% PTU decreased T4 and T3 levels in larval zebrafish (Fig. 8). Treatment with 0.01% PTU did not alter the tissue concentrations of T4, T3, or rT3 at 24 hpf (Fig. 8A). At 72 hpf, the tissue concentration of T4 was decreased more than 10-fold (Fig. 8B). The concentration of T3 was also decreased at 72 hpf; however, this decrease was not statistically significant ($p = 0.08$). At 120 hpf, the tissue

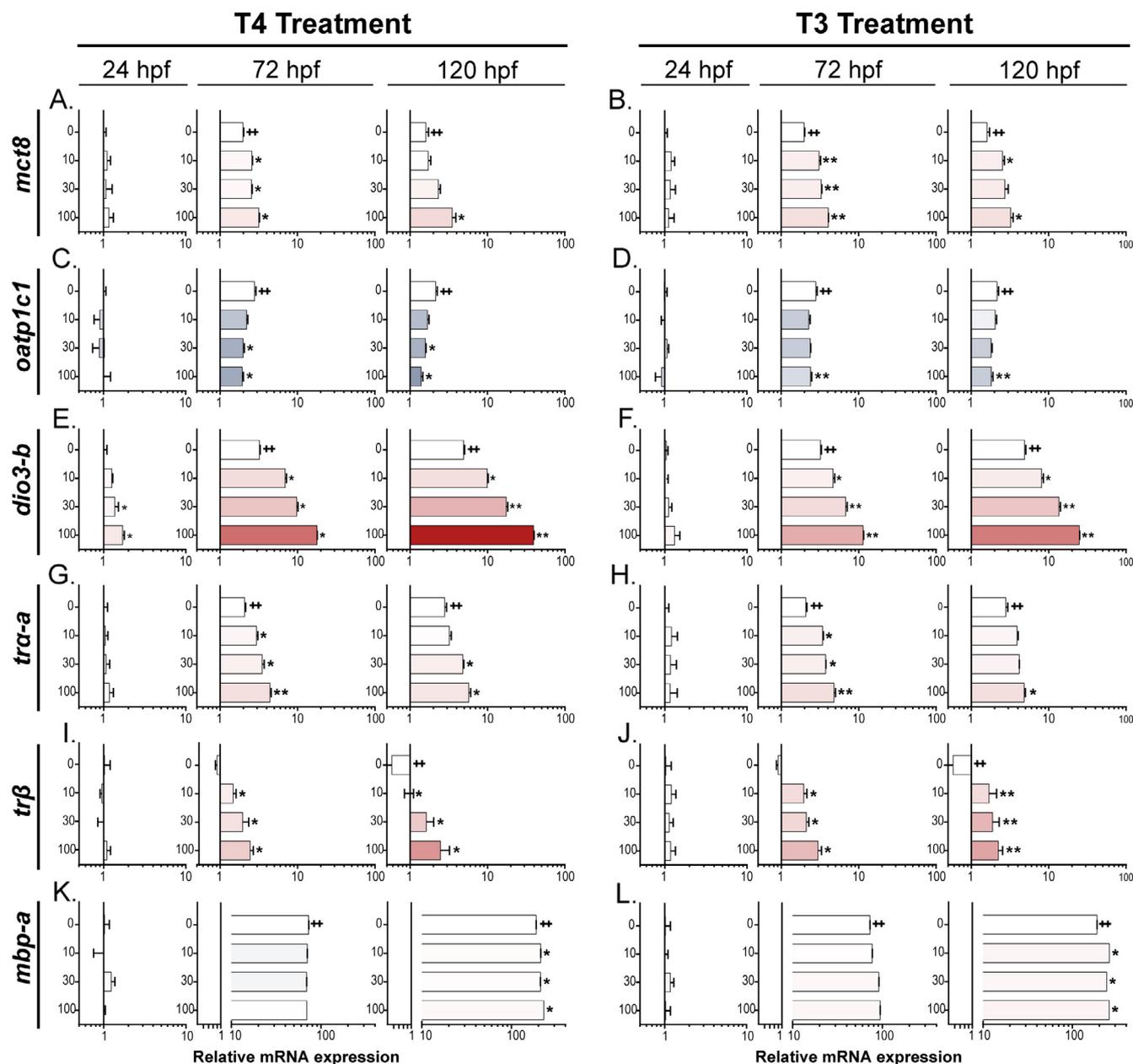


Fig. 7. Changes in gene expression induced by exogenous T4 or T3. Zebrafish embryos were exposed to exogenous T4 or T3 at 10, 30, or 100 nM beginning at 6 hpf. At 24, 72, and 120 hpf, pooled samples were collected to quantify changes in relative gene expression as determined by qRT-PCR using the Pfaffl delta-delta Ct method. Horizontal bars represent the fold expression change relative to the embryo media (EM) control at 24 hpf. Bar color indicates whether TH increased (red) or decreased (blue) gene expression relative to EM controls; the color intensity indicates the relative fold expression change with more intense coloration indicating larger differences in expression relative to EM controls. All samples were normalized to the reference genes β -actin and *elf1a*. Error bars represent the SE ($n = 3$ replicates of 30 pooled embryos per group). *Represents a significant change in expression of EM controls relative to the EM controls at 24 hpf at $p < 0.05$; **, a significant change in expression of T4 or T3-treated fish relative to the EM control at the same time point at $p < 0.05$, ** $p < 0.01$ as determined using REST relative expression software.

concentration of T4 was decreased greater than 100-fold (Fig. 8C). The concentration of rT3 was also significantly decreased at 120 hpf.

Expression of the following genes was quantified in 120 hpf larvae injected with *mct8*MO in the absence or presence of 0.01% PTU and compared to mRNA levels in age-matched fish injected with ctrlMO: *oatp1c1*, *dio2*, *dio3-b*, *tra-a*, *tra-b*, *trβ*, *klf9*, *bdnf*, *shh-a*, *mag*, *mbp-a* (Fig. 9). Expression of these genes at the mRNA level was not altered by *mct8*MO alone, and only *dio3-b* was downregulated by *mct8*MO + 0.01% PTU, exhibiting a 1.4-fold decrease relative to ctrlMO injected fish at 120 hpf ($p < 0.05$) (Fig. 9). Fish in the *mct8*MO + 0.01% PTU group exhibited decreased expression of *bdnf* and *mag*, but these changes were not statistically significant.

3.7. Effects of varying TH levels on *tshβ* mRNA expression and HPT negative feedback

Expression of *tshβ* was assessed in zebrafish at 72 and 120 hpf following exposure to exogenous T4 or T3 or injection of *mct8*MO \pm 0.01% PTU to determine whether these experimental manipulations triggered feedback via the HPT axis (Fig. S3). The mRNA levels of *tshβ* were decreased with exposure to increasing concentrations of T4 and T3 treatment at 72 and 120 hpf. However, because of considerable variability in the measurements, only the downregulation induced by 30 and 100 nM T4 at 72 hpf was significantly different from vehicle controls (Fig. S3A–B). Morpholino knockdown of *mct8*

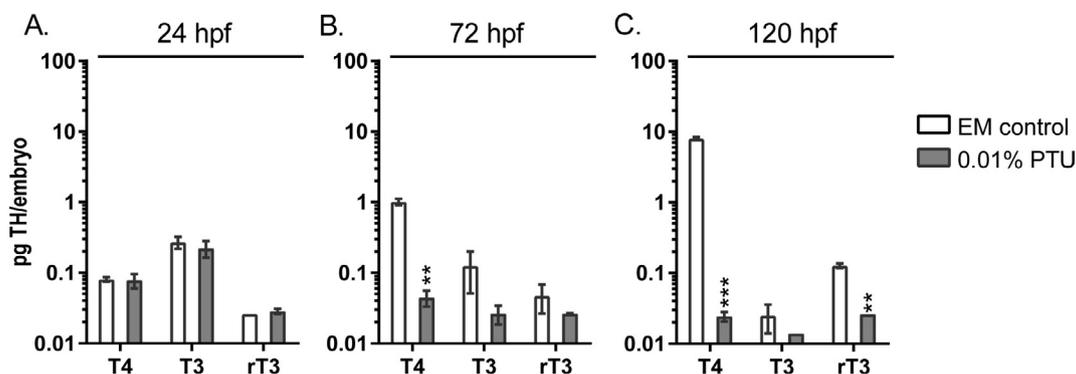


Fig. 8. Concentrations of the thyroid hormones T4, T3, and rT3 in zebrafish larvae treated with 0.01% PTU. Zebrafish were raised in control embryo medium (EM) in the absence or presence of 0.01% PTU. PTU treatment began at 6 hpf and samples were collected and measured at 24, 72, and 120 hpf. Concentrations of T4, T3, and rT3 are presented as the mean pg TH/embryo \pm SE measured from three pooled samples of 150 embryos each ($n = 3$ per group). Significantly different from EM controls at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as determined using unpaired student's t -test.

(*mct8MO*) did not alter *tsh β* transcript levels. In contrast, addition of 0.01% PTU to *mct8MO* injected fish increased *tsh β* mRNA levels 2–7-fold at 120 hpf relative to *ctrlMO* fish (Fig. S3C).

4. Discussion

The current study reports novel data regarding TH signaling in the developing zebrafish, including sensitive LC-MS/MS quantification of T4, T3, rT3, and 3,3'-T2 levels during embryonic and larval development, characterization of the ontogenetic profile of transcripts encoding diverse functional groups of molecules important in TH signaling as well as genes responsive to TH in mammalian models, and the impact of hyperthyroidism and hypothyroidism on these transcriptomic profiles. The findings from these studies indicate that: (1) genes encoding core TH signaling molecules are expressed in the developing zebrafish and are coordinately upregulated during larval stages coincident with an increase in endogenous levels of T4; (2) both T4 and T3 likely regulate gene transcription; (3) molecular targets implicated in mechanisms of TH disruption in mammalian models are expressed in developing zebrafish, and (4) there are likely species-specific differences in the profile of TH-responsive genes in response to changes in TH levels. These findings have significant implications for adapting zebrafish as a model for screening and mechanistic studies of TH disrupting chemicals.

We used an LC-MS/MS methodology previously validated for TH measurements in zebrafish whole embryo/larvae matrix (Chen et al., 2018) to demonstrate that zebrafish embryos at 24 hpf contain abundant THs, including T4, T3, and rT3. T3 concentrations were highest at 24 hpf and then progressively decreased with increasing developmental ages. In contrast, T4 concentrations increased 10-fold and 50-fold from 24 hpf to 72 hpf and 120 hpf, respectively. A previous study that used

ELISA to quantify TH levels, similarly detected TH in developing zebrafish, but at lower concentrations than we observed (Chang et al., 2012). Comparative studies of TH quantification by ELISA vs. LC-MS/MS has demonstrated similar discrepancies and concluded that LC-MS/MS is the more reliable and sensitive method (Kunisue et al., 2011). Despite differences in measured T3 and T4 concentrations, the developmental pattern of T3 vs. T4 levels detected by ELISA in the developing zebrafish (Chang et al., 2012) were similar to what we observed. Collectively, these data suggest that embryonic zebrafish may have reduced capacity for deiodinating T4 to T3, thus, maternally derived T3 may be important for early development. Significant increases in T4 at 72 hpf and 120 hpf are consistent with reports that thyroid follicles become fully functional in the developing zebrafish around 48–72 hpf (Porazzi et al., 2009). We also observed a significant increase in *tsh β* mRNA from 24 hpf to 72 hpf, suggesting that negative feedback pathways become functional at this time as well.

Transcriptomic profiling of the ontogeny of TH signaling molecules, including TH transporters, deiodinases, TRs, RXRs, NCOAs, and NCORs, demonstrated that, with the exception of *tr β* and *ncoa3*, mRNA levels of all these TH signaling molecules increase significantly between embryonic (12–48 hpf) and larval (72–120 hpf) stages. The majority of these genes were significantly upregulated between 24 hpf and 48 or 72 hpf, coinciding with the significant increase in total T4 concentration in larval zebrafish at 72 hpf. The coordinated upregulation of diverse categories of TH-signaling genes coincident with the transition from embryonic to larval development suggests a significant role of THs in the larval development of zebrafish and identifies 48–72 hpf as a developmental window in the zebrafish that may be particularly vulnerable to TH disrupting chemicals. In addition, the transcriptomic profile of TH signaling molecules suggests that many of the putative

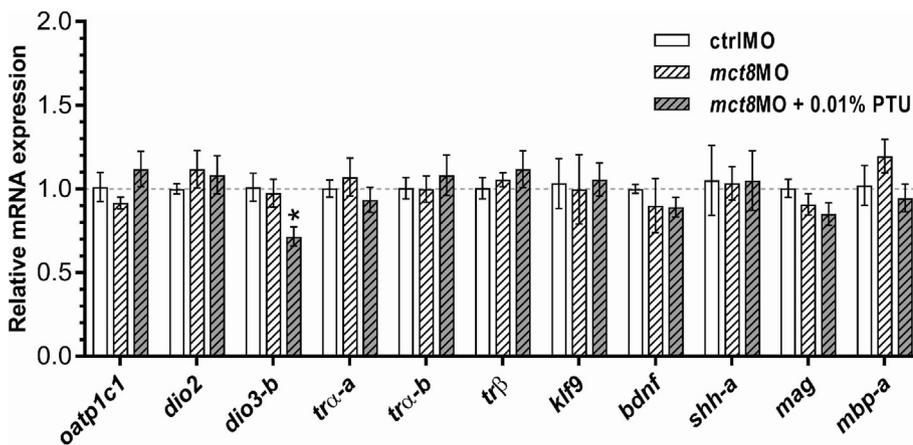


Fig. 9. Effects of decreased TH signaling on gene expression. Zebrafish embryos injected with morpholino targeting *mct8* (*mct8MO*) or control morpholino (*ctrlMO*) at 1–2 cell stage were then raised in embryo media in the absence or presence of 0.01% PTU from 6 hpf to 120 hpf. At 120 hpf, pooled samples were collected to quantify changes in relative gene expression as determined by qRT-PCR using the Pfaffl delta-delta Ct method. All samples were normalized to the reference genes *β-actin* and *elf1a*. Data is shown as the mean fold change in expression relative to *ctrlMO* fish. Error bars are SE ($n = 3$ replicates of 30 pooled embryos per group). * $p < 0.05$ as determined using REST relative expression software.

mechanisms of TH disruption likely also occur in developing zebrafish. Mechanistic studies have focused on chemical interactions with TRs, Dios and TH transporters; but also implicate RXRs, NCOAs, and NCORs as potential targets of TH disrupting chemicals (Macchia et al., 2002; Mengeling et al., 2016; Sherman et al., 1999; Tabb and Blumberg, 2006; Xu et al., 2009). The majority our data on developmental expression trends is consistent with previous reports documenting the expression of TRs, Dios and TH transporters in developing zebrafish (Arjona et al., 2011; Heijlen et al., 2013; Liu and Chan, 2002; Marelli et al., 2016; Walpita et al., 2007; Zada et al., 2017), with a few notable exceptions. For example, previous studies on TR expression patterns suggested that *tra-a* expression is relatively low and does not change until after hatching at 3dpf; whereas, *trβ* expression is relatively consistent between 24 and 120 hpf (Liu and Chan, 2002; Marelli et al., 2016; Walpita et al., 2007). However, we observed a significant increase in expression of *tra-a* at 72 hpf. Our developmental characterization of *mct8* mRNA revealed a peak in expression at 72–96 hpf in contrast to previous studies that identified peak *mct8* transcript levels at 48 hpf (Arjona et al., 2011). In addition, our results for *dio1* and *dio2* mRNA expression indicated significantly larger increases at 72 and 48 hpf, respectively, than previously reported (Walpita et al., 2007).

In contrast to the TH transporters, deiodinases, and TRs, very little characterization of the developmental expression of retinoid-X-receptors (RXRs), nuclear coactivators (NCOAs), or nuclear corepressors (NCORs) in embryonic and larval zebrafish has been conducted; our mRNA expression analysis significantly expands on previous studies (Bertrand et al., 2007; Linney et al., 2011; Waxman and Yelon, 2007). Here, we demonstrate that three RXRs (*rxra-a*, *rxra-b*, and *rxrb-b*), two NCORs, and two NCOAs are expressed in embryonic and larval zebrafish, and their developmental expression patterns are similar to those observed for other TH signaling genes. Thus, transcriptional regulators important in expression of TH-responsive genes in mammalian models are present in developing zebrafish, suggesting that, similar to mammals, these transcriptional regulators are important in TH-mediated development in zebrafish, and chemicals that interfere with their function may interfere with TH-mediated development in zebrafish.

To investigate the effects of hyperthyroidism or TR agonism on transcription of TH signaling molecules, zebrafish were exposed to varying concentrations of exogenous T4 and T3. We observed that expression of six of the sixteen genes investigated was altered at multiple time points in response to multiple TH concentrations. Exposure to either T4 or T3 concentration-dependently upregulated expression of *mct8*, *dio3-b*, *tra-a*, *trβ*, and *mbp-a*, but downregulated expression of *oatp1c1*. These findings confirm a previous report that TR agonists, including T3, upregulate expression of *dio3-b* and another myelin-associated protein, *plp1b* in zebrafish at 48 hpf (Haggard et al., 2018). For the genes we observed to be differentially regulated by exogenous TH, expression was altered by equivalent concentrations of T4 or T3, with a few exceptions: *mct8* and *trβ* mRNA expression appeared to be more sensitive to T3 than T4, while *dio3-b* expression appeared to be slightly more sensitive to T4. These concentration-effect relationships were unexpected because T4 is classically considered to be a prohormone that must be converted to T3 by tissue deiodinases 1 or 2 (*dio1/2*) for biological activity (Cheng et al., 2010). LC-MS/MS measurement of the TH profile in zebrafish tissue following TH treatment demonstrated that T4 exposure increased the larval tissue levels of T4, rT3, and T2, but not T3, and treatment with T3 only increased the levels of T3 and T2. These TH measurements suggest that the effects of T4 are not solely due to T4 conversion to T3. However, it is possible that tissue deiodination of T4 to T3 in target cells is not sufficient to noticeably elevate the whole body T3 concentration yet is sufficient to increase T3-induced signaling within target cells. This may explain the similar sensitivity of most genes to exogenous T4 and T3, but it would not explain the increased sensitivity of *dio3-b* to T4. Alternatively, T4 may directly influence gene expression. Biological activity of T4 has been reported in tissues with low *dio1* and *dio2* activity, suggesting a direct involvement of T4 in TR-

mediated signaling in some tissues (Maher et al., 2016). Moreover, the profile of corepressors and coactivators, which can vary between cell types, has been shown to influence the ability of T4 to induce TR-mediated transcription (Schroeder et al., 2014). In addition, T4 has been reported to influence transcriptional activity through non-genomic pathways (Cheng et al., 2010). These findings suggest that T4 may play a more important role in TH-mediated developmental signaling than previously believed; however, further work is needed to elucidate the relative roles of T4 and T3 in TH signaling in developing zebrafish.

To simulate developmental hypothyroidism, we used a morpholino knockdown of the TH transporter *mct8* combined with treatment with 0.01% PTU to induce TH deficiency. While the extent of morpholino-induced reduction in *mct8* mRNA expression diminished from 24 to 120 hpf, treatment with 0.01% PTU induced a reduction of T4 greater than 10-fold and 100-fold at 72 and 120 hpf, respectively. Thus, zebrafish were significantly hypothyroid throughout the 120 hpf experiment. At 5 dpf, the only gene whose expression was significantly altered by experimental hypothyroidism was *dio3-b*: at 120 hpf, *dio3-b* mRNA levels were significantly decreased relative to controls. Since *dio3-b* functions to convert T4 and T3 to the less active rT3 and T2, respectively, downregulation of *dio3-b* in response to decreased levels of T4 or T3 may be a compensatory mechanism to increase the biological half-life of the active TH forms. Our data from these studies of experimental hypothyroidism are consistent with a previous study demonstrating that *mct8*MO alone did not change expression of *dio1*, *dio2*, *dio3*, or *tsh* (Vatine et al., 2013). Gene expression studies using a transgenic *mct8* knockout line (*mct8*^{-/-}) similarly did not detect significant changes in expression of *dio1*, *dio2*, *dio3*, *oatp1c1*, *trβ*, and *klf9*, but did observe significantly decreased expression of *tra-a*, *tra-b*, and *mbp* at 72 hpf (Zada et al., 2016; Zada et al., 2014). Collectively, these studies suggest the panel of genes we investigated are relatively unresponsive to TH deficiency.

An important finding of our studies is that genes previously identified as TH-responsive in mammalian models, specifically, *mbp-a*, *bdnf*, *shh-a*, *rc3*, *klf9*, and *mag*, are relatively unresponsive to TH-disruption in zebrafish. Genes such as *rc3*, *klf9*, *mbp*, and *mag* are commonly assessed in rodents as an endpoint of TH disruption by environmental chemicals (Bansal et al., 2014; Gilbert et al., 2016; Wang et al., 2011; Zoeller et al., 2000), but in whole-larvae analysis of developing zebrafish, only expression of *mbp-a* was altered following exposure to exogenous TH, and none of these mammalian TH-responsive genes was altered by TH deficiency. This observation may indicate species differences in the TH-mediated transcriptional regulation of these particular genes. An equally plausible hypothesis is that these genes, which have predominantly been used as biomarkers of TH disruption in the developing brain, are expressed in low quantities in neural tissue during development, making it difficult to detect TH-mediated changes when measuring gene expression in the whole larvae. *Dio3-b* mRNA was the most sensitive to TH-disruption, its expression influenced by both developmental hyperthyroidism and hypothyroidism. However, few studies of thyroid hormone disruption in zebrafish or rodents have evaluated expression of *dio3* as an endpoint of TH-disruption (Dong et al., 2013; Haggard et al., 2018; Liu et al., 2016). As the candidate TH-responsive genes that were investigated in this study showed very limited sensitivity to TH-alteration when whole larval tissue was used for qRT-PCR, we suggest that future research should focus on *dio3-b*, and possibly genes that encode myelin-associated proteins.

5. Conclusions

Zebrafish have been proposed as a promising model for studying TH-dependent mechanisms of neurodevelopment, screening chemicals for TH disrupting activity, and elucidating the adverse outcome pathway(s) by which TH-disrupting chemicals interfere with neurodevelopment (Haggard et al., 2018). Many molecular components of the

thyroid axis have been identified in developing zebrafish and found to be homologous to those in higher vertebrates. However, the role of TH in zebrafish development, and in particular neurodevelopment, is not well characterized, which is a significant limitation in adapting this model as a toxicological tool for identifying TH-disrupting chemicals. The current study confirmed that the core signaling components that mediate TH signaling in mammals are expressed during larval zebrafish development and appear to be coordinately regulated. This supports the feasibility of using the larval zebrafish as an experimental model for identifying chemicals that disrupt the expression and/or action of TH signaling. However, the current study also highlights that it will be critical to identify TH-responsive genes specific to the zebrafish for maximizing the potential of this model. Consistent with a recent report from another laboratory (Haggard et al., 2018), our study identifies *dio3-b* and genes encoding for myelin-associated proteins (e.g., *mbp-a* identified in our study, and *plp1b* identified by the Tanguay lab) as candidate transcripts that may serve as biomarkers of TH disruption in the developing zebrafish.

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Appendix A. Supplementary data

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