The molecular mechanisms of thyroid disruption by brominated flame retardants in fish: *in vitro* and *in vivo* studies.

Submitted by

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I certify that all the material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature)……

Abstract

Fish are particularly vulnerable to the exposure of anthropogenic pollutants, with a vast array of endocrine disrupting chemicals (EDCs) introduced into the aquatic environment via sewage discharge, waste disposal and land runoff. Brominated flame retardants (BFRs) are halogenated flame retardants that are used to effectively inhibit the flammability of various materials including plastic products, electrical appliances, construction materials and textiles. BFRs are ubiquitous environmental contaminants and are known to disrupt thyroid hormone (TH) homeostasis in several vertebrate species, including fish. Given the vital role of THs in a wide range of developmental processes and physiological functions, assessing and identifying thyroid disrupting chemicals is crucial for safe guarding the long-term health of humans and wildlife. In fish the molecular mechanisms underlying TH disruption by BFRs and the effects on TH-sensitive tissues during early life stages remains unclear. This has been limited by the lack of fundamental knowledge on the TH system of fish and the difficulties associated with examining transcriptional changes in discrete embryonic-larval tissues. Here I have established the expression profiles of a suite of genes in the hypothalamic-pituitary-thyroid (HPT) axis of zebrafish (*Danio rerio*) during embryonic-larval stages and their regulation by the biologically active TH (3, 5, 3′- tri-iodothyronine; T3). Using molecular tools (whole mount *is situ* hybridisation and RT-PCR), I demonstrate that a number of genes display spatial and temporal expression profiles during embryo/larval development, and their regulation by T3 was tissue- and developmental stage-specific. I subsequently demonstrated that TBBPA and BDE-47, two important BFR compounds, disrupted TH homeostasis at multiple levels of the HPT axis of zebrafish embryo-larvae after short sub-acute exposures. These compounds altered the

expression of genes associated with TH conjugation and clearance, thyroid follicle development and TH transport. In addition, we suggest that TH target genes in the brain, liver, pronephric ducts and craniofacial tissues of zebrafish embryo-larvae may be particularly vulnerable to TBBPA and BDE-47 exposure. It has been proposed that environmental pollutants can disrupt TH signalling in wildlife by disrupting the activity of thyroid receptors (TRs), ligand-binding transcription factors, which mediate the genomic actions of THs. The ability of BFRs to disrupt fish TRs has not yet been examined. Here I developed an *in vitro* reporter gene transcriptional assay for zebrafish thyroid hormone receptors (zfTRα and zfTRβ) in human embryonic kidney cells and investigated their interactions with several BFR compounds. The assays were optimised and validated using the natural TR agonist T3 in cells transiently transfected with two reporter vector constructs, pGL4.24-PAL and pGL4.24-DR4. None of the six brominated flame retardants tested, namely, tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), 2,2′,4,4′,6-penta-bromodiphenyl ether (BDE-100), 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183) and deca-bromodiphenyl ether (BDE-209) had an agonistic effect on zfTRα and zfTRβ activity. These results are consistent with our previous finding which suggests that altered TH homeostasis may be a result of increased metabolism and excretion of THs and/or changes in the production of TH by the thyroid follicles. In conclusion, this investigative work aids the understanding of fundamental TH processes in fish, such as gene expression and regulation, and increases our understanding of the mechanisms and potential targets of BFRs in fish.

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Last but certainly not least, a massive big thank you to my amazing family; Mom, Terje, Jen, Stephen and Barry. Your support, love, encouragement and patience over the last four years have gotten me through the many tough and testing times. I dedicate this work to you guys, though I am sure you never want to hear about flame retardants or fish ever again!

Agus a Dhaid, tú i mo chroí i gcónaí.

Le grá,

Aoife

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Author's Declaration

I, Aoife Parsons, was involved in the following parts of the presented research:

Chapter 2: I planned and carried out the ontogeny work and T3 exposures for all selected target genes via and qRT-PCR. All anti-sense RNA probes used in whole mount in situ hybridization (WISH) assays were synthesised by me with the guidance of Dr. Tetsu Kudoh. DNA-containing plasmids were purchased from Source Bioscience (for *ttr, tshb, thraa* and *thrb* genes) or donated from Dr. Alain Lescure, Université de Strasbourg (*dio1* and *dio2*) and Professor Heather Stapleton, Duke University (*dio3b*). I undertook all WISH assays and subsequent imaging of samples. RT-PCR conditions were optimised with the guidance of Dr. Anke Lange and assays were carried out by me. I carried out all statistical analyses and the writing of the paper (with input from co-authors).

Chapter 3: I planned and carried out the TBBPA and BDE-47 acute and sub-acute exposures. BDE-47 was provided by Ulrika Winnberg, Jorke Kamstra and Kees Swart on behalf of Dr. Juliette Legler from VU University Amsterdam. I performed the WISH and qRT-PCR assays, imaging and data analysis. RNA probes were synthesised and PCR conditions were optimised as per Chapter 1. I wrote the paper with input from co-authors.

Chapter 4: I constructed the zebrafish thyroid receptor alpha plasmid with the guidance of Dr. Anke Lange. The construction of reporter plasmids (DR4 and PAL) and the sub-cloning of TRα and TRβ sequences into mammalian expression vectors

were carried out by Dr. Shinichi Miyagawa (Yokohama City University) and Prof. Taisen Iguchi (Wakayama Medical University). BDE-47, BDE-99, BDE-183 and BDE-209 were provided by Ulrika Winnberg, Jorke Kamstra and Kees Swart on behalf of Dr. Juliette Legler from VU University Amsterdam. Reporter gene assays were planned, carried out and analysed by me with the guidance of Dr. Anke Lange, who also provided in-put on the writing of the paper.

Prof. Charles Tyler and Dr. Tetsu Kudoh contributed to study design and the writing of all research papers.

List of General Abbreviations

EDC	Endocrine Disrupting Chemicals
TDC	Thyroid Disrupting Chemicals
STW	Sewage Treatment Works
BFR	Brominated Flame Retardants
PBDE	Polybrominated Diphenyl Ether
TBBPA	Tetrabromobisphenol A
HBCD	Hexabromocyclododecanes
HCB	Hexachlorobenzene
DTT	Dichlorodiphenyltrichloroethane
TH	Thyroid Hormone
TR	Thyroid Receptor
TSH	Thyroid-Stimulating Hormone
CRH	Corticotropin- Releasing Hormone
rT3	3,3’,5’-triiodothyronie
T2	3, 5-diiodo-L-thyronine
T4	Thyroxine
T3	3,3’,5-Triiodo-L-Thyronine
TG	Thyroglobulin
PAL	Palindromes
IP	Inverse Palindromes
RXR	Retinoid X Receptor

TTR	Transthyretin
TBG	Thyroxine-Binding Globulin
THBP	Thyroid Hormone-Binding Proteins
OATP	Organic Anion Transporter Polypeptides
MCT8	Monocarboxylate Transporter 8
HPT	Hypothalamus-Thyroid-Pituitary
ORD	Outer Ring Deiodination
IRD	Inner Ring Deiodination
LBD	Ligand Binding Domain
DBD	DNA Binding Domain
TRE	Thyroid Response Element
Log Kow	N-Octanol–Water Partition Coefficients
POP	Persistent Organic Pollutant
E&E	Electronics and Electrics
WEEE	Waste Electrical and Electronic Equipment Directive
WISH	Whole Mount *In Situ* Hybridisation
QRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
hpf	Hours Post Fertilisation

CHAPTER 1

General Introduction

1.1 Thyroid Disrupting Chemicals

Over the last two decades there has been concern over the presence of endocrine disrupting chemicals (EDCs) in the environment. The European Commission defines an endocrine-disrupting compound as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or sub population”. To date, attention has largely focused on environmental compounds that interfere with normal hormone signalling in the reproductive system of humans and wildlife (Reviewed in [1-3]). However, xenobiotics which act as thyroid disrupting chemicals (TDCs) have gained increasing attention in recent years.

Thyroid hormones (TH) play key roles in a wide range of physiological functions during the development and adult life stages of vertebrates. Developmental roles include influencing the maturation of tissues including bone (reviewed in [4]), gonads (reviewed in [5]), intestine [6] and the central nervous system (reviewed in [7]) as well as mediating the metamorphic transition from larval to adult stages in fish and amphibians [8]. In adults they modulate growth [9], energy homeostasis [10], cardiac rhythm [11], osmoregulation and the behaviours/physiology associated with rheotaxis and migration in fish [12, 13]. Therefore, maintaining normal thyroid functions is essential for general health and well-being. Even minor alterations in TH levels, particularly during sensitive developmental windows, can have significant acute and long-term health effects [14]. TDC can interfere with thyroid homeostasis by several mechanisms, including; altering THs production by the thyroid gland, TH transport in the blood stream, TH metabolism and/or binding to thyroid hormone receptors (TRs) [15].

Aquatic species are especially vulnerable to xenobiotic chemicals as the aquatic environment receives multiple and considerable chemical contaminant discharges via Sewage Treatment Works (STW) effluents, industrial effluents and agricultural runoff [16]. Fish can be exposed to waterborne contaminants over extensive periods of their lives and even across the entirety of their life. Lipophilic compounds such as polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), phthalates and brominated flame retardants (BFRs) are some of the more well-known examples of TDCs (reviewed in [17]).

The work in this thesis is focused on BFR compounds and their ability to induce TH disruption, in teleost fish, using the zebrafish (*Danio rerio*) model. Here accordingly, we broadly highlight the current knowledge on the structure and function of the TH system in teleost fish and subsequently we examine the relevance of BFRs, in terms of their production and usage, environmental concentrations and their ability to disrupt the TH system.

1.2 Thyroid Hormone System

1.2.1 Thyroid hormone synthesis and transport

In fish, corticotropin-releasing hormone (CRH) from the hypothalamus stimulates the release of thyroid-stimulating hormone (TSH) from thyrotropin cells in the pituitary glands [18]. The thyroid follicles, in response to TSH, take up inorganic iodine from their diet or the water via an NA+/I- symporter system [19]. Unlike the compact thyroid gland found in mammals, the thyroid follicles of teleost fish are found individually or in clusters next to the ventral aorta (e.g., medaka, *Oryzias latipes*),

dispersed in the pharyngeal region (e.g. zebrafish) or associated with the kidney (some freshwater cyprinids) [20]. The trapped iodine in the thyroid follicles is incorporated into large glycoproteins called thyroglobulin (TG) which form di-iodinated residues that enzymatically pair up to form the hormone, thyroxine (T4) [21]. In mammals, the thyroid gland produces T4 and to a lesser extent the biological active 3,3′,5-triiodo-L-thyronine (T3) (ratio 5:1). In contrast, in fish T4 is believed to be the primary TH secreted by the thyroid follicles and the production of T3 is thought to be under the exclusive control of the peripheral tissues [22]. In general circulation, most THs are non-covalently and reversibly bound to thyroid hormone-binding proteins (THBP) such as albumin, transthyretin (TTR), plasma lipoproteins and thyroxine-binding globulin (TBG) [23-25]. In humans the primary TH transporter is TBG while in rodents it is TTR [26]. The dominant transporters in fish are understood to a lesser extent, however it has been shown recently that in some fish species TTR may play a significant role [27, 28]. The transport of plasma TH into target cells is mediated by membrane bound transporters, including the high affinity monocarboxylate transporter 8 (MCT8) and organic anion transporter polypeptides (OATP1c1) [29, 30].

1.2.2 Thyroid hormone regulation

In all vertebrates, the thyroidal system is centrally driven by the hypothalamic-pituitary-thyroid (HPT) axis but also under peripheral control. In fish, both T4 and T3 have a negative feedback effect on TSH secretion by the pituitary [31]. However, the functional activity of TSH is limited to regulating T4 release and iodide uptake by the thyroid follicles [32]. The peripheral levels of THs are tightly regulated by the TH-metabolizing iodothyronine deiodinase enzymes, type I, II and III (D1, D2 and D3)

[33, 34]. D1 and D2 catalyse the conversion of T4 to the bioactive T3 hormone by outer ring deiodination (ORD), whereas D1 and D3 can catalyse the conversion of T4 to the inactive 3,3',5'-triiodothyronie (rT3) by inner ring deiodination (IRD) [35]. Thus D1, a kinetically inefficient enzyme, is capable of both activating and inactivating T4 [36]. In addition, T3-IRD and rT3-ORD can produce the TH metabolite 3, 5-diiodo-L-thyronine (T2). See Figure 1 and 2 for overviews of deiodination pathways and TH regulation and signalling in teleost fishes.

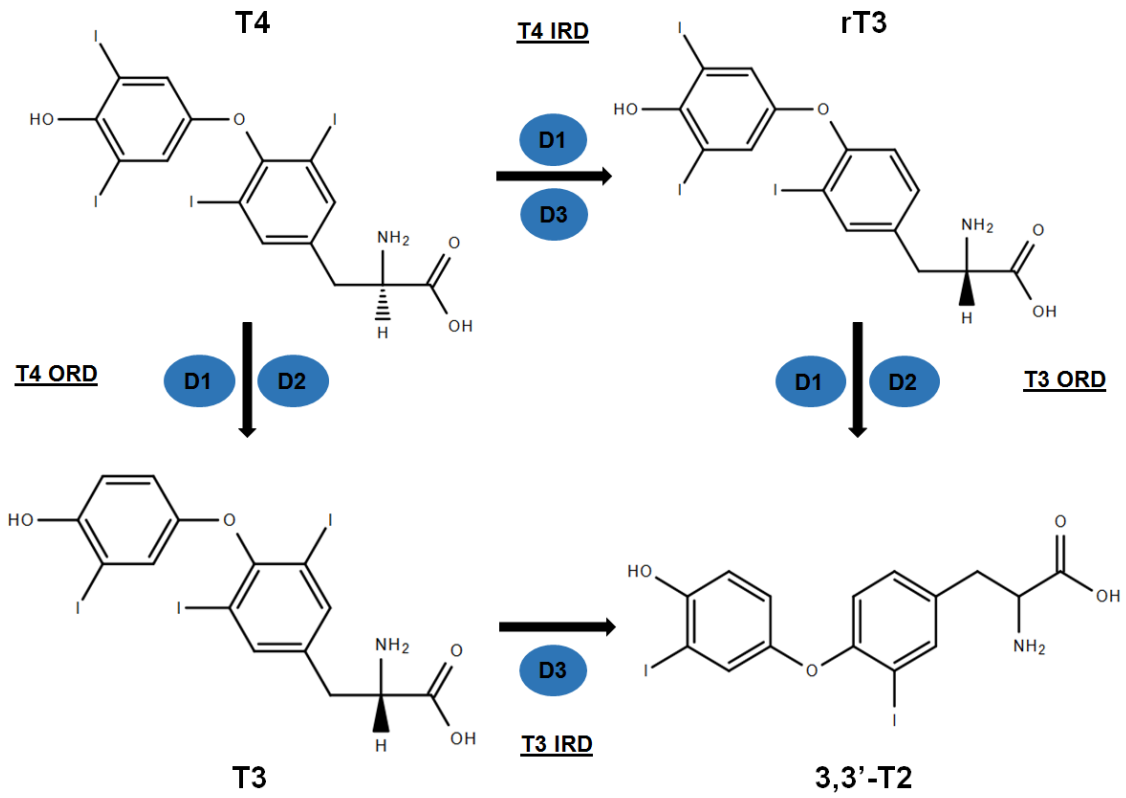

Figure 1: Pathways of outer ring deiodination (ORD) and inner ring deiodination (IRD) of thyroid hormones by the deiodinase enzymes type I, II and III (D1, D2 and D3) in peripheral tissues of vertebrates. T3 = 3,3′,5-triiodothyronine, rT3 = 3,3′,5′-reverse T3, 3,3' T2 = 3,3′-diiodothyronine, T4 = thyroxine.

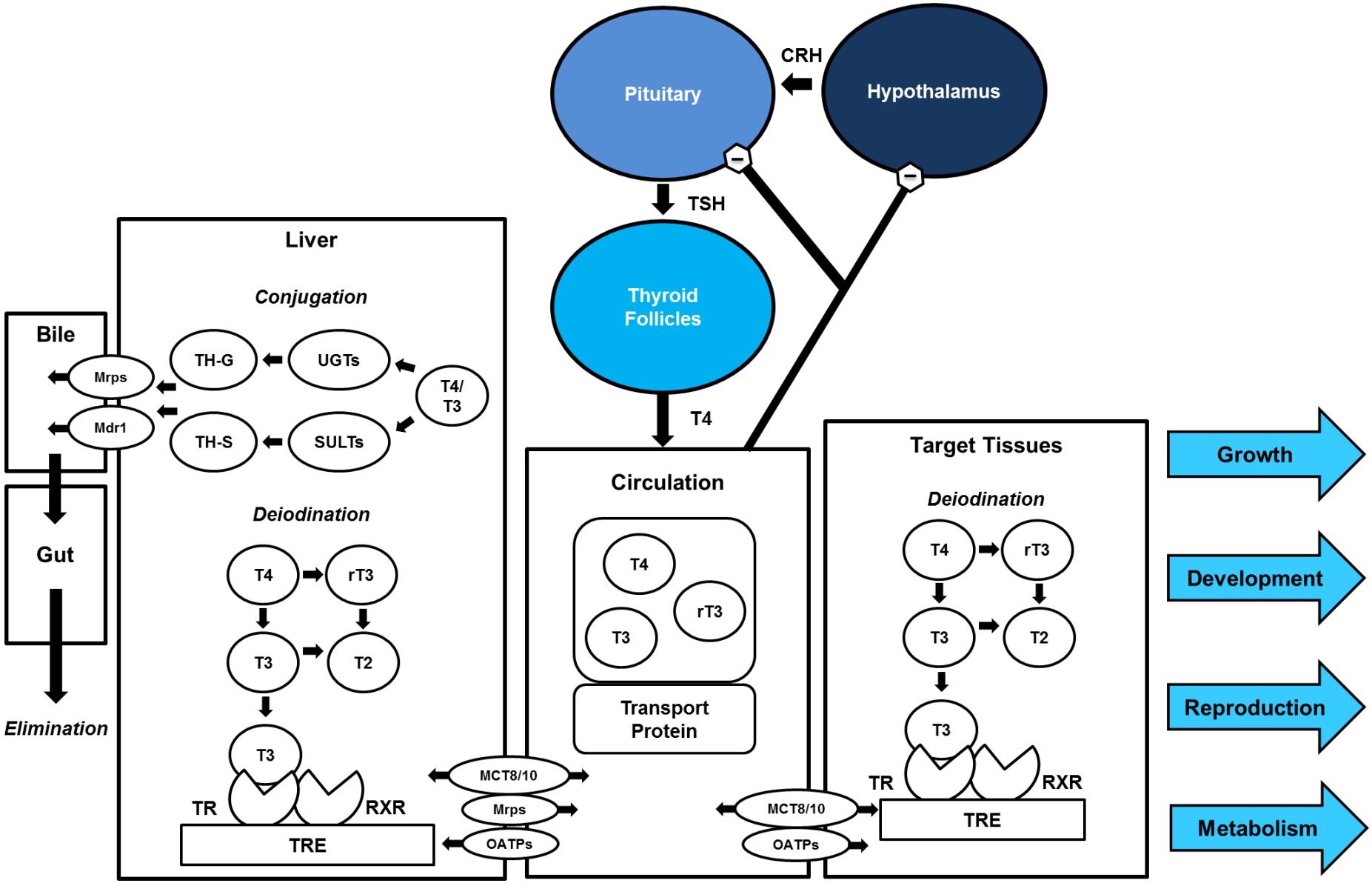
Pituitary
CRH
Hypothalamus
TSH
Thyroid Follicles
T4
Liver
Conjugation
Bile
Mrps
Mdr1
TH-G
UGTs
T4/ T3
TH-S
SULTs
Deiodination
T4
rT3
T3
T2
T3
TR
RXR
TRE
Gut
Elimination
MCT8/10
Mrps
OATPs
Circulation
T4
rT3
T3
Transport Protein
MCT8/10
OATPs
Target Tissues
Deiodination
T4
rT3
T3
T2
T3
TR
RXR
TRE
Growth
Development
Reproduction
Metabolism

Figure 2. Overview of TH regulation and signalling in teleost fishes. CRH = corticotropin releasing hormone; TSH = thyroid stimulating hormone; T4 = thyroxine; T3 = 3,3′,5-triiodothyronine; rT3 = 3,3′,5′-reverse T3; T2 = 3,3′-diiodothyronine; UGT = uridine diphosphate glucuronosyl transferase; SULT = sulfotransferase; TH-G = glucuronidated thyroid hormone; TH-S = sulfated thyroid hormone; Mrp = multidrug resistance associated protein; Mdr1 = multidrug resistance protein 1 or P-glycoproteins; MCT = monocarboxylate transporter; OATP = organic anion transport polypeptide; TR = thyroid hormone receptor; RXR = retinoic x receptor.

1.2.3 Thyroid hormone receptors

The mechanism of TH actions can either be genomic or non-genomic. In this body of work we are principally concerned with the genomic mode of action of THs. Briefly, the non-genomic actions of THs depend on cellular signal transduction systems, cell surface receptors or extra-nuclear thyroid receptors [37]. Genomic actions are mediated by binding to specific TH nuclear receptors (TRs) in the nucleus, which are ligand-dependent transcription factors that bind to T3 (and T4 with low affinity) [38].

The TRs share a common nuclear receptor domain structure, consisting of an N-terminal regulatory domain (termed A/B), a central DNA-binding domain (DBD) where the receptor interacts with the DNA, a less conserved hinge region, the ligand-binding domain (LBD) where TH binds and activates the receptor and the C-terminal region [39]. A functional TR complex is required in order to efficiently bind to the thyroid response element (TRE) in the promotor region of target genes. The TR complex can be in the form of a heterodimer with retinoid X receptor (RXR), a homodimer or monomer (*in vitro* only) [40]. In general, TRE half-sites (AGGTCA) exist in pairs, either composed of direct repeats spaced by four nucleotides (DR-4) or arranged as palindromes (Pal) or inverse palindromes (IP) where no spacing separates the two half-sites [41]. Depending on the conformational state of a TR complex and the type of TRE they bind to, the THs can either promote or repress gene expression [40, 42]. An important property of TRs is their ability to bind TREs constitutively independent of ligand occupancy. Unliganded TRs generally repress basal transcription through the co-repressors, N-CoR or SMRT, which associate with TRs in the absence of their ligands [43]. These co-factors allow the additional recruitment of histone deacetylases, which remodel the structure of chromatin,

causing the repression of target genes. Transcriptional activation occurs when T3 binds with the TRs, inducing conformational changes resulting in the replacement of the co-repressor complex by co-activator proteins [44].

In general, vertebrates have two TR genes encoding thyroid hormone receptor alpha (TRα) and beta (TRβ) [42]. Due to ancestral gene duplication, several fish species have two TRα-encoding genes (*thraa* and *thrab*) as well as a single TRβ gene (*thrb*) [45, 46]. The *thraa* gene encodes two proteins; TRαA1 and TRαA1-2. The TRαA1 isoform has high homology with human TRα1, whereas the TRαA1-2 isoform incorporates an additional 14-amino acid peptide extending its carboxy-terminal "F-domain" that is not found in any other known TR. This extended F domain is reported to reduce the transcriptional activity of TRαA1-2 [44, 47]. TRαB, encoded by the *thrab* gene, lacks a large portion of the N-terminal domain as well as the correlated transactivation function [47]. The TRβ isoforms are all able to bind DNA and retain the T3-dependent transactivation activity.

1.3 Flame Retardants

Flame retardants are used at relatively high concentrations in a wide variety of applications in order to protect against ignition as well as reducing the amount of heat, smoke and toxic gases released during a fire [48]. Rising safety standards and the increasing use of flammable materials has led to a significant growth in the flame retardant market worldwide. In 2013, the annual global consumption of flame retardants was estimated at over 2 million tonnes, with the Asia-Pacific region accounting for approximately 41% of the global demand, followed by North America and Western Europe [49]. There are more than 175 different types of flame

retardants in existence, commonly divided into four major groups: inorganic, organophosphorus, nitrogen-containing and halogenated.

Brominated flame retardants (BFRs) are a large and structurally diverse class of halogenated flame retardants consisting of brominated aliphatics, cyclo-aliphatics and aromatics which were first introduced to the market in the 1970s. The bromine atoms in BFRs delay or suppress the combustion process of materials by eliminating free radicals, thus inhibiting the progression of fire [50]. BFRs are highly effective as a result of their thermal stability at high temperatures and the low volatility of their decomposition products [51]. This high performance efficiency as well as the low production costs of BFRs has made them the second largest group (representing 23%) of flame retardants produced globally [49].

1.4 Commercially Important BFRs: Sources, Applications and Legislation

BFRs are either covalently bound to polymers as reactive flame retardants or more commonly they are incorporated into commercial products as additives, whereby they are physically blended with polymers. The most extensively used BFRs to date have been polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBPA) and hexabromocyclododecanes (HBCDs) [52].

1.4.1. PBDEs

PBDEs are a family of additive flame retardants used abundantly in a variety of commercial and household products [52, 53]. There are 209 possible PBDE congeners that can be divided into 10 congener groups (mono- to decabromodiphenyl ethers). PBDEs consist of two halogenated aromatic rings connected by an ether bridge and their structure resembles that of polychlorinated biphenyls (PCBs). Commercial PBDE mixtures are made up of congeners with varying numbers of bromine atoms on their two phenyl rings and are classified according to their average bromine content; penta-BDEs, octa-BDEs and deca-BDEs [52]. Penta-BDEs, composed mostly of BDE-47 and BDE-99 congeners, were predominantly used in polyurethane foam and textiles [52]. Octa-BDE formulations predominantly consisted of BDE-183, BDE-197 and BDE-203 congeners and were used in thermoplastic resins and in the housings of electrical and electronic (E&E) equipment [52, 54]. Deca-BDE mixtures, which consists mainly of the fully brominated BDE-209 congener, are a general purpose flame retardant used in virtually any type of polymer including polycarbonates, polyester resins, polyolefins, acrylonitrile butadiene styrene (ABS), polyamindes, polyvinyl chloride and rubber [52]. A considerable proportion of a products weight consists of PBDEs, with plastics and polyurethane foam integrating up to 15% and 30% PBDEs, respectively [55-58]. The production and usage of penta- and octa-BDE commercial mixtures were prohibited globally in 2004 and officially labelled as Persistent Organic Pollutants (POPs) by the Stockholm Convention in 2009 [59]. They both met the definition of chemicals considered to be persistent, bioaccumulative, toxic and subject to long-range transportation. In 2008, the use of deca-BDE products in the EU was banned in E&E applications, applying to both manufactured and imported products placed on

the market [60]. However, the use of deca-BDE in Europe was approved for all other applications. Manufacturers in the U.S. agreed to cease production of deca-BDE as of 2013 [61].

1.4.2 HBCDs

HBCDs are additive flame retardants used in polystyrene foams, thermal insulation building materials, upholstery textiles and electronic products [62]. HBCDs are produced in China, Europe, Japan, and the USA with an annual global production of 23,000 tonnes in 2011 making them the third most used BFR worldwide [63]. Commercial HBCD products mainly consist of α-, β-, and γ-diastereoisomers, with γ-HBCD dominating the mixture (>70%) [64]. Since 2013, the production and use of HBCD has been phased out globally after its inclusion on the Stockholm Convention's list of POPs (decision SC-6/13) [65]. However, the European Union was granted a 5 year exception for using HBCD in expanded and extruded polystyrene insulation used in the building industry [66, 67].

1.4.3 TBBPA

TBBPA is the most widely used BFR representing approximately 60% of the total BFR global market [68], amounting to over 120,000 tonnes in 2001 and over 170,000 tonnes in 2004 [69]. Though no official figures are available, these numbers are likely to have increased in recent years as TBBPA has been considered a substitute for certain PBDEs like the octa- and penta-BDE commercial products. The primary application (90%) of TBBPA is as a reactive flame retardant covalently bound to the epoxy and polycarbonate resins of electronic circuit boards. It is also used to a lesser extent (10%) as an additive flame retardant in ABS resin and high-impact

polystyrene, used in automotive parts, pipes, refrigerators and television/computer casings [70]. TBBPA is currently produced in the USA, Israel, Jordan, China and Japan [71]. Though not produced in the EU it can be imported in various forms, either as a primary product or in finished or partially finished products. Currently, there are no restrictions on the production of TBBPA or its derivatives [71]. In the EU it was registered under the Registration, Evaluation, Authorisation & restriction of Chemicals (REACH) framework in October 2010 and the subsequent risk assessment concluded that it presents no risks to human health and the environment when used reactively in printed circuit board applications, but does pose a risk to the aquatic environment when used additively [71]. Under Europe's Waste Electrical and Electronic Equipment Directive (WEEE) directive, TBBPA must be separated from plastics and E&E products prior to recovery and recycling (directive 2002/96/EC) [72].

1.5 Environmental Contamination

There are multiple routes by which BFRs are released into the environment. They can be released from factories during their initial synthesis and/or incorporation into consumer products, during a product's lifetime into the surrounding environment, or released from municipal, hospital or hazardous waste incinerators, electronic recycling facilities, final disposal sites and accidental fires [73].

Physico-chemical properties of BFRs, such as water solubility and octanol-water partition co-efficient (log K_{ow}) are important factors governing their behaviour in the environment. As many BFRs have low water solubility and high log K_{ow} values (Table 1), they tend to bind to particles and associate with particulate matter such as in

dust, sediments, sludge and soils. Therefore, only a very small fraction of the more hydrophobic BFRs are usually found in the dissolved water phase of water bodies. Uptake by humans can occur via several routes including via the diet, ingestion of dust, dermal contact with dust/consumer products, inhalation of air, and/or maternal transfer during embryonic development. Meanwhile aquatic wildlife is exposed to BFRs through uptake of water (in the case of less hydrophobic compounds), through the diet and/or maternal transfer. Depending on their half-lives, BFRs can be extremely persistent compounds in various environmental matrices and are also capable of biomagnifying in food chains.

Table 1: Molecular weight, octanol water partition coefficients (log k_{ow}) and water solubility of commercial PBDE formulations (penta-, octa- and deca-BDE mixtures), TBBPA and HBCD [74-77].

	Molecular Weight (g/mol)	**Log K_{ow} Coefficient**	**Water Solubility (mg/L)**
Penta-BDE (Commercial)	564.8	6.64-6.97	0.013 @ 25 °C
Octa-BDE (Commercial)	801.38	6.29	0.002-0.005 @ 25 °C
Deca-BDE (Commercial)	959.17	6.27	<0.001 @ 25 °C
HBCD (Commercial)	641.7	5.62	0.0024-0.066 @ 20 °C
TBBPA	543.87	5.9	1.26 @ 21 °C

Since BFRs were first detected in Swedish environmental samples in 1979–1981 [78] they have become ubiquitous environmental pollutants with high levels reported in various matrices from both remote and heavily industrialised region of the world. The following sections will highlight the presence and concentrations of PBDEs, HBCDs and TBBPA in different environmental matrices.

1.5.1 PBDEs

Both higher and lower brominated PBDE congeners are found in environmental samples, with the congeners BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-209 most frequently detected, reflecting the most commonly produced technical mixtures (extensively reviewed in [53, 79-82]). Some of the highest concentrations of PBDEs in the environment have been observed in matrices such as suspended solids [4,600 µg/kg dry weight (dw)] [83], sediment (3,250 µg/kg dw) [84], sewage sludge (97,400 µg/kg dw) [84] and raw sewage (up to 2,496 ng/L) [85] from rivers, estuaries and coastal regions in urban areas of Europe, China and the US. PBDEs have also been detected in outdoor air [86] and indoor dust sampled from schools [87], hotels [88], offices [89], cars [90], homes [91] and factories [92] in various countries around the world (reviewed in [93]), with some of the highest concentrations detected in the UK (up to 2,600 µg/kg dw in cars) [87]). In addition, despite their physico-chemical properties suggesting otherwise, several studies report the presence of PBDEs in the dissolved and suspended water phases of rivers, lakes and coastal seawater [94-97]. For the most part, concentrations of PBDEs in water samples are lower than solid environmental matrices, mostly ranging from 0.1-500 pg/L [94-99]. Levels are however significantly higher in effluent samples from wastewater treatment plants (up to 29 ng/L in the US) [100, 101] and

heavily industrialised regions of South Korea (up to 11 ng/L) [102] and China (up to 68 ng/L) [103]. BDE-209 is frequently the predominant congener detected in solid samples (e.g. sewage sludge and sediments) and particulate phases of water samples (accounting for >90% total PBDEs in some samples) [84, 95, 102], as a result of its high degree of hydrophobicity, high molecular weight and subsequent preferentially adsorption to particulate matter. The lower brominated BDE congeners, such as BDE-47, BDE-99, BDE-100 and BDE-154 are often detected at relatively lower concentrations in sewage sludge and sediment samples. Interestingly, as a result of fewer bromine substitutions and lower $\log K_{ow}$ values [104], the lower PBDE congeners are relatively more soluble and are often the mostly commonly detected congeners in the aqueous phase of water samples [95].

In addition, PBDEs have been detected in a large number and variety of biota from all over the world and bioaccumulation has been reported in remote regions with no local point sources or industrial production, such as in the Arctic [105-107]. PBDEs have been detected in a wide range of human samples including blood serum [up to 668 µg/kg lipid weight (lw)] [108], breast milk (up to 2,010 µg/kg lw) [109], placental tissue (up to 17 µg/kg lw) [110], adipose tissue (up to 9,630 µg/kg lw) [111] and liver samples (up to 98 µg/kg lw)[112]. In general the levels of PBDEs in human tissues are substantially higher (by 2 orders of magnitude) in North America than in Europe and Asia [113-115]. However, humans residing in regions of China with heavy electrical waste (e-waste) recycling operations have increasing body burdens of BDE-209 [115-117]. The PBDE congener profiles in human samples vary depending on age, occupation, geographical locations and economics, though BDE-47, -99, -100, -153 and -209 often dominate (reviewed in [115]).

Extremely high levels of PBDEs have been recorded in birds and birds eggs, with the highest levels detected in top predator species such as the Cooper hawk (*Accipiter cooperii*; up to 197 mg/kg lw) [118], Peregrine falcon (*Falco peregrinus*; up to 76 mg/kg lw) [118] and in eggs of Osprey (*Pandion haliaetus;* up to 23 mg/kg ww) [119], Forster's tern (*Sterna forsterii;* up to 62 mg/kg ww) [120] and herring gull (*Larus argentatus;* up to 16 mg/kg ww) [121] from North America. European and Asian birds generally exhibit relatively lower PBDE burdens (in the µg/kg range), though higher levels are increasingly detected in China (reviewed in [122]). BDE-47 tends to dominate the PBDE profiles of aquatic feeding bird species and their eggs, while BDE-153 dominates terrestrial feeding birds [123] and BDE-209 dominates the profile of birds from China [124-126].

PBDEs have been detected in aquatic wildlife living in both freshwater and marine environments. PBDEs have been reported in many marine mammals, including various species of porpoise, dolphin, seals, whales, sea lions and otters (reviewed in [127]). Some of the highest levels have been detected in humpback dolphins (*Sousa chinensis*) from Hong Kong (up to 51,100 µg/kg lw) [128], harbour porpoises (*Phocoena phocoena)* from the UK (up to 15.7 mg/kg lw) [129] and bottlenose dolphins (*Tursiops truncates*) from the US (up to 22,780 µg/kg lw) [130]. The major congeners present in these studies were generally BDE-47, BDE-99 and BDE-100. PBDEs have also been detected in amphibians and reptiles such as sea turtles, alligators and snakes at relatively lower concentrations (ng/kg-µg/kg range), though PBDE levels in a water snake (*Enhydris chinensis*) collected from an e-waste recycling site in China were up to 190 mg/kg lw [131-135]

Some of the highest concentrations of PBDEs in fish are found in carnivorous species positioned at the top of the food webs, such as rainbow trout (*Oncorhynchus mykiss*), perch (*Sander lucioperca*) [136], striped bass (*Morone saxatilis)* and eel (*Anguilla anguilla*) [136] as well as benthivorous species such as barbel (*Barbel* sp) and carp (*Cyprinus carpio*) [137]. The level of PBDEs in fish have been found to be higher in species living in freshwater ecosystems compared to those in marine environments [136]. The highest levels have been reported in fish from the River Scheldt in Belgium (up to 11,500 µg/kg lw) [138], the Hyco River in Virginia (up to 47,900 µg/kg lw) [139] and the Hudson River in New York (up to 37,169 µg/kg lw) [57]. As with human and marine mammal samples, the lower brominated congeners, such as BDE-47, BDE-99 and BDE-100, generally dominate the PBDE profiles in fish samples [57, 58, 133, 140]. The highest reported levels of BDE-209 in fish are relatively low, though certainly not negligible, in comparison to the lower PBDE congeners. For example, high levels of BDE-209 were recorded in fish from the River Vero in Spain (up to 707 µg/kg lw) [141], a reservoir in an e-waste recycling region of China [up to 549 µg/kg wet weight (ww)] [133] and downstream of a WWTP in North Carolina (up to 2,880 µg/kg lw) [84]. Interesting, BDE-209 has been found to dominate the congener profile (> 50% total PBDEs) of sediment associating fish such as roach *(Rutilus rutilus)* [142].

PBDEs have also been detected in a variety of aquatic invertebrate species (e.g. *Crustacea*, *Mollusca*, *Bivalvia* and *Annelida*) and zooplankton (e.g. *Copepoda*, *Rotatoria* and *Copelata*) from around the world [133, 143-148]. High PBDE levels have been reported in zooplankton from the Baltic Sea (up to 4,280 µg/kg lw) [149]

and in invertebrates from Belgium (up to 12.4 µg/kg ww) [147] and China (up to 363 µg/kg ww) [133]. Often the dominant congener in invertebrates is BDE-47 with low or negligible levels of BDE-209 [150]. There are a few exceptions to this which have shown BDE-209 to be an important congener in amphipods [151, 152], hexapod larvae [152] and shrimps [153].

1.5.2 HBCDs

HBCD was first detected in Swedish rivers in 1997 [154] and has since been found in a wide variety of environmental matrices from around the world (reviewed in [155]), including in air sampled in the Arctic [80]. Owing to the high log K_{ow} value, HBCDs are strongly associated with solid particles and high levels have been detected in soils (up to 1,300 µg/kg dw) [156], sediments (up to 7,800 µg/kg dw) [157] and sewage sludge (up to 9,120 µg/kg dw) [158]. Elevated concentrations of HBCDs have also been measured in indoor air from homes (up to 1,300 pg/m^3) and dust from cars, homes and offices (up to 140,000 µg/kg dw) [159]). Relatively high levels of HBCDs have also been detected in the particulate phase of rivers and lakes from around the world. For instance, total HBCD levels of up to 2,100 ng L^{-1} were recorded in the surface waters of the River Kuzuryu in Japan, which in known to receive effluents from textile industries [157]. Much lower HBCD concentrations have been recorded in the dissolved water phase of English lakes (up to 270 pg/L), the Pearl River Delta (up to 82 pg/L) and Polish rivers (>1µg/L) [160-162]. Some of the highest concentrations of HBCDs in environmental matrices have been detected within Europe [154, 158, 163], reflecting the high consumption of HBCD in the region (accounting for approx. 60% of the global market demand in 2001 [52]). HBCD levels in environmental matrices in China and the US are in comparison relatively low, even

in heavily industrialised areas like the Pearl River Delta and the Detroit River [164, 165]. In most cases, γ-HBCD is the dominant diastereoisomer found in many matrices (sediments, soils, sewage sludge) reflecting the high proportion used in HBCD commercial mixtures [160, 166, 167]. However, α-HBCD was the dominant diastereoisomer in airborne particles from urban areas of China [167, 168].

Aside from their association with solid particles, the high log K_{ow} value also results in significant accumulation in biological samples. Compared to PBDEs, relatively few studies have examined the occurrence of HBCDs in human samples, though it has been detected at relatively low levels (ng/kg-μg/kg lw range) in breast milk [169], blood serum [170] and adipose tissue [171]. In general, the highest levels of HBCDs observed in human samples have been reported from European countries including the UK (up to 22 μg/kg lw) [172], Norway (up to 31 μg/kg lw) [173] and Spain (up to 188 μg/kg lw) [174], though similar levels have also been reported from Canada (up to 19 μg/kg lw) [175] and China (up to 38 μg/kg lw) [169].

HBCD have also been detected in numerous birds and bird eggs from around the world, including terrestrial and aquatic species (reviewed in [155]). Some of the highest concentrations have been reported in the muscle tissue of sparrowhawk (*Accipiter nisus*; up to 2,360 μg/kg lw) from the UK [176] and Chinese pond heron (*Ardeola bacchus;* up to 5,058 μg/kg lw) from the Pearl River Delta, China [177].

HBCD have been detected in various marine and freshwater biota from around the world (reviewed in [155, 178]). They have been measured in several marine mammals such as the harbour porpoise [179], common dolphin (*Delphinus delphis*)

[179], California sea lion (*Zalophus californianus*) and Grey seal (*Halicoerus grypus*). As with PBDEs, some of the highest levels of HBCDs in fish have been observed in top predators such as cod (*Gadus morhua*) [180], eels [158], northern pike *(Esox Lucius*) [156] as well as omnivorous feeding fish such as sole (*Solea solea*) and common carp (*Cyprinus carpio*) [181, 182]. Again, HBCD levels are highest in fish sampled from Europe and in e-waste recycling regions of Asia. For example, high HBCD levels were detected in fish from the River Skerne in the UK (up to 10,275 µg/kg lw)[158] *,* the Western Scheldt in the Netherlands (up to 1,113 µg/kg lw) [181], the River Viskan in Sweden (up to 8,000 µg/kg lw) [156] and from natural ponds in South China (up to 2,370 µg/kg lw) [183]. In general, HBCD levels in fish from North America are relatively low [184, 185], though a recent study reported median levels up to 5,420 µg/kg lw in common carp sampled from the Hyco River located in the states of Virginia and North Carolina [182]. HBCD has also been detected in various marine and freshwater invertebrate (e.g. *Cephalopoda*, *Bivalvia*, *Crustacea*, *Echinodermata* and *Annelida*) [186-189].

In most biological samples, including aquatic invertebrates, fish, marine mammals, humans and birds, α-HBCD is the most prominent diastereoisomer detected and can comprise up 90% of the total HBCD measured [155, 190-192].

1.5.3 TBBPA

In its primary application as a reactive flame retardant, TBBPA is perceived to be stable and not easily released from its final product. However, its occurrence in abiotic samples indicates a significant dispersal of TBBPA into the surrounding environment (reviewed in [70, 193]). For instance, elevated levels of TBBPA have

been detected in raw leachates (up to 620 ng/L) [194], soils (up to 7,758 µg/kg dw) [195], sewage sludge (up to 1,329 µg/kg dw) [196], sediments (up to 518 µg/kg dw) [197] and air samples (up to 95 ng/m^3) [198]. TBBPA tends to adhere to suspended particulate matter and is therefore found at very high concentrations in dust. For instance in China, TBBPA has been found in dust from residential homes up to 46,191 µg/kg dw [199], factories up to 9,010 µg/kg dw and offices up to 59,140 µg/kg dw [200]. Despite the fact that TBBPA is highly hydrophobic, it has also been detected in the dissolved water phase of rivers and lakes in the UK (up to 3.3 ng/L) [160], Germany (up to 20.4 ng/L) [201] and at relatively high levels in China (up to 4,870 ng/L) [197, 202]. In general the levels of TBBPA in environmental matrices such as dust, soil and sediments and sludge are highest in Asia, with levels in Europe and the US significantly lower. For instance, the concentrations of TBBPA in dust from residential homes in China, Japan and Korea were found to be 10–100 times higher than the concentrations observed in other countries [203]. Given that Asia accounts for 75% of the global TBBPA market [62] and the large scale e-waste generation in China (11.1 million metric tonnes of domestic e-waste generated in 2012) [204, 205], these levels are not surprising. It is clear that TBBPA levels in biotic matrices (sediments, soils and sludge) are for the most part lower than those reported for PBDEs.

TBBPA has a moderate molecular weight and relatively short half-life [206], and therefore is not considered to be persistent or bioaccumulative [76, 207]. Despite this, TBBPA has been detected in various biotic samples from around the world [158, 208-210]. It has been reported in human tissue samples such as blood serum, placental cord serum, breast milk and adipose tissues [171, 209, 211, 212]. Some of

the highest levels of TBBPA in human breast milk have been reported in China (up to 12.5 µg/kg lw) [213, 214] and France (up to 37.3 µg/kg lw) [212]. Levels in breast milk from women residing in other regions of the world have been found to be relatively low, for instance up to 0.55 µg/kg lw in the US [215] and up to 0.65 µg/kg lw in the UK [172].

The number of studies reporting on the concentrations of TBBPA in bird tissues and bird eggs is limited, with most studies taking place within Europe, and reported concentrations are low. For example, TBBPA has been detected in the eggs of the Peregrine falcon, White-tailed eagle (*Haliaeetus albicilla*), Osprey and Golden Eagle (*Aquila chrysaetos*) in Norway (up to 13 ng/kg ww) [216], and in eggs of the Common tern *(Sterna hirundo*) from Belgium (< 2.9 ng/kg ww) [158]. In addition, TBBPA was detected in Cormorant (*Phalacrocorax carbo*) liver samples from England (up to 14 ng/kg lw) and in the muscle tissue of several bird species in China (up to 1482 µg/kg lw) [177].

TBBPA has repeatedly been detected in numerous fish species from both freshwater and marine environments around the world [158, 160, 217, 218], commonly at concentrations between 0.01-50 µg/kg lw [140, 171, 219-222]. The highest TBBPA levels in fish have been observed in whiting (*Merlangius merlangus*) muscle from the North Sea (up to 245 µg/kg lw) [158]. Interestingly, a recent study by Tang *et al.,* (2015) found that predator fish from an e-waste site in China contained lower concentrations of TBBPA than prey fish [220]. This is contrary to the observed levels of PBDEs in aquatic food webs, for which higher concentrations are generally detected in organisms at higher trophic levels [223]. The number of reports on

TBBPA bioaccumulation in marine mammals are limited, however it has been detected in the blubber of harbour porpoises from coastal waters around the UK (up to 418 µg/kg lw) [158, 224] and bottlenose dolphin from coastal waters around Florida, US (up to 35 µg/kg lw) [171]. Again limited studies have investigated the levels of TBBPA in aquatic invertebrates, though it has been detected in the hermit crab (*Pagurus bernhardus*), common whelk (*Buccinum undatum*), sea star (*Asterias rubens*), Chinese mystery snail (*Cipangopaludina chinensis*) and prawn (*Macrobrachium nipponense)* at levels up to 100 µg/kg lw [158, 225].

It is clear that TBBPA levels in biotic samples are relatively low compared to those of PBDEs and HBCDs (1-2 orders of magnitudes lower). This may be explained by the fact that the release of TBBPA into the environment is limited as a result of its use as a reactive BFR. In addition, the polar nature of TBBPA means that it can be rapidly conjugated in the liver and eliminated from organisms, and therefore the degree to which it bioaccumulates may be limited. Finally, TBBPA has a short biological half-life, and a continuous exposure to this compound is likely to be required in order to maintain a detectable level in an organism. The fact that various studies have detected TBBPA in a suite of organisms from around the world suggests that their exposure to this chemical has been recent and continuous.

1.5.4 Current environmental trends

Frustratingly, but reflecting the paucity of research, relatively few studies have been published to date which have assessed the current levels and/or time trends of BFR compounds in environmental samples (i.e. sampling has taken place in years after the implementation of restrictions/bans on BFR use and production). While there are

some indications that levels of BFRs in human samples from the USA and Europe are decreasing, increasing time trends have been detected in several African countries (reviewed in [79]). In addition, several studies have recently reported levels of PBDEs in dust from countries for which such information to date has been unavailable [226-229]. In general PBDE concentrations have levelled off in sewage sludge and soil samples from Europe and the USA [230, 231], while concentrations of BDE-209 in both regions have increased with levels doubling every 5 years from 1995-2008 in Chicago [231]. Declines in PBDE concentrations in several fish species have also been reported in two studies from the USA [182, 232] and in fish, mammal and birds in the Arctic [233].

Though controls on the use and production of some PBDEs compounds are beginning to yield declines in concentration in some environmental matrices in Europe and the USA, there is still a need to assess time trends in Asia. Similarly, as the use of HBCD has only relatively recently been restricted globally (since 2013), no time trends are yet available in the literature. It is important to note that many older products manufactured prior to the introduction of bans/restrictions, still contain HBCDs and PBDEs and are in service today. These products therefore can act as a continuous source of environmental contamination during their lifetime, after disposal and at e-waste recycling sites [234-240]. Additionally, many BFRs are highly persistent in the environment and even today the constituents of octa- and penta-BDE technical mixtures are still the dominant PBDEs detected in human and wildlife samples [241-246].

It is also noteworthy that the ban on octa- and penta-BDE commercial mixtures has led to a dramatic increase in the production of deca-BDE mixtures in Asia [247]. The continued use of deca-BDE mixtures and the large reservoirs of BDE-209 found in soils and sediments worldwide is a major cause for concern [248]. BDE-209 is highly susceptible to degradation, via photolytic, microsomal and/or metabolic debromination pathways which can yield the lower brominated congeners such nona-, octa- and hepta-BDEs shown to be more mobile and toxic [249-251]. Furthermore, it is often overlooked that the use of HBCD and deca-BDE is still permitted in many materials within Europe, despites restrictions in textiles and E&E products respectively. Finally both the United States and Russia, though signatories on the Stockholm Convention in 2001, have not ratified the Treaty and therefore are not required to take heed of its recommendations on the use of POPs.

1.6 Evidence of Thyroid Disruption by BFRs

Since several BFRs are structurally similar to THs (Figure 3), concerns have been raised about their effect on the thyroid system of both mammalian and non-mammalian vertebrates. A variety of experimental methods (i.e. *in vivo* and *in vitro*) have been developed to help examine the effects of BFRs on thyroid activity. In addition, a number of studies have examined the thyroid disrupting effects of BFRs on humans and wildlife populations, though information on wild fish populations specifically is limited. The following sections highlight the evidence of thyroid disruption by BFRs from both *in vitro* and *in vivo* studies, with the latter largely focusing on studies carried out in fish model species.

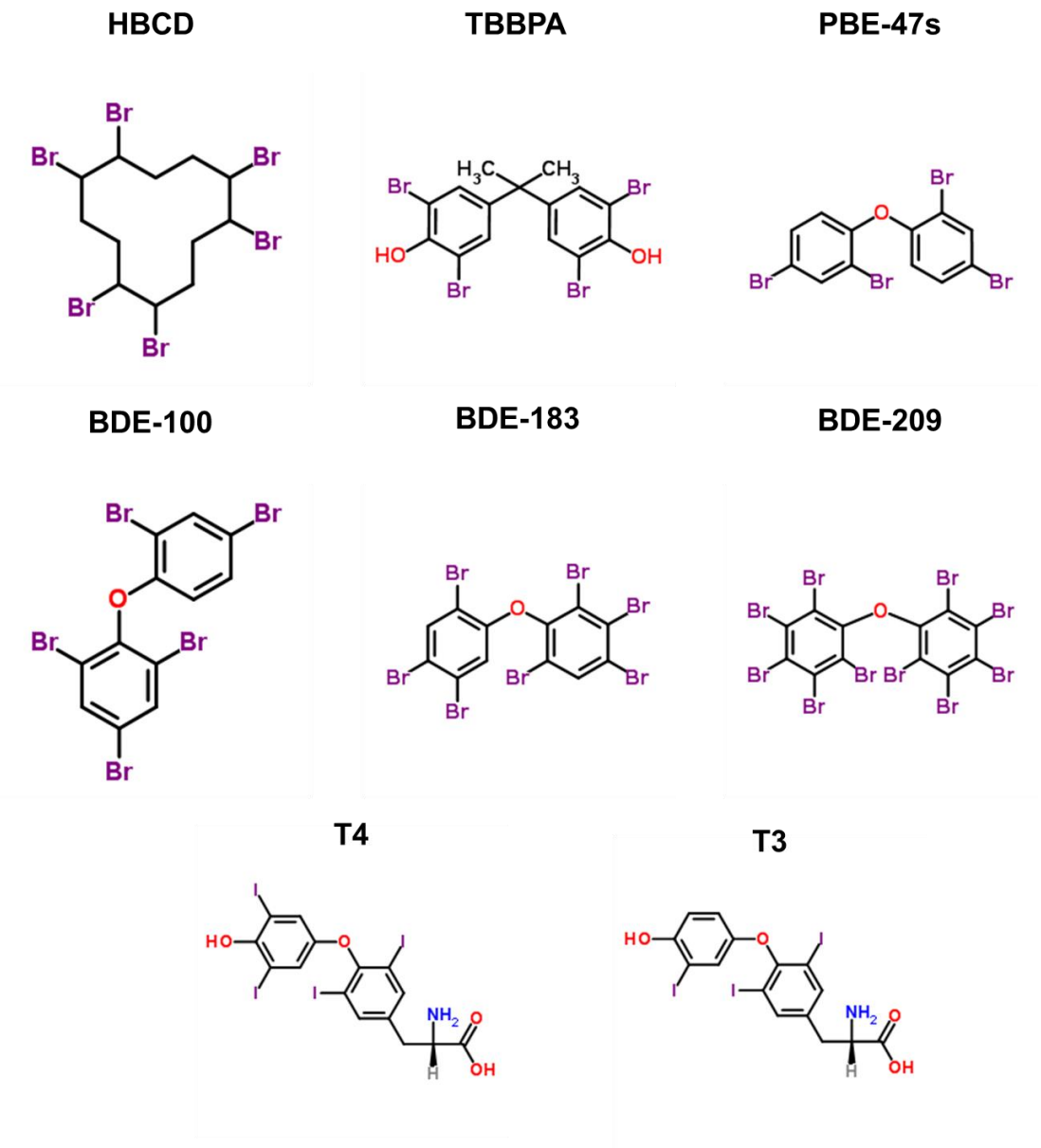

Figure 3: Chemical structure of the BFR compounds tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), 2,2′,4,4′,6-penta-bromodiphenyl ether (BDE-100), 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183) , deca-bromodiphenyl ether (BDE-209) and the thyroid hormones thyroxine (T4) and triiodothyronine (T3).

1.6.1 Evidence of thyroid disruption by BFRs: *in vitro* studies

Several *in vitro* studies have demonstrated that various BFRs have thyroid disrupting capabilities. For instance, an early study by Kitamura *et al.,* (2002) found that TBBPA induced T3-dependent cell proliferation in a rat pituitary tumour cell line (GH3), though no antagonistic activity was observed [252]. Hamers *et al.,* (2006) also used a T-screen assay to test a set of 27 individual BFRs (including TBBPA, HBCDs and 19 PBDE congeners) for their ability to interfere with T3-mediated cell proliferation in GH3 cells. They found that in the absence of T3, α-HBCD, BDE-127 and BDE-185 acted as T3-agonistics on cell proliferation, while in the presence of T3 13 BFRs had a synergistic effect on T3 actions with the most pronounced effects observed for BDE-49, BDE-100, α-HBCD and λ-HBCD [253]. Shriks *et al.,* (2006) carried out a similar T-screen assay with GH3 cells using many of the same BFR compounds and observed no effect on T3-mediated cell proliferation in the absence of T3. However in the presence of T3, BDE-206 acted as an antagonist of cell-proliferation while λ-HBCD potentiated the activity of T3 [254]. A further study by the same research group examined the effect of BDE-206 and HBCD on T3-induced tail tip regression in *Xenopus laevis* tadpoles carried out in organ culture. In the presence of T3, tail tip regression was antagonised by BDE-206 and potentiated by HBCD, while neither compound induced any effect in the absence of T3 [255]. A similar organ culture study also found that TBBPA inhibited the T3-mediated tail regression of *Rana rugosa* [256]. In addition Ibhazehiebo *et al.,* (2011) assessed the impact of 11 different PBDE compounds on T4-mediated dendrite arborisation of cerebellar Purkinje cells and found that BDE-209 inhibited the development of Purkinje cells in the presence of T4 [257].

As previously mentioned, it is suspected that BFRs may disrupt TH levels by binding with TRs and altering the transcription of downstream target genes. The effect of BFRs on TR-mediated thyroid signalling has been examined in various mammalian cell-based assays [256-261]. For instance, a comprehensive study carried out by Kojima *et al.,* (2009) examined the effect of several PBDEs, PBDE metabolites and TBBPA on human TR activity using a CHO-K1 cell line. They found none of the 16 compounds tested showed any agonistic effect on TR activity but 4-HO-BDE-90 inhibited both the TRα- and TRβ-mediated transcriptional activity induced by T3 [259]. Kitamura *et al.,* (2005) examined the thyroid activity of TBBPA by means of a binding assay with TRs from the rat pituitary cell line MtT/E-2, as well as a TH-dependent reporter assay in the Chinese hamster ovary cell line (CHO-K1). They demonstrated that TBBPA did not exhibit an agonistic effect on human TRα- and TRβ-mediated transcriptional activity, but did inhibit the binding of T3 to the TRs and had an antagonistic effect on the activity of T3 on the two TRs [256]. A reporter gene assay performed in a green monkey kidney fibroblast cell line (CV-1) also found that BDE-209, -100, -153 and -154 were capable of supressing human TR transcriptional activity. The authors demonstrated that in the case of BDE-209 this may be a result of the partial dissociation of the TRs from TRE, though this was not observed for the other congeners tested [257]. In addition Freitas *et al.,* (2011) found that two hydroxy-PBDEs (4-OH-BDE-69 and 4-OH-BDE-121) induced TR-mediated transcription in a reporter gene assay carried out in the GH3 cell line (constitutively expresses both rat TR isoforms). Interestingly, the same study found TBBPA to be a TR agonist at low concentrations and a TR antagonist at higher concentrations [258]. In the only study to examine the effect of BFRs on amphibian TR activity, Schriks *et al.,* (2007) found that BDE-206, HBCD and BDE-28 had no agonistic effect on

Xenopus laevis TR activity. BDE-206 was observed to have antagonist effect on both TRα- and TRβ-mediated transcriptional activity, while BDE-28 potentiated the effect of T3 on TRβ activity [261].

Taking the above studies together, there appears to be considerable discrepancies reported from various mammalian and amphibian derived *in vitro* assays with regards to the effect of BFR compounds on TR-mediated transcription. This may be a result of the extensive range of BFR compounds tested, the different species and receptor types (TRα or TRβ) examined, as well as the different cell systems and reporter constructs utilised. To date, the effect of BFRs on the transcriptional activation of fish TRs is limited. One recent study, however, assessed the effect of TBBPA on the transcriptional activity of TRs derived from the Japanese medaka (*Oryzias latipes*) and found that TBBPA inhibited the T3-induced activation of TRα [262]. It is clear that further work on the mechanisms of thyroid disruption by BFRs is required in order to assess their potential health and environmental risks, which cannot be predicted from investigating TR activation in one or two vertebrate species.

One of the major drawbacks of using *in vitro* system is that cell systems often do not account for metabolism of chemicals or co-factors, nor can they (*in vitro*) represent the thyroid system as a whole given its complex functions, interactions and reactions. However, *in vitro* assays have several advantages over *in vivo* test methods. For instance, *in vitro* studies are considered more ethical in terms of using less test animals; they tend to generate less waste and are cheaper and more rapid than *in vivo* systems. In addition, interpreting the mechanistic pathways of toxicity

from *in vivo* tests can prove difficult, whereas in general *in vitro* studies allow for a more focused examination of a single mechanistic action.

1.6.2 Evidence of thyroid disruption by BFRs: *in vivo* studies

Many studies examining the potential thyroid disrupting ability of environmental contaminant have traditionally evaluated thyroid status by measuring circulating plasma T4 and T3 levels. For instance, in human epidemiology studies, altered plasma TH concentrations in adults were associated with PBDE levels in serum and dust [263-265]. Similarly, concentrations of BDE-47 were negatively correlated with circulating concentrations of free T4 in blood plasma of white whales (*Delphinapterus leucas*) from Svalbard [266]. Numerous *in vivo* studies on mammals and birds have also found that oral exposures to several individual PBDE congeners, PBDE technical mixtures, HBCD and TBBPA resulted in reduced circulating levels of T3 and/or T4 [267-277].

The thyroid system maintains normal physiological functioning by responding to TH perturbations with changes in TH production from the thyroid gland/follicles and in the peripheral tissues [278, 279]. Such integrated compensatory responses in the central HPT axis and in peripheral target tissues make it challenging to evaluate mechanisms of action for thyroid disruptors. For instance in fish, significant increases in T4 levels do not necessarily indicate an adverse effect, as T3 function may be unchanged as a result of the action of deiodinase enzymes in target cells [32]. Consequently the mechanisms of thyroid disruption by BFRs are not well understood. Nonetheless, several general explanations have been proposed including interference with deiodinase enzyme activity/expression; enhanced

metabolism and elimination of THs; altered expression and activity of plasma transporters and membrane bound transporters; altered genomic signalling via TRs; and altered thyroid gland histology and morphology. In the following sections we give an overview of the *in vivo* studies carried out in fish to date which have increasingly focused on changes in TH signalling along the entire HPT axis of model organisms, as well as changes in circulating levels of TH, in order to shed light on the thyroid disrupting capabilities of BFRs.

1.6.2.1 PBDEs

In one of the earliest studies in fish, reduced circulating levels of free T4 and T3 were recorded in the plasma of lake trout (*Salvelinus namaycush*) dosed via the diet with a mixture of 13 PBDE congeners (e.g., BDE-28, -47, -99, -100, -153, -154, -209) for 56 days [280]. Dietary exposure to the commercial penta-BDE mixture also suppressed total T4 levels in the European flounder (*Platichthys flesus*) after 101 days, however no change in total T3 levels were detected [281]. Zebrafish adults had increased T3 and T4 levels following a 30-day exposure to DE-71, a penta-BDE commercial mixture [282]. Similarly, a 14-day exposure to DE-71 via the water reduced T4 levels in zebrafish larvae and upregulated the transcription of several genes in the HPT axis including those encoding; D1, D2, TG, the sodium iodine symporter (NIS), nk2 homeobox 1a (NKX2.1a*)* and paired box 8 (Pax8) [283]. Interestingly a later study by the same research group, examining the effects of long term DE-71 exposure on adult zebrafish and their progeny, reported elevated T4 levels in female adults accompanied by elevated expression of genes encoding corticotropin-releasing factor (CRH) and TSHβ in the brain following a 5 month waterborne exposure.

Similarly, elevated levels of both T4 and T3 were observed in the F1 generation [284].

Several studies have also examined the effect of BDE-47 on the thyroid system of various fish species. For instance, exposure to BDE-47 for 21 days reduced T4 levels in fathead minnows (*Pimephales promelas*) but had no effect on T3 levels. In the same study, T4 alterations were accompanied by elevated mRNA transcripts encoding TSHβ in the pituitary, while elevated and reduced levels of mRNA transcripts encoding TRα and TRβ, respectively, were detected in the brain but not the liver [285]. Chan and Chan (2012) found that zebrafish larvae exposed to BDE-47 via the water for 4 days had elevated transcript levels for the genes encoding TG, thyroid peroxidase (TPO), TRα, TRβ, TSHβ, and TTR [286]. While in the same study, exposure to BDE-47 for 4 days up-regulated the transcription of the genes encoding NIS and TSHβ in zebrafish embryos [286]. Interestingly, a later study found that exposure to BDE-47 (at lower concentrations to the Chan & Chan study) had no effect on the transcription of several genes in the HPT axis of zebrafish larvae after a 5 day exposure via the water, though the analogues 6-OH-BDE-47 and 6-MeO-BDE-4 did elicit transcriptional changes [287]. Furthermore, BDE-47 had no effect on the thyroid condition (follicular cell height, colloid depletion, angiogenesis) of pubertal zebrafish following dietary exposures for 40, 80 and 120 days [288]. A recent paper by Zhao *et al.,* (2016) examined the thyroid disruption induced by a life-cycle exposure to BDE-47 in adults and offspring of zebrafish. They found that BDE-47 decreased whole-body T4 levels but increased T3 levels in both F0 and F1 generations. In F0 adults and F1 embryos, BDE-47 exposure led to an up-regulation of genes encoding CRH, TSHβ, Pax8, UGT1ab, D2 and TG while in F0 adults only

the expression of the gene encoding TTR was down-regulated [289]. A recent study by Dong *et al.,* (2013) examined the effect of the hydroxylated metabolite 6-OH-BDE-47 on the tissue-specific expression pattern of the deiodinase encoding genes in zebrafish [290]. They found that exposure resulted in a significant up-regulation of *dio1* mRNA expression in the periventricular region of the brain and *dio3b* mRNA expression in the pronephric ducts of 22 hpf embryos [290].

Several studies have shown that the higher brominated PBDE congener, BDE-209, is also capable of disturbing both TH levels and gene expression in the HPT axis of several fish species. For example, rainbow trout exposed to BDE-209 for 21 days via a single intraperitoneal injection had reduced circulating levels of free T4 and T3 [291]. Meanwhile, zebrafish larvae exposed to BDE-209 for 14 days via the water had reduced T4 levels and enhanced T3 levels [292]. These alterations in TH levels were accompanied by enhanced mRNA transcripts encoding several genes including TRα, TRβ, TSHβ, D1, D2, NIS, TG, CRH, Nkx2.1a and Pax8, while mRNA transcripts encoding TTR and UGT1ab were suppressed [292]. Chinese rare minnow (*Gobiocypris rarus*) larvae exposed to BDE-209 for 21 days via the water had elevated levels of mRNA transcripts encoding D2 and NIS [293]. Adult rare minnows in the same study, following exposure to BDE-209 for 21 days, had increased mRNA transcripts encoding TRα, D2, NIS and TTR in the liver and decreased levels of D2 and NIS in the brain [293]. Juvenile fathead minnows exposed to BDE-209 for 28 days via the diet, had reduced rates of outer and inner ring deiodination of T4 which were accompanied by evidence of thyroid follicle hypertrophy [294]. A later study by the same research group examined the effects of a 28-day dietary exposure to BDE-209 on TH levels and signalling in male adult fathead minnows. BDE-209 reduced

circulating total T4 and T3 levels, reduced outer ring deiodination of T4 and elevated the mRNA transcript levels of genes encoding deiodinases (D1 and D1), nuclear thyroid receptors (TRα and TRβ), and membrane transporters (MCT8 and OATP) in the brain and liver in patterns that varied with time and dose [295].

1.5.2.2 HBCDs

Limited studies have been conducted on the effect of HBCD exposure on the thyroid system of fish. In one of the earliest studies, thyroid axis disruption was examined in juvenile rainbow trout fed with one of the three different HBCD diastereoisomers (α, β or γ) for 56 days. While all three HBCD diastereoisomers reduced free T4 levels and T4 outer ring deiodination, disruption of the thyroid axis was most evident in fish exposed to γ-HBCD, as indicated by the additional increase in free T3 levels and increase in thyroid epithelial cell height [296]. The same group later demonstrated that β- and γ-HBCD increased type II outer ring deiodinase enzyme activity in the liver of rainbow trout following a 32 day treatment via the diet [297]. Conversely, TH levels and thyroid histology of European flounder were not affected by a chronic exposure (78 days) to HBCD via the diet and/or sediment [298].

1.5.2.3 TBBPA

Information related to TBBPA's ability to disrupt the expression of genes in the HPT axis of fish is relatively limited. A study by Chan & Chan (2012) reported that a 4 day waterborne exposure to TBBPA resulted in the transcription of TSHβ to be down-regulated in zebrafish embryos while the transcription of TRα, TSHβ and TTR was up-regulated in zebrafish larvae [286]. A more recent study found that TBBPA up-

regulated the transcription of TRα and down-regulated the transcription of TPO in zebrafish larvae following a 5 day waterborne exposure [299].

1.7 Biological Significance of Developmental Thyroid Disruption

The consequences of TH disruption depend on the developmental stage in which the disturbance begins. In adult humans, hypothyroidism results in a number of non-specific signs such as constipation and fatigue, dry skin, muscle cramps and menorrhagia. The formation of thyroid tumours is an additional effect of long-term hypothyroidism in adult rodents. The permanent and adverse effects of developmental disruption of thyroid functions are well documented in the literature [300]. Deficiencies in THs during development are associated with irreversible damage to virtually all organ systems (reproductive, cardiovascular, pulmonary, skeletal and central nervous systems). Below we will briefly discuss the role of THs in the development of central nervous system, the cardiovascular system and the skeletal system.

1.7.1 The central nervous system

In mammals, THs are known to play a critical role in the maturation of the developing brain and control numerous physiological processes in adult brains [301]. Studies on rodents have demonstrated that normal brain development requires the presence of THs for cell migration, dendrite and axon outgrowth, synapse formation, myelination and gliogenesis [302]. Therefore TH deficiency, when established during critical periods of neural development, whether severe, moderate or transient, may cause serious damage to the cyto-architecture and organization of the brain, including

altered cell migration, delay in maturation of neurons and glial cells, reduction of synapses, myelination deficits and changes in the number of particular cell populations. The cerebellum, neocortex and hippocampus are particularly vulnerable to TH disruption. In humans, developmental hypothyroidism can lead to cretinism, a syndrome associated with severe mental and physical retardation, while hyperthyroidism is associated with impaired cognitive and emotional functions, as well as psychiatric symptoms (reviewed in [303]). The effects of THs on fish brain development are understood to a lesser extent, though a recent study has demonstrated that zebrafish maternal THs, while not essential for the early specification of the neural epithelia in embryos, had profound effects on the later dorsal specification of the brain and spinal cord, as well as neuron specification [304].

1.7.2 The skeletal system

THs are one of the major regulators of skeletal development and remodelling in vertebrates, including flatfish [305] and teleost [8, 306]. A euthyroid status is essential for normal bone development, growth and maintenance [307]. While the hormonal control of cellular differential within fish bones has not been fully elucidated, current studies in higher vertebrates indicate that THs are essential to proliferating chondrocytes, osteoblastic bone marrow stromal cells and osteoblasts. In humans congenital and juvenile hypothyroidism results in delayed skeletal development and bone age with short stature. Meanwhile juvenile hyperthyroidism is characterized by accelerated skeletal development and rapid growth but an early cessation of growth and persistent short stature (reviewed in [307, 308]).

1.7.3 The cardiovascular system

The role of TH in regulating the cardiac system in adults is well established. THs control heart output by regulating tissue oxygen consumption, vascular resistance, blood volume, cardiac contractility, and heart rate (reviewed in [11]). In adults, hyperthyroidism is characterized by an increase in resting heart rate, blood volume, stroke volume, myocardial contractility, and ejection fraction. Conversely, hypothyroidism leads to lower cardiac output, decreased stroke volume, decreased vascular volume, and increased systemic vascular resistance. In addition to their well-known effect on cardiac function in adults, THs are critical regulators of foetal and postnatal cardiac growth and development. In mammals, congenital hypothyroidism results in cardiac anomalies such as atrial septal defects [309] and prenatal exposure to THs is necessary for the adaptive responses in gene expression necessary for postnatal heart development [310]. Postnatally, THs are involved in enhancing cardiac contractile functions, hemodynamic alterations (increases in blood pressure, stroke volume and cardiac output) and cardiac morphology (reviewed in [311]).

Given the key role of TH in early developmental processes, it is essential to consider how TH actions can potentially be disrupted by environmental pollutants. Therefore, it is surprising to observe that fish studies have largely focused on the effects of BFRs on the thyroid system in juvenile and/or adult life stages. It is also important to note that the effects of TDCs in the developing organism can be independent of TSH, and instead can result from altered tissue levels of T4 and T3 [312]. Despite this fact, only one study to date has focused on tissue-specific effects of BFRs on the expression of key genes in the TH system during early embryonic-larval stage. This

information is crucial in order to better understand the potential targets and biological significance of thyroid disruption by these BFRs.

The zebrafish was the study organism used throughout this body of work. There are many advantages to the zebrafish model. It is small (adults 4-5cm in length), easily maintained in the laboratory and has a short completion time for embryogenesis (72 hour hatch) allowing for high throughput toxicity studies and facilitates studies on reproduction. In addition, the zebrafish is widely used in the field of developmental biology as it has a sequenced genome and eggs have a transparent chorion membrane, allowing the observation of developing embryos and examination of gene pathways. The *in vivo* studies carried out during this PhD utilised zebrafish *casper*s, which are mutants (*nacre* and *roy*) lacking melanocytes and iridophores and therefore have almost entirely transparent bodies. The use of the *casper* mutants allows us to clearly visualise gene expression patterns within tissues of embryo-larvae (via whole mount *in-situ* hybridisation), which may otherwise be obstructed by pigment cells in the non-mutant *wik* strains.

1.8 Aims of this Thesis

BFRs are found at high concentrations in the aquatic environment, including in the dissolved and particulate phase of water bodies, sediments and contained within the bodies of many aquatic species. While the vast majority of research into the biological effects of BFRs has been carried out in mammals and amphibians, fish are also highly vulnerable to exposure. It is clear that several BFR compounds are capable of interacting with TH-signalling pathways in several fish species, however,

the molecular mechanism of action and tissue targets are not fully understood. This PhD aimed to help bridge this knowledge gap in two ways:

1. My thesis sets out to increase our knowledge of the TH system during early fish life stages through establishing the ontogenies and expression patterns of a number of key constituents of the TH system. An improved understanding on the spatial and temporal dynamics of the TH system is needed to better establish the tissues and life stages which may be vulnerable to the effects of BFRs.
2. Examine the tissue targets and molecular mechanism of thyroid disruption caused by exposure of fish early life stages to a selection of BFRs.

In this thesis, the following experiments were carried out to address these aims.

Chapter 2: Expression dynamics and tissue localisation of genes in the hypothalamic-pituitary-thyroid (HPT) cascade and their responses to thyroid hormones in zebrafish embryo-larvae.

This chapter addressed the following specific aims and hypotheses;

- Establish mRNA transcript levels and tissue expression patterns of thyroid-related genes in zebrafish embryo-larvae between 24-120 hours post fertilisation (hpf).
- Exogenous T3 alters mRNA transcript levels and tissue expression profiles of thyroid-related genes at multiple developmental stages in zebrafish embryonic-larvae.

In this work, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and whole mount *in-situ* hybridisation (WISH) assays were set up for genes of interest in the HPT axis. Newly fertilised zebrafish embryos were collected and fixed at several early developmental stages (between 24 and 120 hpf). qRT-PCR and WISH assays were then applied in order to examine gene expression profiles at each developmental stage. This allowed for the most appropriate life stages to be used in subsequent experiments. Subsequent experiments involved exposing zebrafish embryos to T3 via the water for three time periods from the point of fertilization; 48, 96 or 120 hpf, and changes in the expression of the selected HPT genes were measured via qRT-PCR (on whole body extracts for each of the developmental stages) and WISH (to assess changes in tissue expression patterns of a more limited selection of genes at two of the developmental stages).

Chapter 3: The molecular effect mechanisms and tissue targets of two important BFR compounds, TBBPA and BDE-47, in embryo-larval life stages of zebrafish. This chapter set out to address the following specific hypotheses:

- Sub-acute exposure to TBBPA and BDE-47 alters whole body mRNA transcript levels of genes in the HPT axis of zebrafish during early embryonic-larval development.
- Sub-acute exposure to TBBPA and BDE-47 alters tissue-specific expression of genes in HPT axis of zebrafish during early embryonic-larval development.
- TBBPA alters whole body and tissue specific mRNA expression in a developmental stage specific manner.

To address these hypothesis zebrafish embryos were exposed to TBBPA via the water for three time periods from the point of fertilization; 48, 96 or 120 hours post fertilisation and effects on the expression of several genes in the HPT axis assessed. This was undertaken using qRT-PCR on whole body extracts for each of the developmental stages and via WISH (for tissue-specific effects at two of the developmental stages). Zebrafish embryo-larvae were also exposed to BDE-47 for a single time period only; 96 hours post fertilisation and examined the effect on whole body transcript levels and expression patterns of thyroid related genes using qRT-PCR and WISH.

Chapter 4: Development of a transactivation reporter gene assay for zebrafish thyroid receptors TRα and TRβ and an investigation into their interactions with brominated flame retardants.

The overall aim of this chapter was to examine one of the potential mechanistic pathways of BFR-induced thyroid disruption in zebrafish, with the following specific hypothesis;

- Exposure to BFR compounds alters zebrafish TR-mediated transcriptional activity

In order to address this hypotheses, *in vitro* reporter gene transactional assays for two zebrafish thyroid receptor (zfTRα and zfTRβ) were developed in human embryonic kidney cells (HEK). These assays were subsequently optimised and validated by assessing the activity of both receptors upon exposure to the natural TR agonist T3. Using the optimised reporter gene assays, the effect of six brominated flame retardant compounds (TBBPA, HBCD, BDE-47, BDE-100, BDE-183 and BDE-209) on TR-mediated transcription were examined.

The final chapter of this thesis (Chapter 5), provides a critical overview on the main findings of my thesis studies, the challenges faced and discusses future prospects for research into mechanistic effects of brominated flame retardants and the broader area of ecotoxicology.

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Chapter 2

Expression dynamics and tissue localisation of genes in the hypothalamic-pituitary-thyroid (HPT) cascade and their responses to 3,5,3′-tri-iodothyronine in zebrafish *(Danio rerio*) embryo-larvae

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2.1 Abstract

Some chemicals in the environment disrupt thyroid hormone (TH) systems leading to alterations in developmental processes, but their effect mechanisms are poorly understood and 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 the expression profiles of a suite of genes in the hypothalamic-pituitary-thyroid (HPT) axis of zebrafish (*Danio rerio*) between 24-120 hours post fertilisation (hpf) using whole mount *in situ* hybridisation (WISH) and qRT-PCR (of whole body extracts). Thyroid receptors (*thraa* and *thrb*) were differentially expressed both spatially and temporally, with *thraa* expression observed in brain, lower jaw and intestinal tract, branchial arches and pectoral fins while *thrb* expression was observed in the brain, otic vesicles, liver and lower jaw. The TH deiodinase enzymes (*dio1, dio2* and *dio3b*) were expressed in the liver, pronephric ducts and brain, though the expression patterns differed depending on the life stage examined. In addition, *dio1* and *dio2* were both expressed in the intestinal bulb (96-120 hpf), while d*io2* was also observed in the pituitary (48-120 hpf). Zebrafish embryo-larvae were subsequently exposed to sublethal concentrations of 3,5,3′-tri-iodothyronine (T3; 30 and 100 µg L^{-1}) for 48, 96 or 120 hpf and transcriptional changes of genes in the HPT axis were quantitatively measured (via qRT-PCR) and qualitatively assessed (via WISH). T3 exposure caused the transcription of *thraa*, *thrb*, *dio3b*, *chrb* and *pax8* to be up-regulated and the transcription of *dio1*, *dio2*, *ugt1ab* and *tshb* to be down-regulated with both life stage and tissue-specific responses observed. T3 induced *thraa* expression in the pineal gland, pectoral fins, brain, somites, branchial arches and pronephric ducts in 48 hpf embryos and in the brain, pineal gland, pectoral fins, jaw cartilage, liver and intestine of 120 hpf larvae. T3 enhanced *thrb*

expression in the brain of 48 embryos, while in 120 hpf larvae *thrb* expression was also induced in the jaw cartilage and intestine and suppressed in the liver. T3 exposure suppressed the transcription of *dio1* and *dio2* in the liver, brain, gastrointestinal tract and craniofacial tissues, while *dio2* signalling was also suppressed in the pituitary gland. Meanwhile *dio3b* expression was induced by T3 exposure in the jaw cartilage, pectoral fins and brain. These results suggest a role for THs in the development of various zebrafish tissues but also highlight the vulnerability of numerous tissues to the exposure of environmental thyroid disrupting chemicals.

2.2 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 (Reviewed in [1-3]). The growing awareness of the role of thyroid hormones (TH) during development has led to an increasing concern over environmental contaminants which act as thyroid disrupting chemicals (TDCs). TDCs are xenobiotics that alter the structure or function of the thyroid gland, alter TH regulatory enzymes or change circulating or tissue concentrations of THs [4]. Lipophilic compounds such as polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), phthalates and brominated flame retardants (BFR) are known to influence thyroid functions (reviewed in [5]). Aquatic and semi-aquatic species, including fish and amphibians are especially vulnerable to TDCs, as the aquatic environment receives large volumes of thousands of chemical contaminants via Sewage Treatment Works (STW) effluents, industrial discharges and agricultural runoff [6]. Uptake of TDCs in

to the bodies of these animals can occur via several routes, including through the water via the skin, gills and diet or via maternal transfer during embryonic development.

Thyroxine (T4) is the primary TH secreted by the thyroid follicles of teleost fish, while the production of biologically active 3,3′,5-triiodo-L-thyronine (T3) is thought to be under the exclusive control of the peripheral tissues [7]. The genomic actions of THs depends 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, a process that can either enhance or repress transcription [8]. Signalling through this pathway is highly sensitive to changes in TH concentration and the consequences of an abnormal thyroid function have been well recognised [9]. TH activity is tightly regulated by the TH-metabolising iodothyronine deiodinase enzymes, type I, II and III (D1, D2 and D3), which can change TH signalling in individual tissues as well as controlling serum thyroid concentrations [10, 11]. D2 catalyses the outer ring deiodination (ORD) of the prohormone T4 to produce the bioactive T3 [12]. In contrast, D3 catalyses the inner ring deiodination (IRD) of T4 and T3 producing the inactive metabolites reverse T3 (rT3) and 30, 3-T2, respectively. D1 is a kinetically inefficient enzyme that is capable of both activating and inactivating T4 [13].

THs play a crucial role in a wide range of physiological and developmental processes of vertebrates. In teleost fish, developmental roles include mediating the metamorphic transition from larval to adult stages and influencing maturation of tissues including bone, gonads, intestine and the central nervous system [14-18]. In

adults they modulate growth, energy homeostasis, cardiac rhythm, osmoregulation and the behaviours/physiology associated with rheotaxis and migration [19, 20]. Consequently even minor alterations in TH levels, particularly during sensitive developmental windows, can have significant acute and potentially long-term health effects. Although significant advances regarding thyroid disruption have been achieved in recent years, several aspects remain unresolved and this has been hindered by the lack of fundamental knowledge on the thyroid system in fish including the characterisation of basal gene expression and their responses to altered TH levels.

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 [21]. To better understand the role of THs in teleost fish and the potential biological impacts and mechanisms of TDC, detailed information is needed on the ontogeny of expression of genes in the HPT axis, their tissue localisations and their regulation by THs. In this study, we characterised the expression of a suite of genes in the HPT axis of zebrafish *(Danio rerio)* from 24 to 120 hours post fertilisation (hpf) using a combination of quantitative reverse transcription polymerase chain reaction (qRT-PCR) on whole body extracts and whole-mount *in situ* hybridisation (WISH) to identify the tissue expression pattern dynamics of these genes. We further undertook to examine the regulation of these genes by THs at key developmental stages by administrating zebrafish embryo-larvae with exogenous T3 via the water. The objective of this work was to establish whether T3 differentially regulated target gene mRNAs and by doing so identify

tissues and life stages which may be particularly vulnerable to thyroid mimicking chemicals.

2.3 Materials and Methods

2.3.1 Materials and reagents

3,3',5-Triiodo-L-thyronine (T3; CAS 6893-02-3) (purity ≥ 95%) was purchased from Sigma-Aldrich Inc. T3 stocks were dissolved in dimethylsulfoxide (DMSO).

2.3.2 Maintenance of zebrafish and embryo collection

Adult zebrafish [*casper* (*mitfa^w2; roy^a9*) mutant strain] were obtained from breeding stocks at the University of Exeter. The *casper* mutant zebrafish was employed in this work because they lack all melanocytes and iridophores, facilitating the visualisation of gene expression via WISH. Fish were maintained at 28 ± 1°C in a 12:12 hour light: dark cycle in a closed flow-through system. Embryos were collected approximately 1 hour post fertilisation (hpf) from breeding colonies, washed twice with culture water with the addition of methylene blue (10^{-5}%) to prevent fungal growth as described in Nusslein-Volhard, 1996 [22]. No known effects of methylene blue on thyroid signalling in fish are reported in the literature to the best of our knowledge. Eggs were incubated in culture water without methylene blue. Culture water was aerated artificial freshwater made according to the ISO-7346/3 guidelines (ISO water diluted 1:5, pH 6.5-7.5, air saturation 95-100%) [23]. Embryos were examined under a stereomicroscope and only fertilised embryos were selected for subsequent procedures.

2.3.3 Developmental expression of genes in the HPT axis of zebrafish

Approximately 100 fertilised embryos were maintained in 100 ml of embryo culture water, replacing half of the culture water daily. At 24, 48, 72, 96 and 120 hpf, 80 embryo-larvae were fixed using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4ºC overnight. Samples were subsequently washed with PBS, dechorionated and stored at -20ºC in 100% methanol for use in subsequent WISH analyses. Twenty embryo-larvae from the same developmental stages were pooled, frozen in liquid nitrogen and stored at -80ºC for use in subsequent qRT-PCR analyses. Each experiment was carried out in triplicate and repeated three times.

2.3.4 Acute toxicity of T3

T3 exposures were carried out at 3, 10, 30, 100 and 300 µg L^{-1} (DMSO at 0.01% v/v of the culture medium). Control groups were incubated in DMSO at 0.01%. Twenty fertilised embryos were selected for each treatment group and half of the exposure solutions were replaced every 24 hours with freshly prepared solutions. Exposures were conducted for 96 hours from fertilisation. The number of dead embryo-larvae and phenotypic deformities were recorded every 24 hours. Experiments were carried out in triplicate and repeated three times (n=9 per treatment group).

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

To examine the TH regulation of gene transcripts in the HPT axis of zebrafish during early development, embryo-larvae were exposed to exogenous T3 and gene transcript changes and tissue expression patterns were analyzed using qRT-PCR and WISH. T3 exposures were carried out at concentrations of 30 µg L^{-1} and 100 µg

L^{-1} (with DMSO at 0.01% v/v of the culture medium). Control groups were incubated in DMSO at 0.01%. Half of the exposure solution was replaced every 24 hours. Fifty embryos were exposed for each treatment group and the exposures were conducted from no later than 2 hours post fertilisation to 48, 96 and 120 hours post fertilisation. At each developmental stage, 40 individuals from each treatment were fixed in PFA overnight at 4°C, washed and dechorinated in PBS and stored at -20°C in 100% methanol for WISH experiments and 10 embryo-larvae from each treatment group were pooled, frozen in liquid nitrogen and stored at -80°C for qRT-PCR analyses. Experiments were carried out in triplicate and repeated three times.

2.3.6 Development of WISH RNA probes

WISH was used to examine tissue-specific changes in the expression of genes of interest in the HPT axis including: thyroid receptors (*thraa and thrb),* thyroid stimulating hormone *(tshb,* deiodinase enzymes *(dio1, dio2 and dio3b)* and transthyretin *(ttr).* DNA-containing plasmids were purchased from Source Bioscience (for *ttr, tshb, thraa* and *thrb* genes) or donated from Dr. Alain Lescure, Université de Strasbourg (*dio1* and *dio2*) and Professor Heather Stapleton, Duke University (*dio3b*). Plasmids were cultured overnight in LB broth at 37°C in shaker and cultures were purified as per Thermo Scientific GeneJet Plasmid Midiprep Kit (#K0481 Fermentas). DNA sequences were confirmed by Source Bioscience and the direction of gene insert was obtained by BLAST (NCBI). Plasmids were then digested at 37°C for 2 hours using the appropriate restriction enzymes (Table S1). DNA samples were purified by phenol chloroform extraction and recovered by standard precipitation with

ethanol and the purified DNA was run on a 1% agarose TAE gel by electrophoresis to confirm probe integrity and stored at -20°C until required for WISH (Fig. S1A).

To prepare the transthyretin (*ttr*) and deiodinase type III (*dio3b*) DNA template, a PCR-based technique was used. Plasmid DNA (10 ng) was used as a template in a PCR reaction using GoTaq-Polymerase (Promega) to obtain a full coding *ttr* sequence (Genbank accession number NM_001005598.2) of 744 base pairs (bp). The following PCR conditions were employed: 95°C for 2 minutes, followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 48°C for 30 seconds and elongation at 72˚C for 1 minute and a final extension at 72˚C for 5 minutes, using the forward: ATG GCG AAA GAA GTG ATT and reverse: GGA TAG AAA TGG TGC TTT primers. Plasmid DNA (10 ng) was used as a template in a PCR reaction using DreamTaq DNA Polymerase (Thermo Scientific) to obtain the full coding *dio3b* sequence (Genbank accession number NM_001177935.3) of 809 bps. The following PCR conditions were employed: 95°C for 2 minutes, followed by 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 69°C for 30 seconds and elongation at 72˚C for 1 minute and a final extension at 72 ˚C for 10 minutes using the forward: ATG GAG ATG CTG CAG GGC TCC GCA GGT GTG and reverse: ATG CTA GAC GTG CAG CAC CGC GGA primers. The downstream-primers contained an artificially introduced T3-promoter (GCA ATT AAC CCT CAC TAA AGG) at the 5`-end to enable synthesis of antisense transcripts. DNA was purified using (Thermo Scientific GeneJET PCR Purification Kit, #K0701) and stored at -20°C. The integrity of *dio3b* and *ttr* DNA was confirmed on a 1% agarose TAE electrophoretic gel (Fig. S1B) and DNA was stored at -20°C. DNA sequences were

obtained by Source Bioscience and verified using BLAST (NCBI) and Clustal Omega alignment software (EMBL-EBI; Fig. S2 and S3).

The purified DNA samples, from both vector and PCR methods, were used to synthesise RNA probes using digoxigenin (DIG) RNA labelling mix (Roche), transcription buffer x5 (Fermentas), RNAse inhibitor (Roche), water and the appropriate polymerase (T7, T3 or SP6) and incubated at 37°C for 5 hours. Probes were then treated with DNAse at 37°C for 1 hour and purified by lithium chloride precipitation. RNA probe integrity was confirmed by agarose gel electrophoresis (Fig. S4), an equal volume of hyper buffer was added and the probes stored at -20°C with subsequent use at 10ul in 1 ml.

2.3.7 WISH

Fixed embryos were rehydrated through as a series of methanol dilutions -75%, 50%, and 25% in PBS before being washed with PBS + 0.1% Tween20 wash (PBTw) for 10 minutes. Embryo-larvae were subsequently treated with protinease K: 48hpf stage for 22min (1x strength), 72 hpf stage for 30 mins (2x strength), 96-120 hpf stages for 40 mins (3x and 4x strength, respectively). Embryos were then washed repeatedly with PBTw to stop the digestion process, fixed for 1 hour in PFA and rinsed again in PBTw. A pre-hybridisation step was performed in which embryos were placed in hybridisation buffer for 3 hours and subsequently incubated with the appropriate probe (diluted 1:100) overnight at 65ºC. The embryos were then washed in 50% formamide 2XSSC, 0.1%Tween20 wash for 30-60 minutes at 65ºC, followed by a 2XSSC 0.1%Tween20 wash at 65ºC for 30 minutes and then 2x 30 minutes

wash at 65ºC with 0.2XSSC 0.1%Tween 20. Non-specific binding was reduced with the addition of blocking solution (Blocking solution+5%NGS) for 3 hours. Embryos were then incubated in anti-DIG antibody (Roche) (x5000, diluted 1/100 with blocking solution) for 3 hours at room temperature. A series of 4x 30minutes of PBTw washes were conducted before a 10 minute wash with AP buffer (Tris 0.1M pH 9.5, NaCl 0.1M, $MgCl_2$ 50mM, Tween20 0.1%). Embryos were transferred to a 24-well plate and placed in BM-Purple AP Substrate (Roche). Staining reactions were carried out at room temperature until signal or background staining became visible. Staining times varied depending on the probe and developmental stage tested (Table S2 and S3). Staining reactions were terminated by rinsing embryos in PBTw and fixing in fresh PFA. Embryos were observed and photographed using Nikon SMZ1500 microscope equipped with a digital camera.

2.3.8 Transcript profiling

qRT-PCR was used to quantify the transcript profiles, in whole zebrafish samples, of a number of target genes including: thyroid receptors α and β (*thraa* and *thrb*), thyroid-stimulating hormone (*tshb*), deiodinase enzymes type I, II and III (*dio1*, *dio2*, *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). Total RNA was isolated from whole homogenised zebrafish samples using Tri-Reagent following the manufacturer's instructions. The total RNA concentration was quantified using a NanoDrop spectrophotometer and purity estimated by obtaining an absorbance ratios (A260/A280 = 1.8-2.0 and A260/A230 = 2.0-2.2) for each sample. To remove genomic DNA contamination, RNA (0.5 µg/µl) was treated with 1 µg RQ1 RNase-

Free DNase (Promega, UK) and subsequently reverse transcribed to complementary DNA (cDNA) using random hexamers (Eurofins MWG Operon, Germany) and M-MLV reverse transcriptase (Promega), according to the manufacturer's instructions. Following reverse transcription, synthesised first strand cDNA was diluted 1:2 in HPLC water and stored at -20C until used for real-time qPCR.

Specific oligonucleotide primers for each of the selected genes in the HPT axis were designed according to the full length sequences published in GenBank and using Beacon Designer software (Table S4). Primers were purchased from Eurofin and diluted to working solutions of 10 µM. BioRad CFX96 Manager was used to optimise annealing temperatures (Ta) for selected primer pairs. Specificity was confirmed by melt curve analysis and the detection range, linearity and amplification efficiency (E) was established using serial dilutions of zebrafish larvae cDNA. In all experiments, cDNA samples were amplified in triplicate. A no-template control was included in every assay to screen for contamination of reagents. The mRNA levels of each target gene were calculated by the comparative CT method and normalised with an endogenous reference gene, ribosomal protein L8 (*rpl8*) [24]. The formula was as follows:

$$RE = (E\ Ref)^{Ct\ Ref} / (E\ Target)^{Ct\ Target}$$

where RE is relative gene expression, ref is the housekeeping gene, target is the gene of interest, and E is PCR amplification efficiency for that particular gene. Ontogeny results are shown as mRNA levels relative to *rpl8* mRNA, while T3 exposure results are expressed as fold changes relative to the control. In order to assess the stability of the housekeeping gene, *rpl8* CT values were compared across

each developmental stage and values for T3 treatments were compared with control values (Fig. S5 and S6).

2.3.9 Data analyses

Statistical analyses were conducted in R. Gene expression data was first scrutinised by Chauvenet's criterion to detect outliers for each gene and these were subsequently removed [25]. Difference in gene transcripts in the HPT axis across developmental stages were analysed using general linear models (GLM) with Gaussian error structures for each gene of interest. Minimum adequate models were derived by model simplification using F tests based on analysis of deviance. When a significant effect was identified, pairwise comparisons to determine which groups differed were conducted using Tukey's honest significant difference (HSD) post hoc test. A similar approach of model simplification of general linear models was used to examine the effect of T3 on gene transcripts. The differences between the proportion of mortalities and deformities following exposure to T3 were assessed using generalised linear models with binomial error structures. Minimum adequate models were derived by model simplification using Chisq tests based on analysis of deviance. F and Chisq tests refer to the significance of removing terms from the models. All statistical models were checked for homoscedasticity and normality of residuals and data was log-transformed where necessary to ensure linearity. For all statistical analyses, differences were considered significant at $p<0.05$.

2.4 Results

2.4.1 Developmental expression of genes in the HPT axis of zebrafish

The ontogeny studies showed that expression levels of the two TR genes (*thraa* and *thrb)* differed from each other both temporally and spatially during the early stages of zebrafish development. Via qRT-PCR *thraa* and *thrb* transcripts were detected throughout embryogenesis and early larval life stages, but there were varying patterns of expression. At 24 hpf, *thraa* mRNA levels were relatively low but they increased by 3.3- fold at 48 hpf ($p<0.001$), with no further increase at 72 hpf. Thereafter (96 and 120 hpf), *thraa* mRNA levels, though elevated, were significantly lower than 72 hpf ($p<0.01$; Fig. 1A). At 24 hpf, *thraa* signalling was detected ubiquitously in the brain and head region (Fig. 1B). Subsequently, at 48 hpf and 72 hpf, *thraa* expression signals were observed in the branchial arches of the head, pectoral fins and throughout the brain (Fig. 1C-D). Expression in these tissues continued between 96 hpf and 120 hpf, with additional and/or enhanced signalling detected in the cartilage of the lower jaw, intestinal tract and brain (Fig. 1E and 1F).

Levels of *thrb* transcripts were higher than for *thraa* transcripts throughout all of the developmental stages tested ($p<0.01$; Fig. S7A). *Thrb* transcripts were at their lowest level at 24 hpf and there was a progressive increase (by 2- fold) up to 72 hpf ($p<0.001$). Thereafter (96 and 120 hpf) *thrb* transcripts remained stable (Fig. 2A). *Thrb* expression was seen in the brain throughout all of the developmental stages tested. At 24 hpf a highly localised *thrb* signal was seen in lateral tissues of the midbrain-hindbrain boundary immediately caudal to the eyes and in the prospective fin buds (Fig. 2B-Bi). In addition, signalling was detected in a small group of cells in

the region of the otic vesicle at life stages between 24-120 hpf (Fig. 2B-E). At 48 and 72 hpf embryo-larvae, *thrb* was expressed in the pectoral fins and/or pronephric ducts (Fig. 2C-D). In 96 and 120 hpf larvae, *thrb* was expressed in the liver and cartilage of the lower jaw (Fig. 2E-F)

The mRNAs encoding all three of the deiodinase enzymes (*dio1, dio2* and *dio3b*) were also found to be spatially and temporally regulated during early zebrafish development. *Dio1* mRNA levels were low at 24 hpf and increased significantly at 48 hpf, (by 2- fold; $p<0.001$) and again at 72 hpf (by a further 2-fold; $p<0.01$). Between 96 hpf and 120 hpf *dio1* transcription increased significantly again by a further 3- and 1.5- fold respectively ($p<0.001$; Fig. 3A). During the embryonic stages of development, *dio1* was not seen to be well localised in any specific tissue, as seen by WISH (Fig. 3B-C), but there was a very weak *dio1* signal detected in the brain (Fig. 3C). At 96 hpf, however, *dio1* was expressed strongly in the brain, liver, intestinal bulb and pronephric ducts (Fig. 3D) and this expression pattern persisted at 120 hpf, with additional expression observed in intestine (Fig. 3E).

At 24 hpf, levels of *dio2* mRNA were 3- fold lower than for *dio1* transcripts ($p<0.001$; Fig. S7B). Transcription of *dio2* increased significantly at 48 hpf ($p<0.001$), with mRNAs elevated by 8- fold, with a further elevation at 72 and 96 hpf (by a further 2-fold; $p<0.001$). At 120 hpf, *dio2* mRNA levels further increase ($p<0.05$: Fig. 4A). *Dio2* signalling was detected by WISH in the brain and pituitary gland of zebrafish at 48 hpf (Fig. 4B) and between 72 hpf and 120 hpf, additionally in the liver, pronephric ducts, intestinal bulb and intestine (Fig. 4D-F).

Throughout all of the developmental stages tested (24 hpf to 120 hpf), *dio3b* mRNA levels were significantly higher than for *dio1* and *dio2* ($p<0.001$; Fig. S7B). The first significant change detected in *dio3b* mRNA was at 72 hpf, where mRNA was elevated by 2.5- fold above those at 24 and 48 hpf. By 96 and 120 hpf, *dio3b* mRNA levels had increased by approximately 4.5- fold ($p<0.001$). A strong *dio3b* signal was detected initially by WISH in the pronephric ducts of 24 hpf embryos (Fig. 5A), but this signal weakened in 48 and 72 hpf embryo-larvae (Fig. 5B-D). At 72 hpf *dio3b* expression was also detected in the prospective liver and brain (Fig. 5D). In 96 and 120 hpf larvae a strong *dio3b* expression signal was detected in the liver and yolk syncytial layer (Fig. 5E-F).

At 24 hpf, *ttr* mRNA levels were relatively low and transcript levels decreased at 48 hpf ($p<0.05$; Fig. 6A). In 24 and 48 hpf embryos, no tissue-specific *ttr* staining pattern was observed (Fig. 6B-C). Transcription of *ttr* increased significantly at 72 hpf and 96 hpf ($p<0.001$). At 120 hpf, *ttr* RNA levels remained high but no further increases were observed. WISH data revealed a strong *ttr* expression signal in the left lobe of the liver at 72 hpf (Fig. 6C), with a greater and more expansive level of expression in the liver at 96 and 120 hpf (Fig. 6D-E).

C*rhb* mRNA levels were low at 24 hpf but were elevated at 48 hpf (by 14- fold; $p<0.001$) and increased further at 72 and 96 hpf ($p<0.001$ and $p<0.05$ respectively; Fig. 7A). At 120 hpf, *chrb* levels remained elevated but stable. The levels of *tshb* mRNA were lowest at 24 hpf, increasing significantly up to 96 hpf (by 26- fold; $p<0.001$) with levels at 120 hpf remaining elevated but reduced compared to 48 and 72 hpf stages ($p<0.05$) (Fig. 7B). WISH failed to detect *tshb* signalling in zebrafish

embryo-larvae between 24 and 96 hpf, but at 120 hpf *tshb* signal was observed in the pituitary gland of a limited number of larvae (<5%) (Fig. S8A-E). *Pax8* mRNA levels were higher at 48 and 72 hpf (by 2.7- and 1.9- fold) compared to levels at 24 hpf ($p<0.01$), with reduced levels observed at 96 and 120 hpf ($p<0.01$; Fig. 7C). *Ugt1ab* mRNA levels were higher at 48 hpf compared to 24 hpf (by 1.5- fold; $p<0.05$) and increased significantly at 72 and 96 hpf ($p<0.001$; Fig. 7D). At 120 hpf, *ugt1ab* levels remained elevated but stable.

2.4.2 Acute toxicity of T3

T3 had no significant effect on mortality rates in the whole dose range tested (10-300 μg L^{-1}) compared with the controls (Fig. 8Ai), therefore no 96-LC-50 value was calculated. The number of zebrafish larvae presenting morphological deformities increased significantly in eggs exposed to 30 μg T3 L^{-1} (61.55 ± 8.95%), 100 μg T3 L^{-1} (73.88 ± 8.37%) and 300 μg T3 L^{-1} (92.87 ± 2..73%) and a dose-response relationship was observed ($p<0.05$; Fig. 8Aii). At 72 hpf and 96 hpf, larvae exposed to these higher T3 concentrations presented with deformities such as oedema, curved spine, reduced pigmentation, swollen yolk sac, small eyes and craniofacial deformities (Fig. 8B-C). The lowest-observable-effect concentration (LOEC) in terms of deformities in zebrafish embryo-larvae after 96 hours was 30 μg T3 L^{-1}.

2.4.3 T3 treatment alters gene transcription in the HPT axis

The effects of T3 exposure were quantified at 48, 96 and 120 hpf via qRT-PCR and by WISH at specific developmental stages (*thraa* and *thrb* at 48 hpf and 120 hpf, *dio1*, *dio2*, *dio3b* and *ttr* at 48 hpf and 96 hpf) as determined by the ontogeny

studies. Exposure to exogenous T3 resulted in a 2-3 fold higher level of *thraa* mRNA at the developmental life stages studied with no differences between the different T3 treatment levels (30 µg L^{-1} vs 100 µg L^{-1}) ($p<0.001$; Fig. 9A). After a 48 hour exposure, T3 (30 and 100 µg L^{-1}) induced *thraa* mRNA in the pineal gland, pectoral fins, branchial arches, pronephric ducts, pronephros, brain and the somites compared with controls (Fig. 9B-D). After 120 hours, exposure to 30 and 100 µg T3 L^{-1} resulted in a strong (and concentration related) signal induction for *thraa* in the pineal gland, lower jaw cartilage, liver, pectoral fins and intestine and an enhanced expression in the brain compared to a weak signal in the brain of control larvae (Fig. 9E-G).

At all of the developmental stages tested, the transcription of *thrb* was significantly elevated by exogenous exposure to T3 (Fig. 10A). *Thrb* levels were 2- fold, 3- fold and 4.9- fold higher in T3 treated groups at 48 hpf ($p<0.05$), 96 hpf ($p<0.001$) and 120 hpf ($p<0.001$) than in controls, respectively. In addition, there were no differences in mRNA levels between the T3 treatments at any of the developmental stages tested. At 48 hpf, *thrb* was up-regulated in the brain (Fig. 10B-D) and at 120 hpf in the lower jaw and intestine. Within the brain tissues, there was a pronounced effect of T3 on the hindbrain. At 120 hpf *thrb* expression appeared reduced in the liver of 100 µg T3 L^{-1} treated individuals compared to controls (Fig. 10E-G).

There were no effects of T3 on the expression of *dio1* at 48 hpf, but there was a concentration-dependent suppressive effect at both 96 hpf and 120 hpf ($p<0.001$; Fig. 12A). Similarly at 48 hpf, no change in the expression pattern of dio1 was observed in WISH assays following T3 treatments (Fig. S9A-C). At 96 hpf *dio1*

expression occurred in the liver, intestinal bulb and swim bladder, and also in the brain and jaw cartilage (Fig. 11B). Exposure to T3 (30 and 100 µg L^{-1}) resulted in a reduced *dio1* expression in the liver, swim bladder, intestinal bulb and jaw cartilage compared with controls (Fig. 11C). At 100 µg T3 L^{-1}, *dio1* expression was also lower in the brain (Fig. 11D).

For *dio2* at 48hpf there were no effects of T3 exposure on whole body transcript levels as determned by both qRT-PCR (Fig. 12A) or on expression in the brain as determined by WISH (Fig. 12B-D). However T3 treatment resulted in a reduced *dio2* expression in the pituitary gland. In the control groups, *dio2* expression was observed in the pituitary of 96% of embryos, but in only 65% and 48% of embryos treated with 30 and 100 µg T3 L^{-1}, respectively and any observed staining in the pituitary of embyros was reduced visually in these T3 treatments (Fig. 12Bi-Di). At 96 hpf exposure to T3 resulted in a reduced level of *dio2* as assesed by qRT-PCR ($p<0.01$; Fig. 12A). Similar to 48 hpf embryos, *dio2* signalling in the pituitary was also suppressed by T3 in 96 hpf larvae. *Dio2* signalling was detected in the pituitary of 54% of control individuals compared to only 4% and 0% of individuals treated with 30 and 100 µg T3 L^{-1}, respectively (Fig. 12Ei-Gi). There was also a reduced *dio2* signalling in the liver, intestinal bulb, intestine and brain of larvae (Fig. 12E-G).

T3 caused a significant induction of *dio3b* transcription in the whole embryo-larvae for all developmental stages tested ($p<0.001$). At 48 hpf and 96 hpf, mRNA levels were approximately 4.5- fold higher in both T3 treated groups relative to controls. At 120 hpf mRNA levels were further elevated by 7- and 10- fold in the 30 and 100 µg T3 L^{-1} groups, respectively (Fig. 13A). At 48 hpf a strong *dio3b* induction was

observed in the pectoral fins and the pronephric ducts in T3 treated animals (Fig. 13B-G). At 96 hpf, T3 (30 and 100 µg T3 L^{-1}) treatment resulted in a strong induction of *dio3b* expression in the brain, lower jaw cartilage, yolk syncytial layer, the caudal fin and pectoral fins, and an apparent reduction in expression in the liver (Fig. 13H-J).

At 48 and 120 hpf, exposure to exogenous T3 had no effect on whole body *ttr* levels (Fig. 14A). At 96 hpf, whole body levels of *ttr* mRNA appeared reduced but this was not statisically significant (Fig. 14A). However hepatic expression of *ttr* appeared suppressed in whole mount expression samples (Fig. 14B-D). At 48 hpf *ttr* tissue localisation was not detected using WISH (Fig. S9D-F).

Treatment with T3 (100 µg L^{-1}) significantly increased *crhb* mRNA levels at 96 hpf ($p<0.01$) but no effect was observed at 48 and 120 hpf (Fig. 15A). At 48 hpf, T3 treatment (30 and 100 µg L^{-1}) significantly reduced *tshb* transcript levels ($p<0.05$), though no effect at 96 and 120 hpf (Fig. 15B). For *pax8*, after 48 hours, treament with T3 (30 µg L^{-1}) resulted in enhanced mRNA levels ($p<0.05$), but no treatment related effects were detected in subsequent life stages (96 hpf and 120 hpf; Fig. 15C). For *ugt1ab*, T3 (100 µg L^{-1}) significantly suppressed transcription after 120 hpf ($p<0.05$) but no effect was observed at 48 and 96 hpf (Fig. 15D).

2.5 Discussion

Low but physiologically relevant levels of maternally derived THs are deposited in developing fish eggs and all the genes required for TH signalling are expressed early

in zebrafish embryonic development [15, 26, 27]. In order to understand the role of many of the key components of the thyroid axis during early fish development, an understanding of their regional and developmental patterns of expression is needed and yet this remains largely undefined. Here we assessed the ontogeny and tissue specific expression patterns of genes in the HPT axis of zebrafish and examined the transcriptional regulation of these genes by T3 at selected developmental stages.

2.5.1 Developmental expression of TR mRNAs in zebrafish embryo-larvae

The data we obtained from qRT-PCR and WISH assays confirmed the differential expression of the TR-encoding genes, *thraa* and *thrb*, in a spatio-temporal manner during early zebrafish development. Consistent with previous findings, we found that transcripts for both *thraa* and *thrb* were detected at relatively low levels in 24 hpf zebrafish embryos [28]. The transcription of both receptors subsequently increased between 48-72 hpf, a period known to correspond with the development of fully functioning thyroid follicles and the embryonic to larval transitory phase [28, 29]. Throughout all of the developmental stages examined the *thraa* transcript levels were significantly lower than *thrb* levels, consistent with previous studies [28]. *Thraa* transcripts appear to be the predominant TR in the early phase of zebrafish embryogenesis (up to ~ 5 hpf) [30].

An important element of our study involved examining the expression pattern of *thraa* and *thrb* transcripts in the later stages of embryogenesis and larval development. WISH revealed the co-expression of *thraa* and *thrb* in several zebrafish tissues, including the brain (24-120 hpf), pronephric ducts, (48-72 hpf),

liver (72-120 hpf), pericardia (72 hpf), craniofacial tissue (120 hpf) and pectoral fins (72 hpf) raising the possibility of co-operative roles in mediating the actions of THs in these tissues at distinct developmental stages. Consistent with our findings, previous studies have observed the expression of both TRs in the brain of zebrafish [31, 32] and aligns with the well-known role of THs during vertebrate brain development [9]. Similarly our results are consistent with the role of THs in influencing the development of the fin (pectoral, pelvic and dorsal) and cranial skeletal tissues in several fish species [33-35]. The specific role and target genes of the different TRs in these tissues are however not yet fully understood. For example, while TRs are co-expressed in osteoblasts and growth plate chondrocytes of several mammal bones, including limb bones [36], TRα has been shown to be functionally dominant during mice skeletal development [37, 38]. Whether this is the also the case during fish development remains to be determined. Again both receptors are required for the correct differentiation of the mammal cerebellum [39, 40], but TRα1 and TRβ1 are differentially expressed in the granular and Purkinje cell layers, respectively [41]. Future work should focus on determining the regional expression patterns of TRs at the cellular level.

The otic vesicles/inner ears are responsible for hearing, balance and sensing acceleration in vertebrates [42]. Interestingly, we detected a specific *thrb* expression domain in the otic vesicles of zebrafish embryo-larvae, suggesting a role for THs in the development of this tissue. Similarly, TRβ2 has been shown to mediate the effect of THs on auditory functions in mammals influencing cellular differentiation in the cochlea (inner ear), while TRα also made minor contributions [43]. Here, however, *thraa* was not detected in the otic vesicle of the developing zebrafish. Either *thraa*

expression levels were extremely low and consequently not detected by WISH, or perhaps the mechanisms of TH action in the otic vesicles of zebrafish are mediated principally by TRβ receptors. THs have also long been suggested to be a regulator of intestinal development [44-46]. Indeed the TH-dependent intestinal remodelling during amphibian metamorphosis is well characterised [47]. We detected a specific *thraa* expression domain in the intestinal bulb of zebrafish larvae (96-120 hpf), consistent with the role of TRα isoforms in meditating T3-dependent functions in intestinal epithelial cells of rodents [46].

2.5.2 Developmental expression of deiodinase mRNAs in zebrafish embryo-larvae

The deiodinase enzymes play a key role in regulating both the systemic and peripheral levels of THs in vertebrates by selectively removing iodide from T4 and its derivatives, thus activating or inactivating these hormones [11]. In zebrafish embryo-larvae we found that the transcript levels of *dio1*, *dio2* and *dio3b* mRNA*s* varied with developmental stage and *dio3b* levels were consistently high relative to *dio1* and *dio2* levels. This pattern is consistent with D3's role in deactivating THs and the largely accepted view that D3 shields tissues against high levels of THs (both maternal and zygotic) during vertebrate development [48]. While the abundance of *dio3b* mRNAs during early life stages of fish has been examined previously (using pooled embryo-larval samples) [48], relatively little is known about the tissue-specific *dio3b* expression patterns. Here using WISH, we observed d*io3b* expression in the pronephric ducts (24-72 hpf), liver (72-120 hpf) and brain (48-72 hpf) of zebrafish embryo-larvae. Similar to our findings Dong *et al.,* (2013) observed *dio3b* expression in the pronephric ducts and brain of zebrafish embryos from the 6 somite stage to 24

hpf [49]. D3 proteins in the liver and pronephric ducts are likely involved in controlling systemic TH levels, while the expression of *dio3b* in the brain suggests D3 regulates local T3 levels during distinct neurodevelopmental windows. Indeed, D3 has recently been demonstrated to play a significant role in multiple elements of embryonic development including muscle, liver and intestine development, swim bladder inflation and general growth rates [48].

D2 proteins are considered to be the major TH-activating enzymes, catalysing the conversion of T4 to the bioactive T3 by ORD and thereby maintaining TH homeostasis at a systemic and local level [10]. Though D1 proteins are capable of activating and deactivating THs (by ORD and IRD, respectively), recently it has been proposed that the primary role of D1 in fish is to activate THs [50]. Nevertheless, there has been much debate over the importance of D1 in catalysing the conversion on T4 to T3 in fish [10, 51, 52]. D1 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 [53, 54]. Interestingly, here we found *dio1* transcript levels were often higher than *dio2* transcript levels in zebrafish embryo-larvae (24, 96 and 120 hpf), suggesting D1 proteins might well have a significant role in maintaining TH homeostasis. Indeed we observed that the *dio1* and *dio2* expression domains overlapped in zebrafish larvae, consistent with the findings of previous studies [49]. At 96 and 120 hpf, *dio1* and *dio2* were co-expressed in the liver, pronephric ducts, intestinal bulb, intestine and brain of larvae, [49]. Similar to the findings of Walpita *et al.,* (2010), our results suggest that D1 and D2 proteins co-operate to control systemic T3 levels and/or local T3 availability during development [54]. It should be noted however that we did

also observe a distinct *dio2* expression pattern in the pituitary gland of zebrafish embryo-larvae (48-120 hpf), suggesting that D2 alone regulates the availability of T3 in the pituitary and thereby influences the negative feedback loop of the central HPT axis.

2.5.3 T3 regulates TR transcription in zebrafish embryo-larvae

THs regulate a variety of cell functions by facilitating or inhibiting gene transcription, yet little is known about TH's regulation of genes in the HPT axis of teleost fish during early life stages. Here we examined the tissue and developmental stage specific regulation of a suite of thyroid related genes by T3. Exogenous T3 elevated *thraa* and *thrb* mRNA levels in whole body zebrafish embryo-larvae samples, reflecting the presence of TREs in their promotor regions, with *thrb* transcription more sensitive to T3 exposure than *thraa*. In agreement with our findings, Liu *et al.,* (2000) showed that T3 (5 nM) up-regulated the expression of TRα1 and TRβ1 in zebrafish embryo-larvae after 72 and 144 hour exposures post fertilisation, with TRβ1 more responsive than TRα1 [30]. Interestingly a study by Walpita *et al.,* (2007) found the transcription of *thrb* to be unresponsive to T3 (5nM) in zebrafish embryos exposed up to 75 hpf [26]. The same study also found that *thraa* transcription was unresponsive to T3 when exposed between 8-36 hpf and at 75 hpf, but mRNA levels increased at 48 hpf [26]. A study with sea bream larvae (*Sparus aurata*) found that T3 treatment (via the diet) did not alter TRα expression at any time point during the experiment (after 7, 18 or 31 days of treatment), though TRβ expression was up-regulated after 18 days but was not effected after 7 or 31 days of treatment [55]. Taken together these results suggest that the transcription regulation of TRs by T3 in

zebrafish early life stages may be stage specific. Indeed here, though T3 induced *thrb* transcription across all developmental stages, the effect was more pronounced at 96 hpf compared to 48 and 120 hpf (6- fold increase compared to 2- and 4- fold increases, respectively). Conversely, the induction of *thraa* by T3 was relatively constant across developmental stages, with mRNA levels consistently elevated by ~2.5- fold compared to controls. Since zebrafish larvae at 96 hpf have recently undergone the embryonic-larval transition, a TH-dependant metamorphic event associated with increased endogenous TH, this may explain the observed differences in *thrb* responsiveness to T3 at this stage. It also raises the possibility that TH signalling, mediated by TRβ receptors, at 96 hpf (coinciding with embryo-larval transition) may be particularly vulnerable developmental window in terms of altered TH status [55].

Not only does T3 display a differential regulation of TRs across early developmental stages but also appears to dynamically regulate their expression across different tissues. Indeed we have shown that 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), *thraa* and *thrb* expression in the brain was particularly sensitive to T3 exposure. In addition, we found that *thraa* and *thrb* expression was markedly induced by T3 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, examined TH-regulation of TR transcripts in a limited number of tissues in adult vertebrates, including fish. For instance exogenous T3 elevated both TRα and TRβ expression in the liver and brain

of adult fathead minnows (*Pimephales promelas*), and elevated TRβ transcripts in the gonads [56]. Interestingly, in adult goldfish (*Carassius auratus*), T3 down-regulated TRα1 and TRβ expression in the gonads [57]. Meanwhile, T3 exposure increased TRαA and TRβ transcripts in the liver (males) and brain (males and females) of striped parrotfish (*Scarus iseri*), while TRαA and TRαB were also up-regulated in the gonads (males) [58]. Taken together these results suggest that fish exhibit tissue-specific TR transcriptional changes in response to T3 exposure and that alterations are dynamic, with discrepancies observed between species, sexes and life stages.

Here we observed the induction of *thraa* by T3 in the pineal gland of zebrafish embryo-larvae. To our knowledge, this is the first demonstration of *thraa* induction by T3 in the pineal gland of teleost fish. The pineal gland is the site of melatonin production and influences the light–dark rhythm in most vertebrates including fish [59]. Apart from its involvement in circadian rhythm, melatonin has also been implicated in regulating smoltification, reproduction and the immune system in fish [60, 61]. Early studies on catfish found a potential role of the pineal gland in controlling thyroidal uptake of iodine and circulating levels of THs [62, 63]. Interesting, administration of melatonin to catfish (*Clarias batrachus)* decreased plasma levels of both T3 and T4 [62]. The induction of *thraa* in the pineal gland of zebrafish embryo-larvae as observed here suggests a relationship between the pineal gland and the thyroid system. Perhaps the pineal gland and/or melatonin play a role in regulating TH synthesis as a compensatory response to exogenous T3 exposure in teleost fish. If this is the case it would be interesting to examine whether

altered pineal gland signalling may be a mechanistic pathway for thyroid disruption or may in fact be a target of thyroid disruption by environmental chemicals.

2.5.4 T3 regulates deiodinase transcription in zebrafish embryo-larvae

Our results provide a comprehensive examination of TH-mediated deiodinase transcriptional regulation in zebrafish embryo-larvae and indicate that zebrafish exhibit tissue- and developmental stage-specific changes in deiodinase expression patterns following T3 treatment. Here, *dio1* mRNA levels were unaffected by T3 after 48 hours, but were suppressed after 96- and 120- hour exposures, specifically in the craniofacial tissue, brain, liver and gastrointestinal tract. Previous studies examining the effect of T3 on deiodinase expression in larval and adult fishes have found mixed results. For example, T3 treatment reduced *dio1* transcript levels in whole sea bream larval samples [55] and in the livers of adult killifish (*Fundulus heteroclitus*) [64]. Conversely, no effect of T3 treatment was observed in whole zebrafish embryo samples [26] or in the gonad, liver and brain of adult striped parrot fish [58]. As far as we are aware, our findings are the first demonstration of tissue-specific regulation of *dio1* expression by THs in fish early life stages. It is important to note, however, that in contrast to the T3- mediated suppression of *dio1* mRNA levels observed here across multiple zebrafish tissues, in mammals TH exposure is associated with increased *dio1* transcription [10, 13, 65]. Therefore the mechanisms for teleost *dio1* regulation appears to be divergent from that of mammalian *dio1* regulation, emphasising the importance of understanding the thyroid system of fish, particularly since there is a growing interest in using small teleost fish model species in ecotoxicology research into TDCs.

Here we found that T3 exposure suppressed *dio2* transcript levels in 96 hpf zebrafish larvae. Indeed several previous studies have found that hyperthyroidism, induced by T3 administration, reduced *dio2* mRNA levels/D2 activity in the liver, brain, gonads or whole body samples of various teleost fish including zebrafish [26], sea bream [55], rainbow trout [66, 67], striped parrotfish [58] and killifish [64]. Here, we additionally demonstrated that T3 suppressed *dio2* expression across multiple tissues in zebrafish embryo-larvae, including the pituitary, liver, brain, gastrointestinal tract and craniofacial tissues. These results are consistent with D2's functional role in converting T4 into T3 and support the idea that changes in *dio2* transcript levels help to regulate T3 status in peripheral tissues. It is also important to consider that the reduced *dio2* expression in the pituitary may be a compensatory mechanism aimed at regulating the central HPT axis. The pituitary gland is the site of thyroid-stimulating hormone production and given the negative-feedback mechanism regulating this production, reduced conversion of T4 to T3 (as a result of reduced D*2* activity/ levels) in the pituitary may serve to increase local T4 levels and therefore inhibit TH release by the thyroid follicles.

Dio3b expression in zebrafish embryo-larvae was highly responsive to TH treatment at all stages tested here. Similar results were observed in other fish species such as striped parrotfish *(Scarus iseri)* [58], trout (*Oncorhynchus mykiss*) [66] and sea bream (*Sparus aurata)* [55]. The overall response pattern of *dio3b* to increasing TH levels is consistent with its proposed function of protecting tissues from high levels of TH as mentioned previously. The induction of *dio3b* was observed in the jaw cartilage, pectoral fins and brain in 96 hour larvae, suggesting these tissues are particularly sensitive to elevated levels of THs at this stage of development.

Interestingly *dio3b* expression was down-regulated in the liver following T3 treatment, perhaps demonstrating the switch from systemic to local TH inactivation.

In summary, the present data demonstrates the differential expression patterns of several key genes in the HPT axis of zebrafish embryo-larvae across multiple tissues and developmental stages. In addition, this study showed that T3 regulated the expression of several TH-related genes during zebrafish embryonic-larval life stages, with early developmental windows and multiple target tissues vulnerable to altered TH signalling. Taking into consideration the fact that various environmental chemicals are capable of altering circulating TH levels in fish and other vertebrates, these data suggests that multiple TH-target tissue and/or TH-dependent processes may be susceptible to the effects of thyroid disrupting chemicals.

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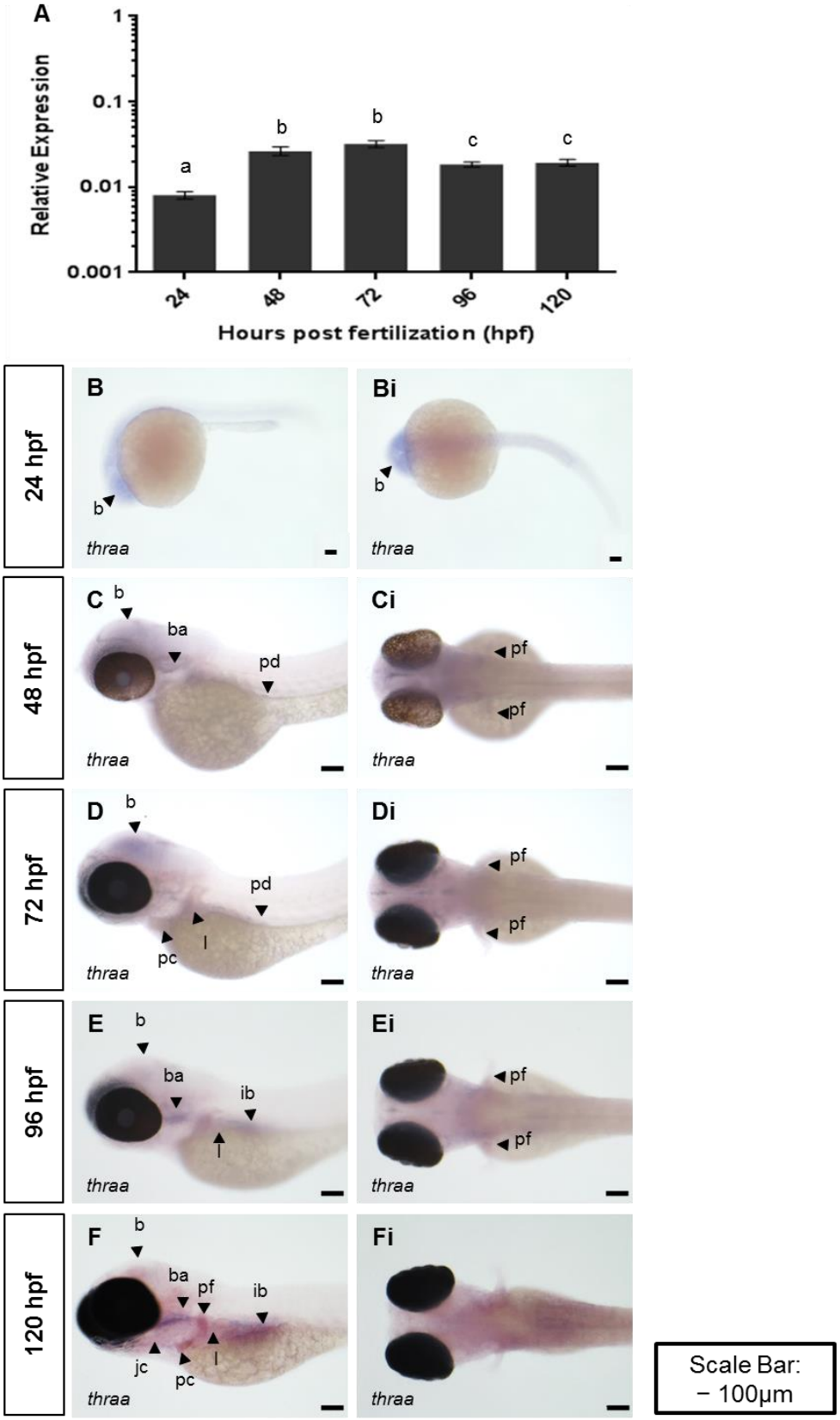
Figures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Relative Expression
1
0.1
0.01
0.001
a
b
b
c
c
24
48
72
96
120
Hours post fertilization (hpf)
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
B
Bi
C
Ci
D
Di
E
Ei
F
Fi
b
ba
pd
pf
l
pc
ib
jc
thraa
Scale Bar:
– 100µm

Figure 1. (A) Transcript profile of thyroid receptor alpha (*thraa)* in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 7-9 homogenised pooled samples per developmental stage. Different letters indicate significant differences between developmental stages (Tukey's HSD post hoc test; $p<0.05$). (B-F) Tissue expression pattern of *thraa* mRNA in zebrafish embryo-larvae between 24-120 hpf. Images are representative of three experiments (B, n=32; C, n=32; D, n=21; E, n=30; F, n=29). Lateral (B-F) and dorsal (Bi-Fi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. ba=branchial arches, pf=pectoral fins, b=brain, l=liver, pc=pericardia, jc=jaw cartilage, ib=intestinal bulb, pd=pronephric ducts. Scale bar=100 μm.

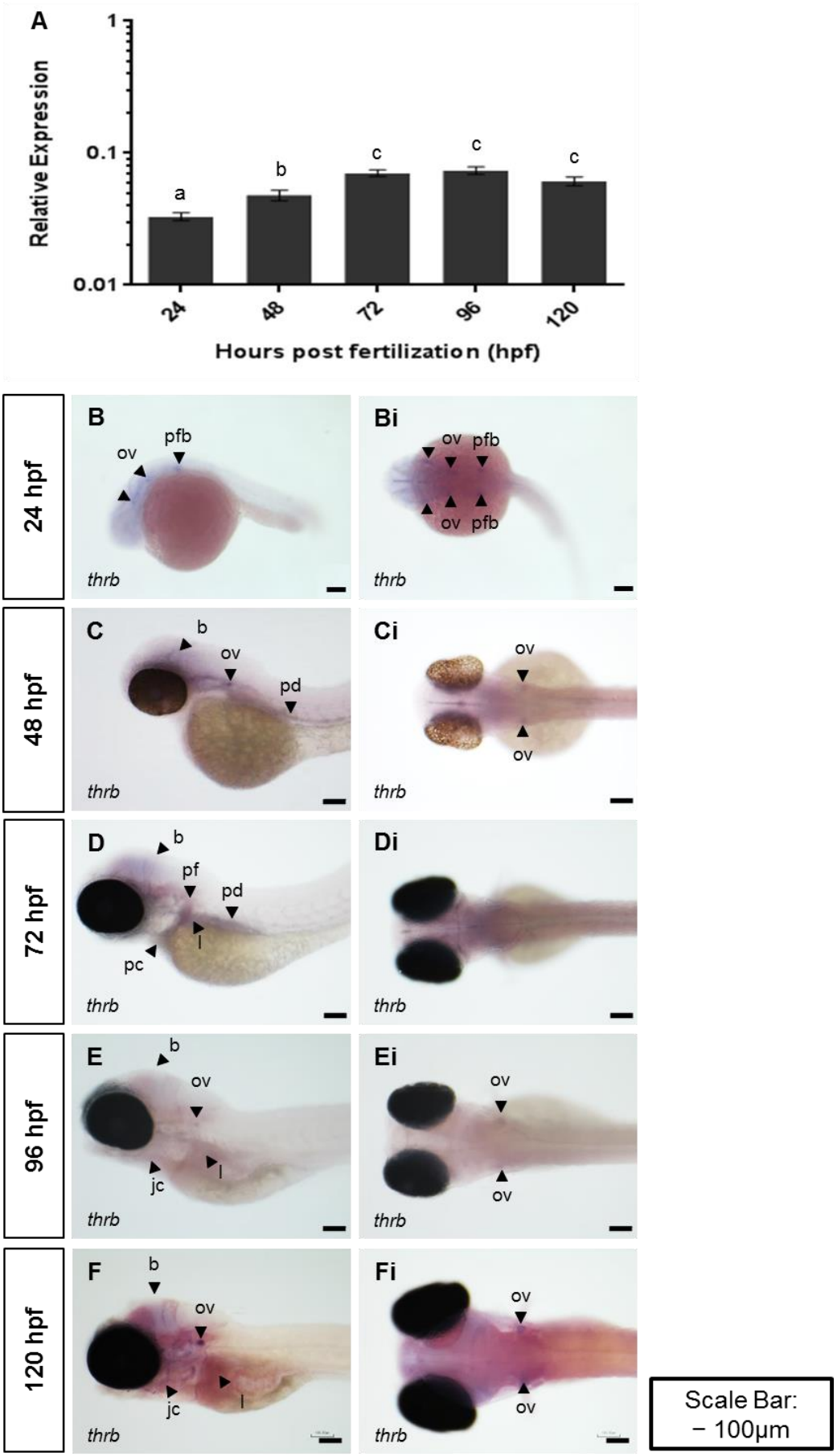
A
Relative Expression
1
0.1
0.01
a
b
c
c
c
24
48
72
96
120
Hours post fertilization (hpf)
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
B
Bi
C
Ci
D
Di
E
Ei
F
Fi
ov
pfb
b
pd
pf
l
pc
jc
thrb
Scale Bar:
− 100µm

Figure 2. (A) Transcript profile of thyroid receptor beta (*thrb)* in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 7-9 homogenised pooled samples per developmental stage. Different letters indicate significant differences between developmental stages (Tukey's HSD post hoc test; $p<0.05$). (B-F) Tissue expression pattern of *thrb* mRNA in zebrafish embryo-larvae between 24-120 hpf. Images are representative of three experiments (B, n=27; C, n=34; D, n=22; E, n=26; F, n=27). Lateral (B-F) and dorsal (Bi-Fi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, l=liver, jc=jaw cartilage, ov=otic vesicle, pfb=prospective fin bud, pc=pericardia, pd=pronephric duct. Scale bar=100 μm.

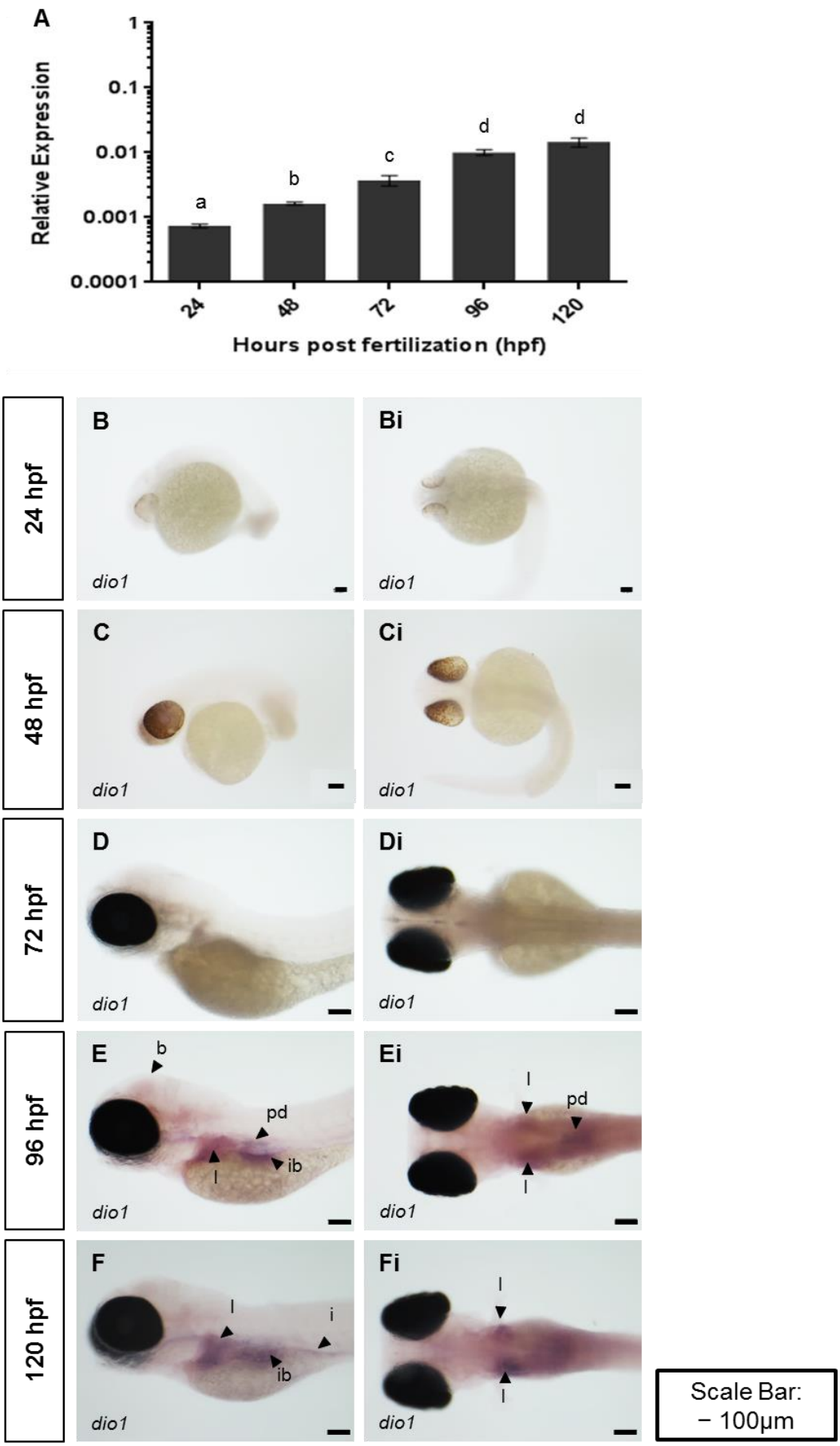
A
Relative Expression
1
0.1
0.01
0.001
0.0001
a
b
c
d
d
24
48
72
96
120
Hours post fertilization (hpf)
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
B
Bi
C
Ci
D
Di
E
Ei
F
Fi
dio1
b
pd
ib
l
i
Scale Bar:
– 100µm

Figure 3. (A) Transcript profile of deiodinase type I (*dio1*) in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per developmental stage. Different letters indicate significant differences between developmental stages (Tukey's HSD post hoc test; $p<0.05$). (B-F) Tissue expression pattern of *dio1* mRNA in zebrafish embryo-larvae between 24-120 hpf. Images are representative of three experiments (B, n=24; C, n=28; D, n=19; E, n=34; F, n=38). Lateral (B-F) and dorsal (Bi-Fi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, l=liver, p=pronephric ducts, ib=intestinal bulb, i=intestine. Scale bar=100 µm.

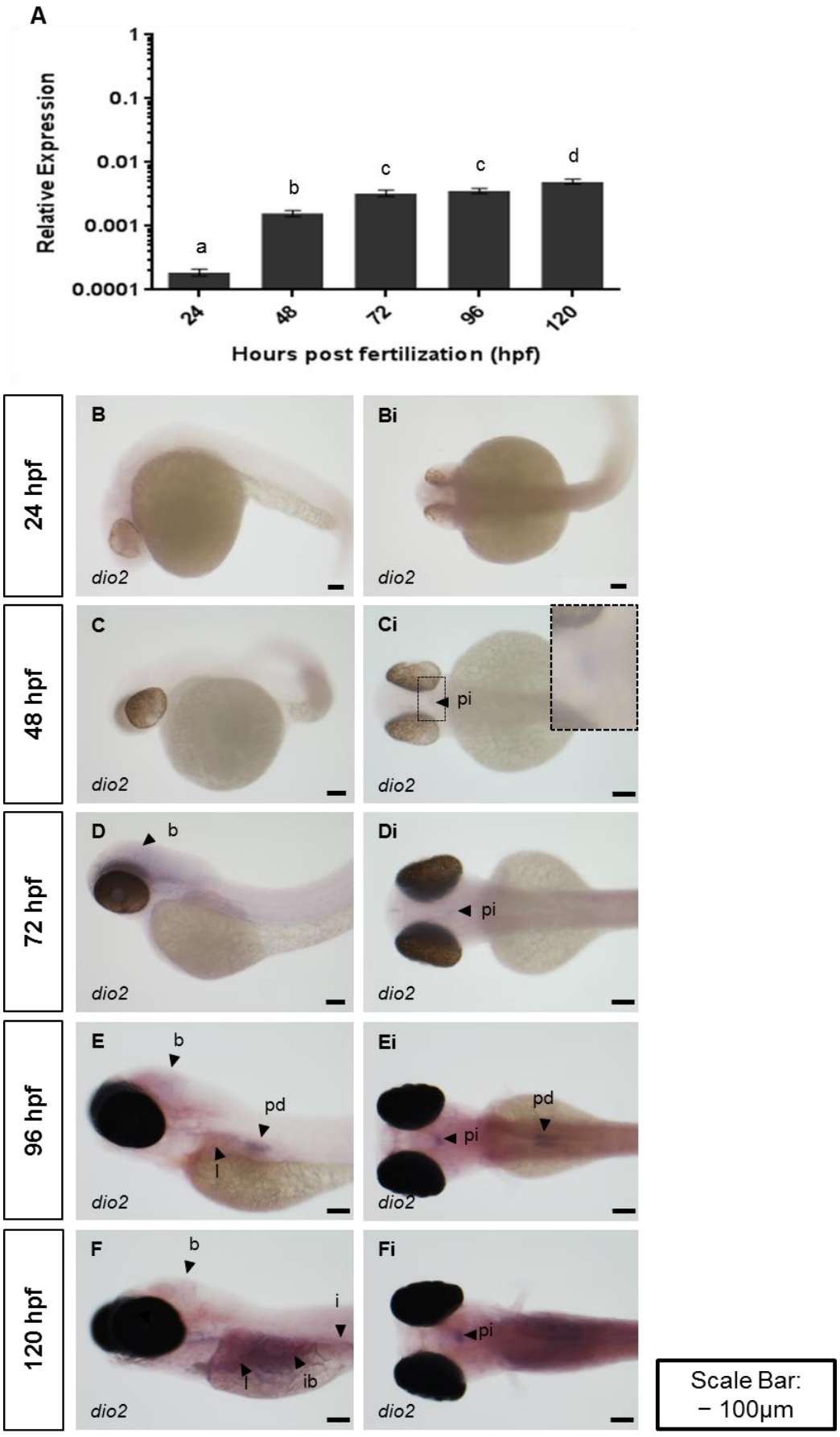
A
Relative Expression
1
0.1
0.01
0.001
0.0001
a
b
c
c
d
24
48
72
96
120
Hours post fertilization (hpf)
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
B
Bi
C
Ci
D
Di
E
Ei
F
Fi
dio2
b
pi
pd
l
i
ib
Scale Bar:
– 100µm

Figure 4 (A) Transcript profile of deiodinase type II (*dio2*) in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per developmental stage. Different letters indicate significant differences between developmental stages (Tukey's HSD post hoc test; $p<0.05$). (B-F) Tissue expression pattern of *dio2* mRNA in zebrafish embryo-larvae between 24-120 hpf. Images are representative of three experiments (B, n=31; C, n=29; D, n=28; E, n=30; F, n=31). Lateral (B-F) and ventral (Bi-Fi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, l=liver, p=pronephric ducts, ib=intestinal bulb, i=intestine, pi=pituitary. Scale bar=100 µm.

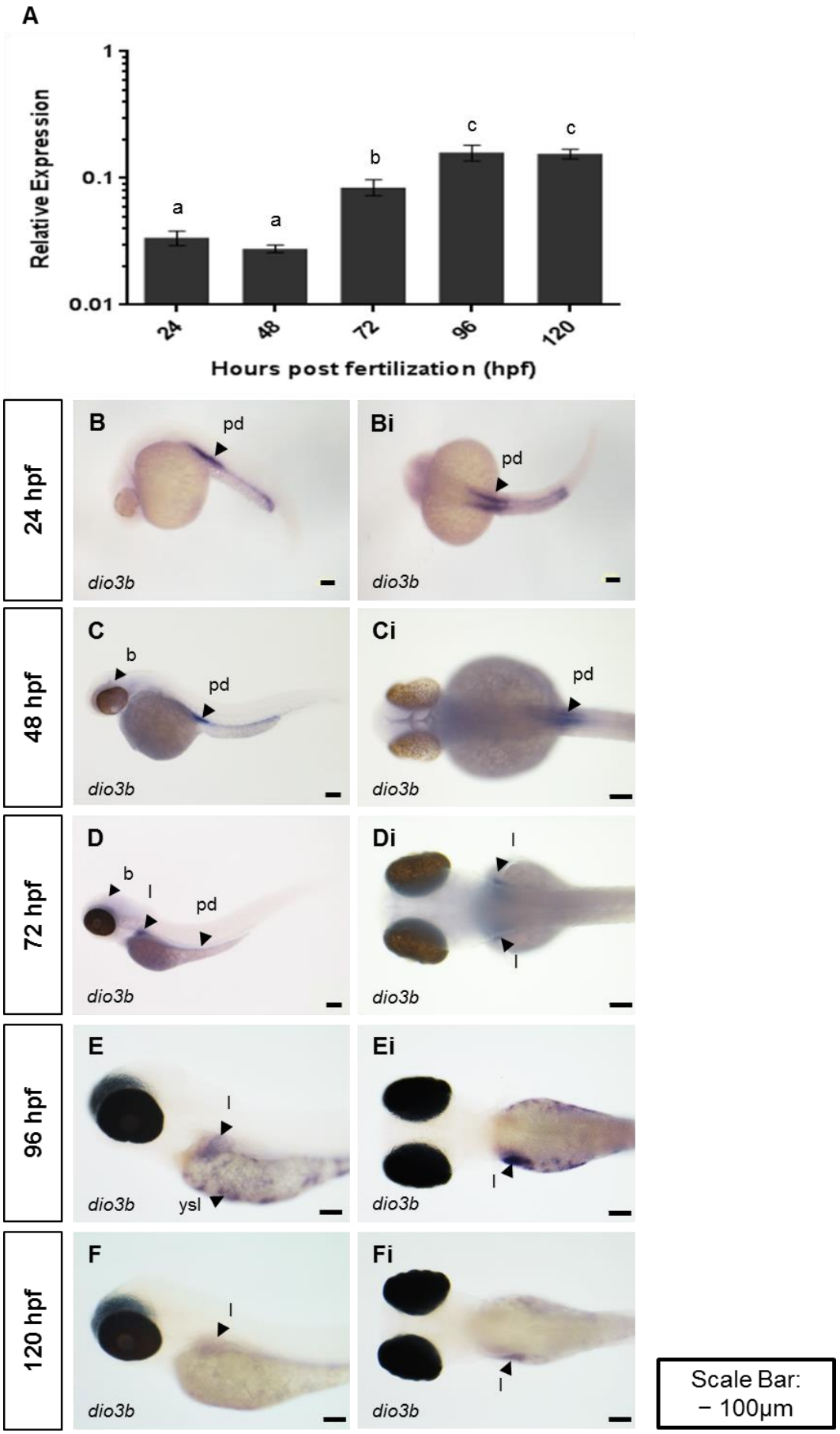
A
Relative Expression
1
0.1
0.01
a
a
b
c
c
24
48
72
96
120
Hours post fertilization (hpf)
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
B
pd
dio3b
Bi
pd
dio3b
C
b
pd
dio3b
Ci
pd
dio3b
D
b
l
pd
dio3b
Di
l
l
dio3b
E
l
dio3b
ysl
Ei
l
dio3b
F
l
dio3b
Fi
l
dio3b
Scale Bar:
− 100µm

Figure 5. (A) Transcript profile of deiodinase type III (*dio3b*) in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per developmental stage. Different letters indicate significant differences between developmental stages (Tukey's HSD post hoc test; $p<0.05$). (B-F) Tissue expression pattern of *dio3b* mRNA in zebrafish embryo-larvae between 24-120 hpf (B, n=19; C, n=36; D, n=18; E, n=22; F, n=44). Lateral (B-F) and dorsal (Bi-Fi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. . l=liver, p=pronephric ducts, ysl=yolk syncytial layer, b= brain. Scale bar=100 µm.

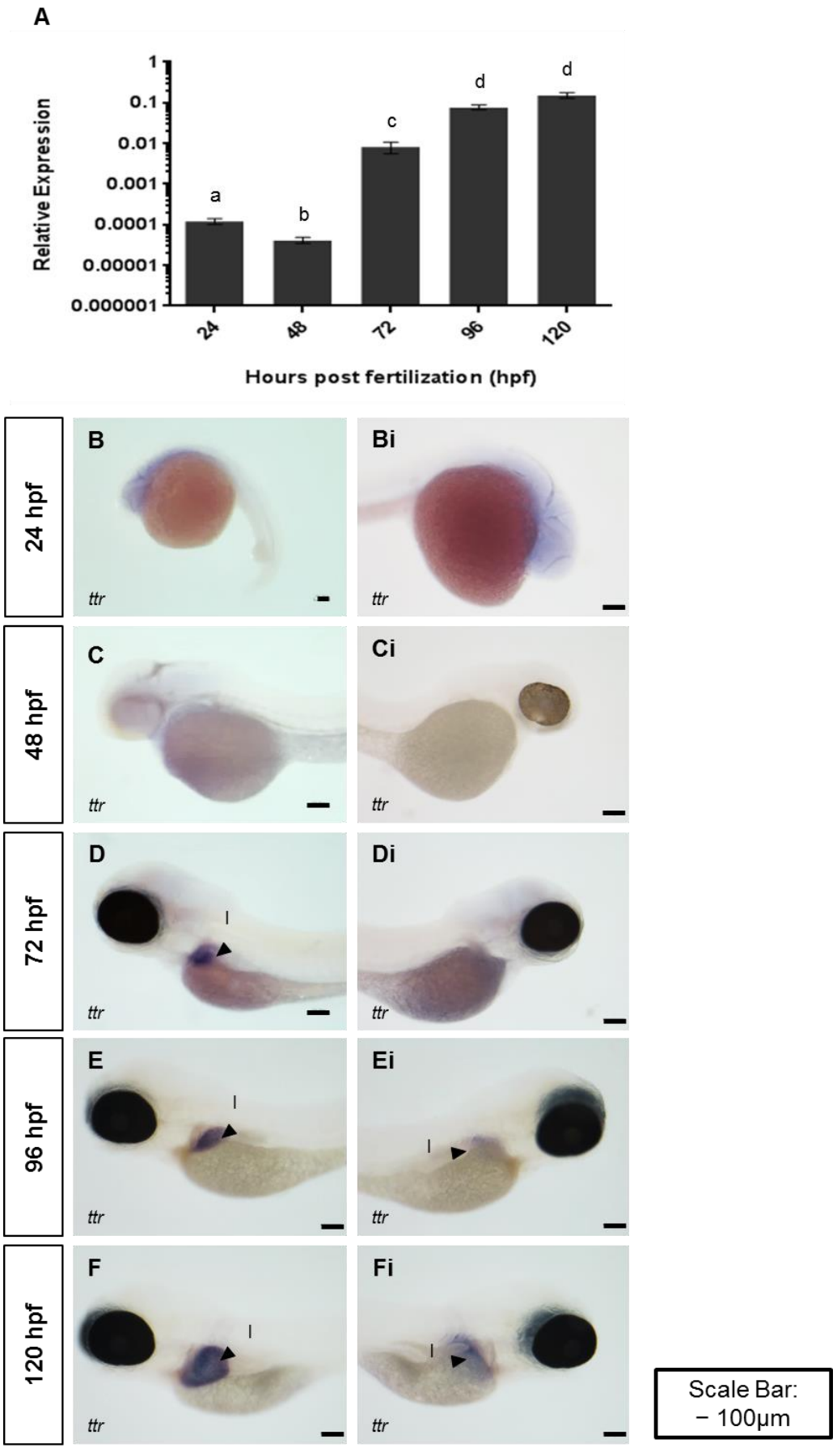
A
Relative Expression
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
a
b
c
d
d
24
48
72
96
120
Hours post fertilization (hpf)
24 hpf
48 hpf
72 hpf
96 hpf
120 hpf
B
Bi
C
Ci
D
Di
E
Ei
F
Fi
ttr
l
Scale Bar:
– 100µm

Figure 6. (A) Transcript profile of transthyretin (*ttr)* in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per developmental stage. Different letters indicate significant differences between developmental stages (Tukey's HSD post hoc test; $p<0.05$). (B-F) Tissue expression pattern of *ttr* mRNA in zebrafish embryo-larvae between 24-120 hpf. Images are representative of three experiments (B, n=25; C, n=38; D, n=25; E, n=23; F, n=50). Lateral views of whole embryo-larvae are shown with anterior to the left (B-F) or anterior to the right (Bi-Fi) and focal areas of expression are indicated by black arrowheads. l= liver. Scale bar=100 µm.

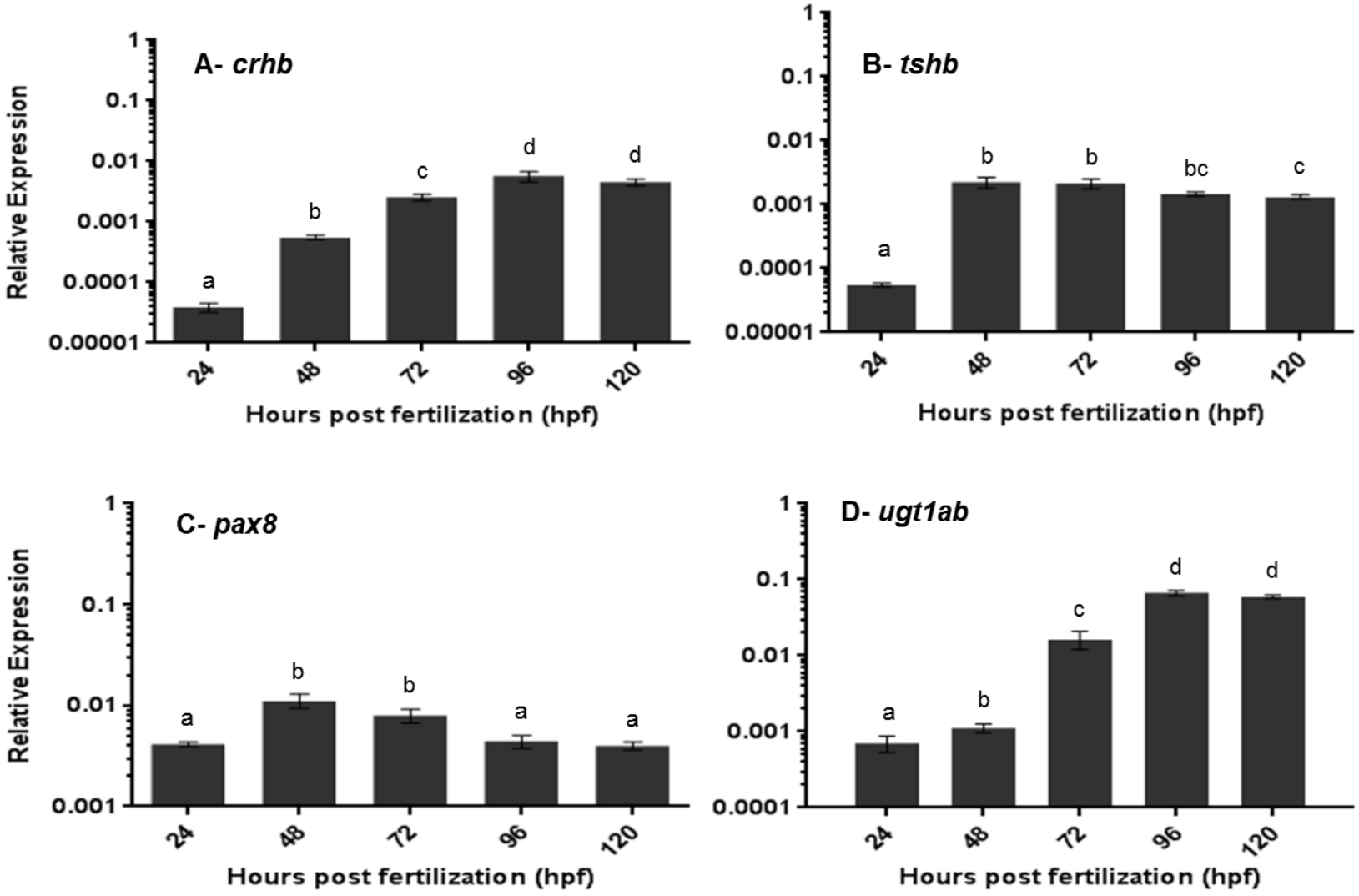
A- crhb
B- tshb
C- pax8
D- ugt1ab
Relative Expression
Hours post fertilization (hpf)
24
48
72
96
120
a
b
c
d
bc

Figure 7. Transcript profile of A) corticotropin-releasing hormone (*crhb),* B) thyroid-stimulating hormone beta (*tshb), C)* paired box 8 (*pax8)* and D) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* in whole zebrafish at 24, 48, 72, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 7-9 homogenised pooled samples per developmental stage. Different letters indicate significant differences between developmental stages (Tukey's HSD post hoc test; $p<0.05$).

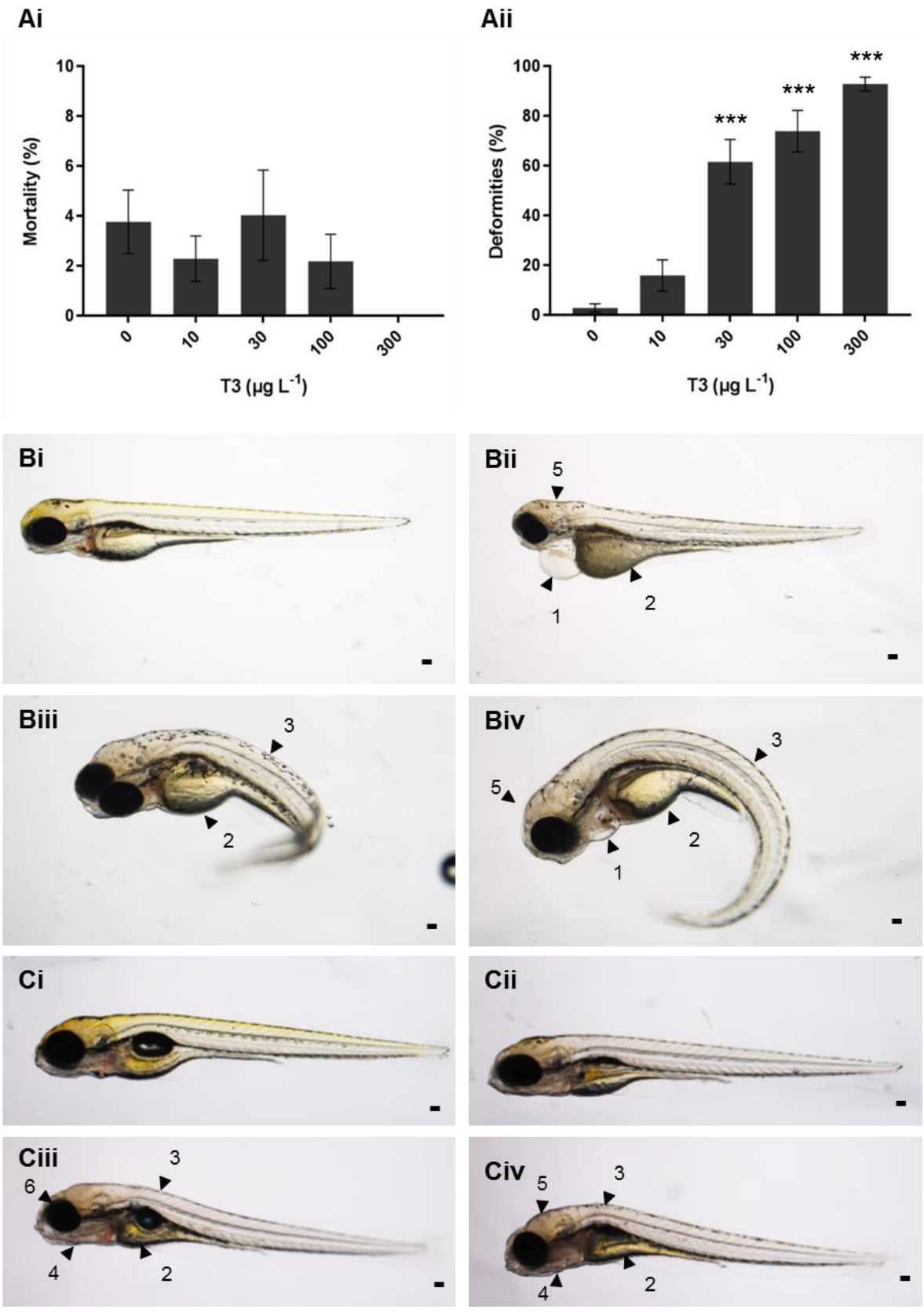
Ai
Mortality (%)
10
8
6
4
2
0
0
10
30
100
300
T3 (µg L-1)
Aii
Deformities (%)
100
80
60
40
20
0
***
***
***
0
10
30
100
300
T3 (µg L-1)
Bi
Bii
5
1
2
Biii
3
2
Biv
3
5
2
1
Ci
Cii
Ciii
3
6
4
2
Civ
5
3
4
2
Scale Bar:
− 100µm

Figure 8. (A) The effect of T3 on (i) percentage mortality and (ii) percentage of morphological deformities in zebrafish larvae following exposure for 96 hours post fertilisation (hpf). The results are represented as means ± SEM. Statistics were carried out using generalised linear models (GLSD; R) with model details reported in Table S6. Asterisks represent significant differences between treatment groups compared to the control group (***p<0.001). (B) Images of zebrafish embryo-larvae at 72 hpf exposed to (i) 0 µg T3 L^{-1}, (ii) 30 µg T3 L^{-1}, (iii) 100 µg T3 L^{-1} and (iv) 300 µg T3 L^{-1}. (C) Images of zebrafish larvae at 96 hpf exposed to (i) 0 µg T3 L^{-1}, (ii) 10 µg T3 L^{-1}, (iii) 100 µg T3 L^{-1} and (iv) 300 µg T3 L^{-1} T3 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.

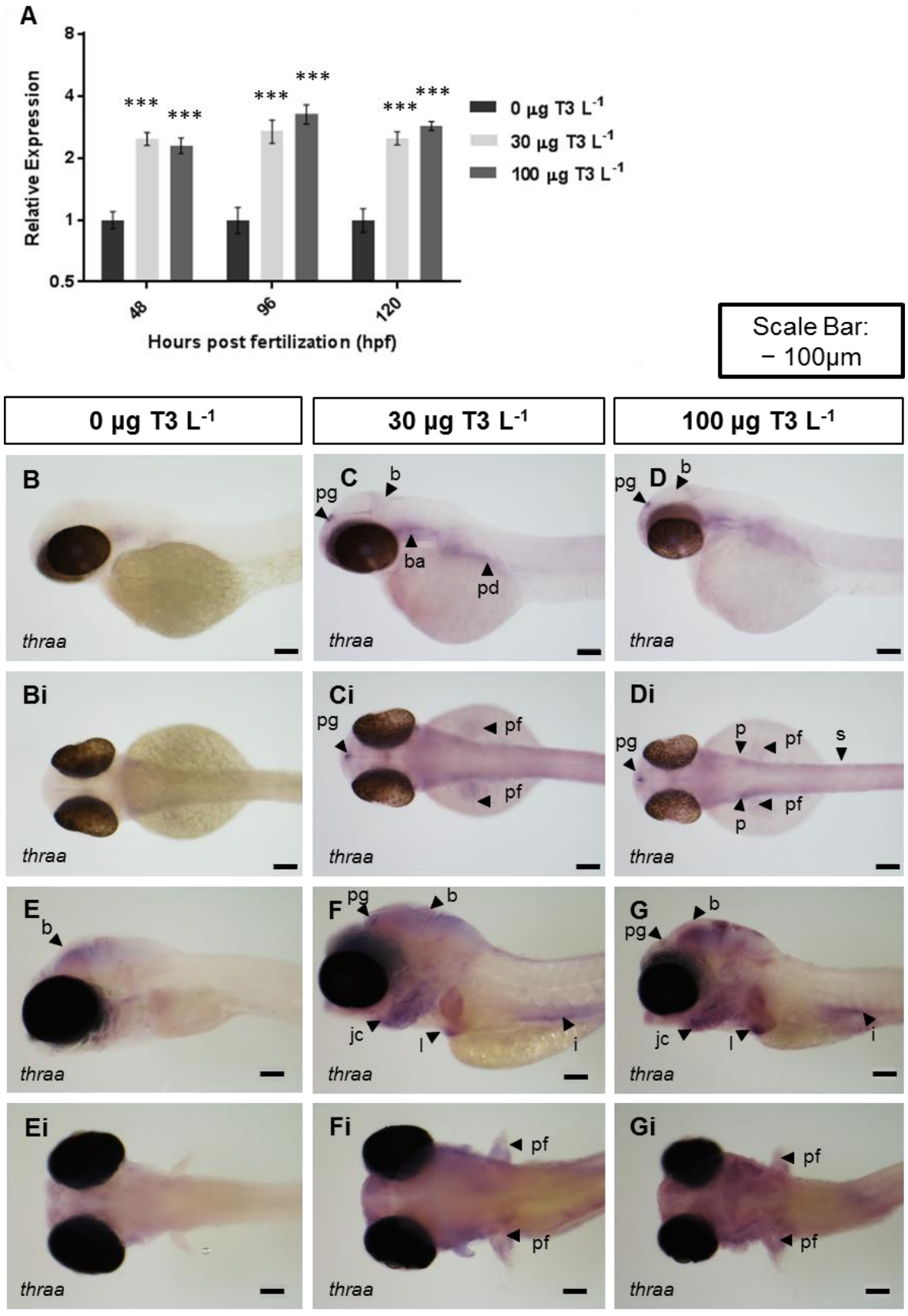
A
Relative Expression
8
4
2
1
0.5
***
***
***
***
***
***
48
96
120
Hours post fertilization (hpf)
0 µg T3 L-1
30 µg T3 L-1
100 µg T3 L-1
Scale Bar:
– 100µm
0 µg T3 L-1
30 µg T3 L-1
100 µg T3 L-1
B
C
b
pg
ba
pd
D
b
pg
Bi
Ci
pg
pf
pf
Di
pg
p
pf
s
pf
p
E
b
F
pg
b
jc
l
i
G
b
pg
jc
l
i
Ei
Fi
pf
pf
Gi
pf
pf
thraa

Figure 9. (A) Transcript profile of thyroid receptor alpha (*thraa*) in whole zebrafish following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001. Tissue expression pattern of *thraa* mRNA in zebrafish embryo-larvae at 48 hpf (B-D) and 120 hpf (E-G) treated with T3 (0, 30 and 100 µg L^{-1}). Images are representative of three experiments (B, n=25; C, n= 26; D, n=23; E, n=31; F, n=41 and G, n=28). Lateral (B-G) and dorsal (Bi-Gi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads.. pg=pineal gland, ba=branchial arch, pf=pectoral fins, b=brain, jc=jaw cartilage, l=liver, i=intestine, p=pronephric tubules, p=pronephros, s=somites. Scale bar=100 µm.

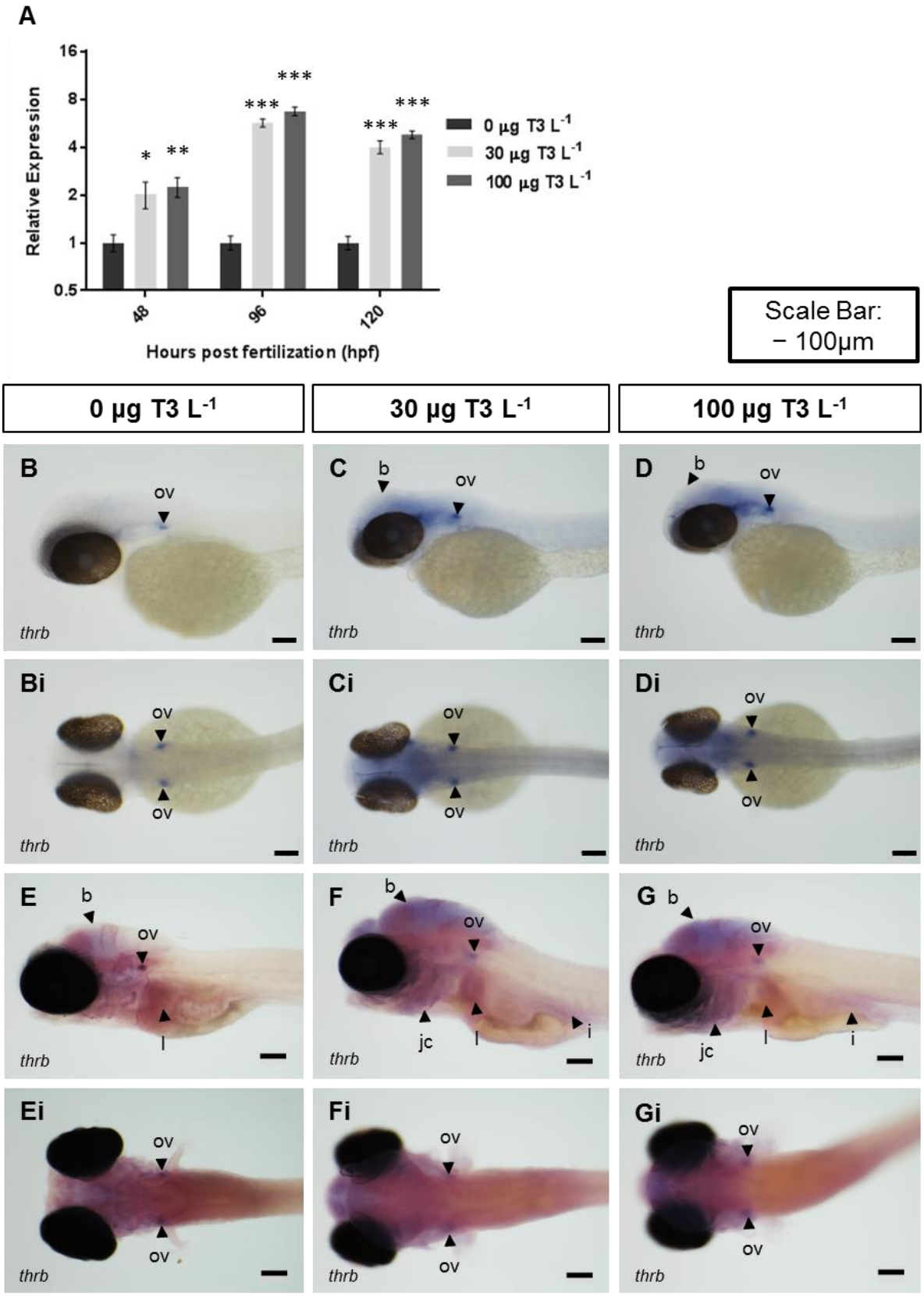

Figure 10. (A) Transcript profile of thyroid receptor beta (*thrb*) in whole zebrafish following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post

fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised pooled samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001. Tissue expression pattern of *thrb* mRNA in zebrafish embryo-larvae at 48 hpf (B-D) and 96 hpf (E-G) treated with T3 (0, 30 and 100 µg L^{-1}). Images are representative of three experiments (B, n=21; C, n=27; D, n=24; E, n=20; F, n=17 and G, n=20). Lateral (B-G) and dorsal (Bi-Gi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, jc=jaw cartilage, l=liver, i=intestine, ov=otic vesicle. Scale bar=100 µm.

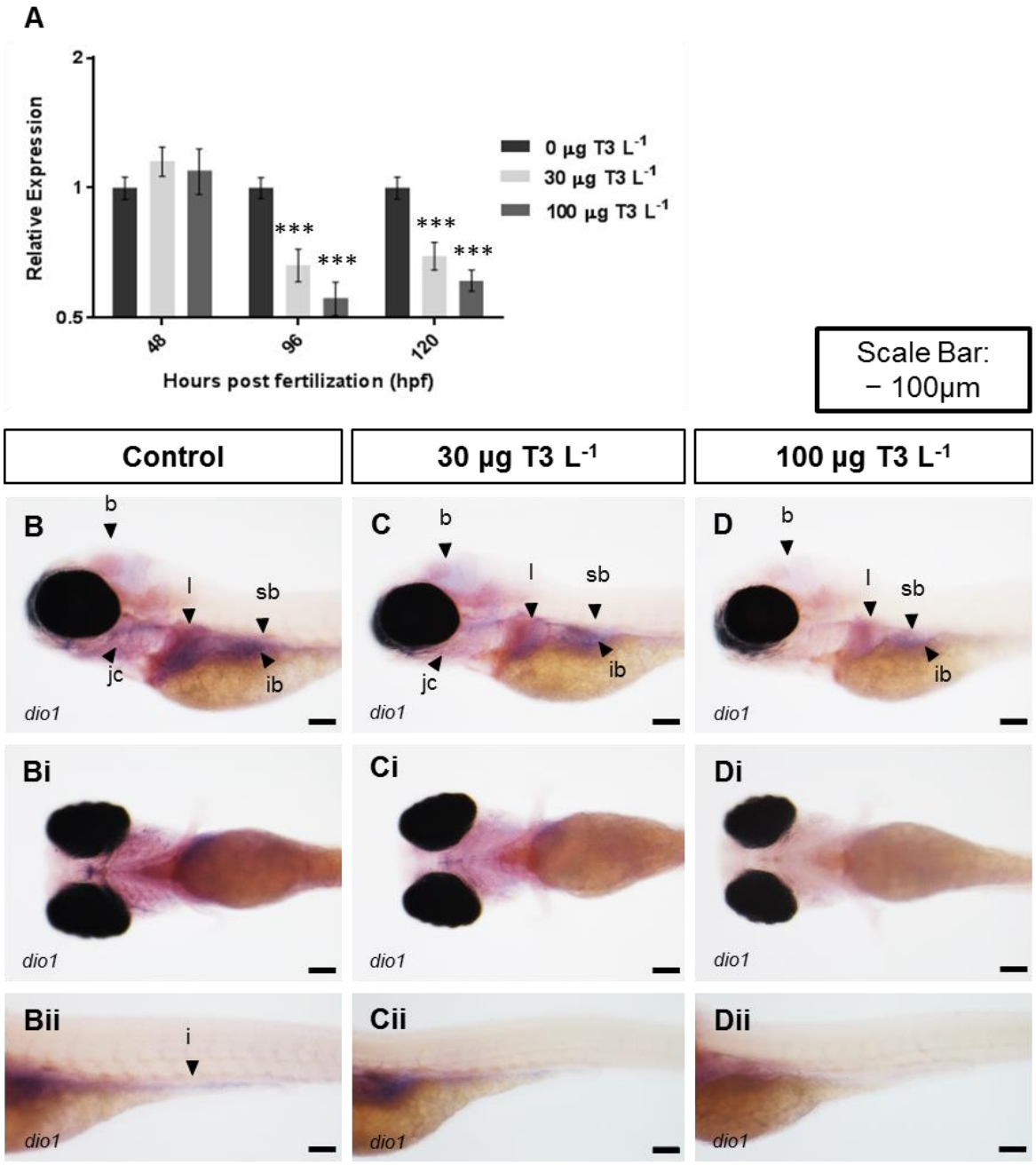

Figure 11. (A) Transcript profile of deiodinase type I (*dio1*) in whole zebrafish following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by

Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001. Tissue expression pattern of *dio1* mRNA in zebrafish embryo-larvae at 96 hpf (B-D) treated with T3 (0, 30 and 100 µg L^{-1}). Images are representative of three experiments (B, n=31; C, n=25 and D, n=30). Lateral (B-D; Bii-Eii) and ventral (Bi-Di) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, jc=jaw cartilage, l=liver, i=intestine, sb=swim bladder, ib=intestinal bulb. Scale bar=100 µm.

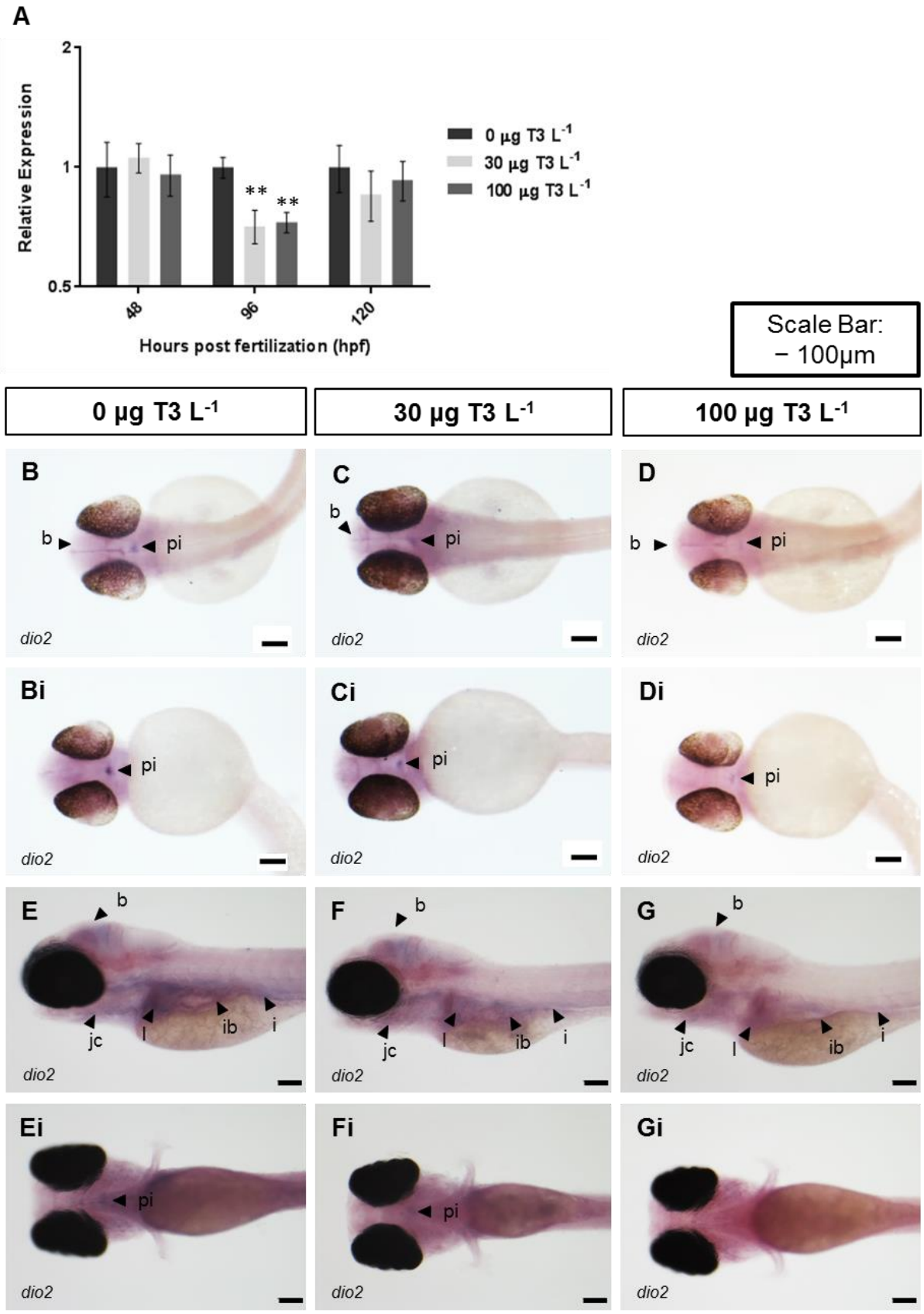
A
Relative Expression
2
1
0.5
**
**
48
96
120
Hours post fertilization (hpf)
0 µg T3 L-1
30 µg T3 L-1
100 µg T3 L-1
Scale Bar:
– 100µm
0 µg T3 L-1
30 µg T3 L-1
100 µg T3 L-1
B
C
D
Bi
Ci
Di
E
F
G
Ei
Fi
Gi
b
pi
jc
l
ib
i
dio2

Figure 12. (A) Transcript profile of deiodinase type II (*dio2*) in whole zebrafish following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001. Tissue expression pattern of *dio2* mRNA in zebrafish embryo-larvae at 48 hpf (B-D) and 96 hpf (E-G) treated with T3 (0, 30 and 100 µg L^{-1}). Images are representative of three experiments (B, n=24; C, n=29; D, n=21; E, n=24; F, n=23 and G, n=26). Dorsal (B-D), ventral (Bi-Gi) and lateral (E-G) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, l=liver, ib=intestinal bulb, pi=pituitary gland, p=pronephric tubules, jc=jaw cartilage. Scale bar=100 µm.

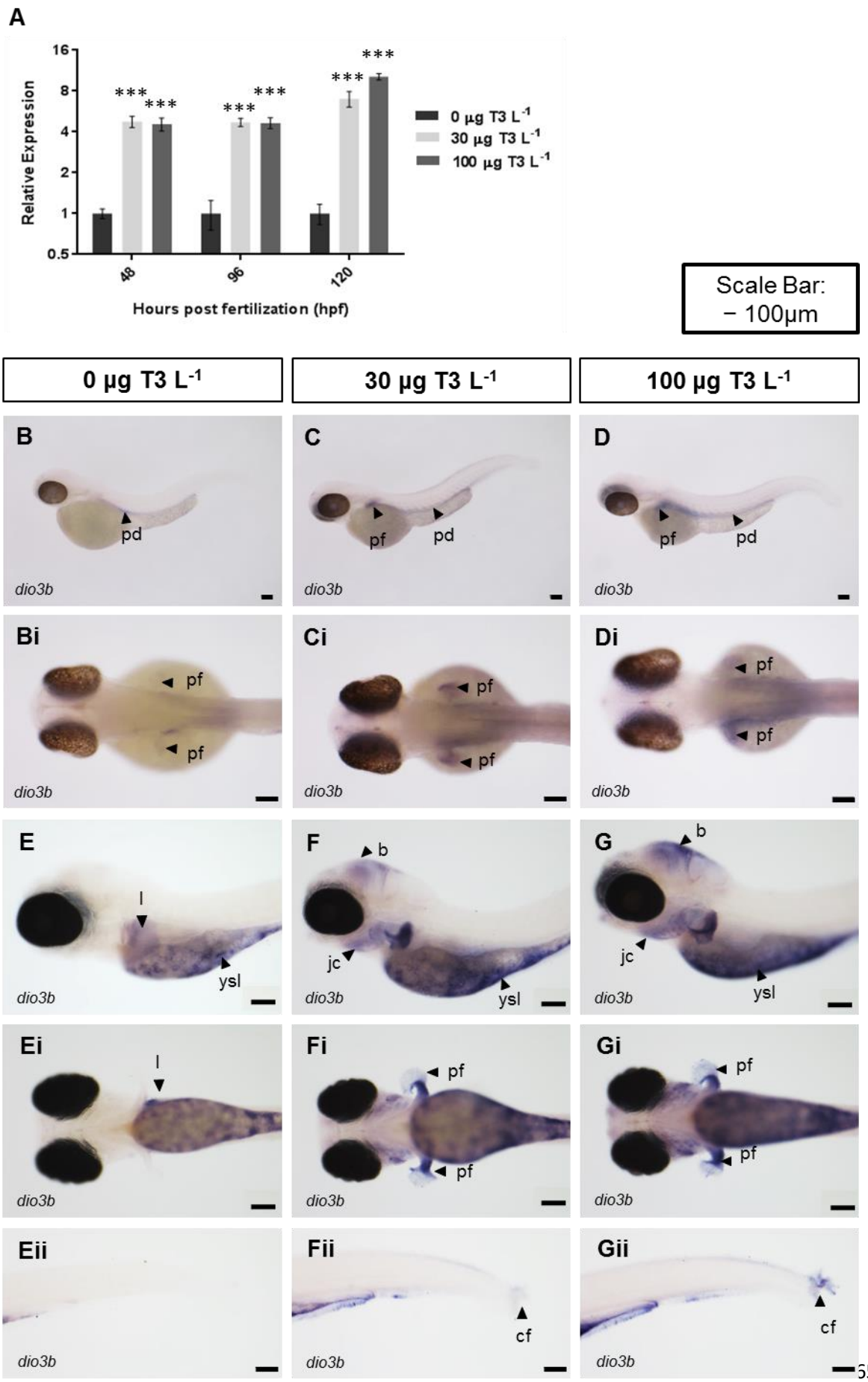
A
Relative Expression
16
8
4
2
1
0.5
***
***
***
***
***
***
48
96
120
Hours post fertilization (hpf)
0 µg T3 L-1
30 µg T3 L-1
100 µg T3 L-1
Scale Bar:
– 100µm
0 µg T3 L-1
30 µg T3 L-1
100 µg T3 L-1
B
pd
dio3b
C
pf
pd
dio3b
D
pf
pd
dio3b
Bi
pf
pf
dio3b
Ci
pf
pf
dio3b
Di
pf
pf
dio3b
E
l
ysl
dio3b
F
b
jc
ysl
dio3b
G
b
jc
ysl
dio3b
Ei
l
dio3b
Fi
pf
pf
dio3b
Gi
pf
pf
dio3b
Eii
dio3b
Fii
cf
dio3b
Gii
cf
dio3b

Figure 13. (A) Transcript profile of deiodinase type III (*dio3b*) in whole zebrafish following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001. Tissue expression pattern of *dio3b* mRNA in zebrafish embryo-larvae at 48 hpf (B-D) and 96 hpf (E-G) treated with T3 (0, 30 and 100 µg L^{-1}). Images are representative of three experiments (B, n=28; C, n=35; D, n=34; E, n=32; F, n=33 and G, n=35). Lateral (B-G; Eii-Gii), dorsal (Bi-Di) and ventral (Ei-Gi) views of whole embryo-larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. pf=pectoral fins, b=brain, l=liver, p=pronephric tubules, pg=pineal gland, m=mouth, cf=caudal fin, jc=jaw cartilage, ysl= yolk syncytial layer. Scale bar=100 µm.

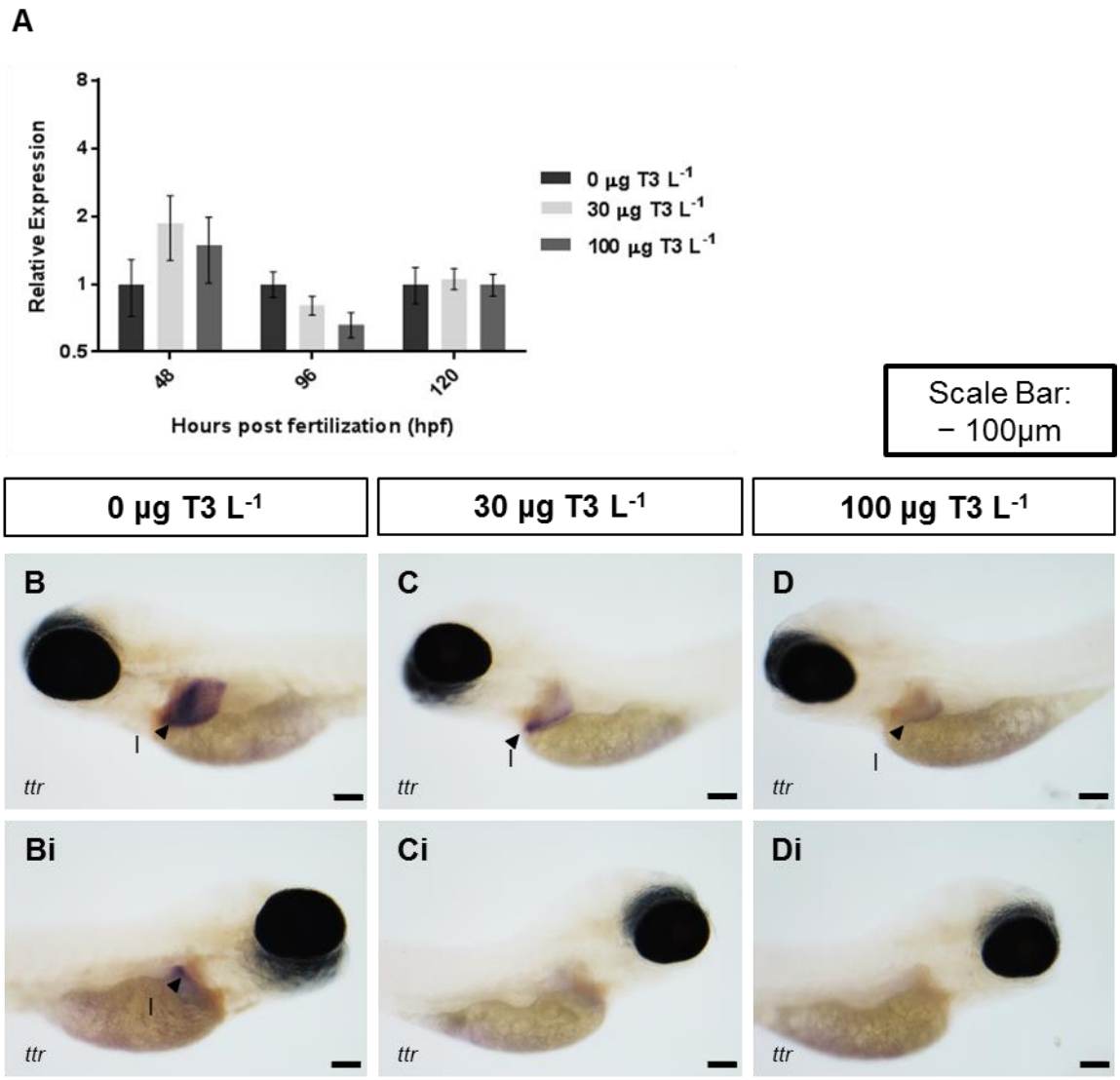

Figure 14. (A) Transcript profile of transthyretin (*ttr)* in whole zebrafish following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 8-9 homogenised pooled samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-D) Tissue expression pattern of *ttr* mRNA in

zebrafish larvae at 96 hpf treated with T3 (0, 30 and 100 µg L^{-1}). Images are representative of three experiments (B, n=24; C, n=35 and D, n=31). Lateral views of whole larvae are shown with anterior to the left (B-D) and right (Bi-Di) and focal areas of expression are indicated by black arrowheads.. l=liver. Scale bar=100 µm.

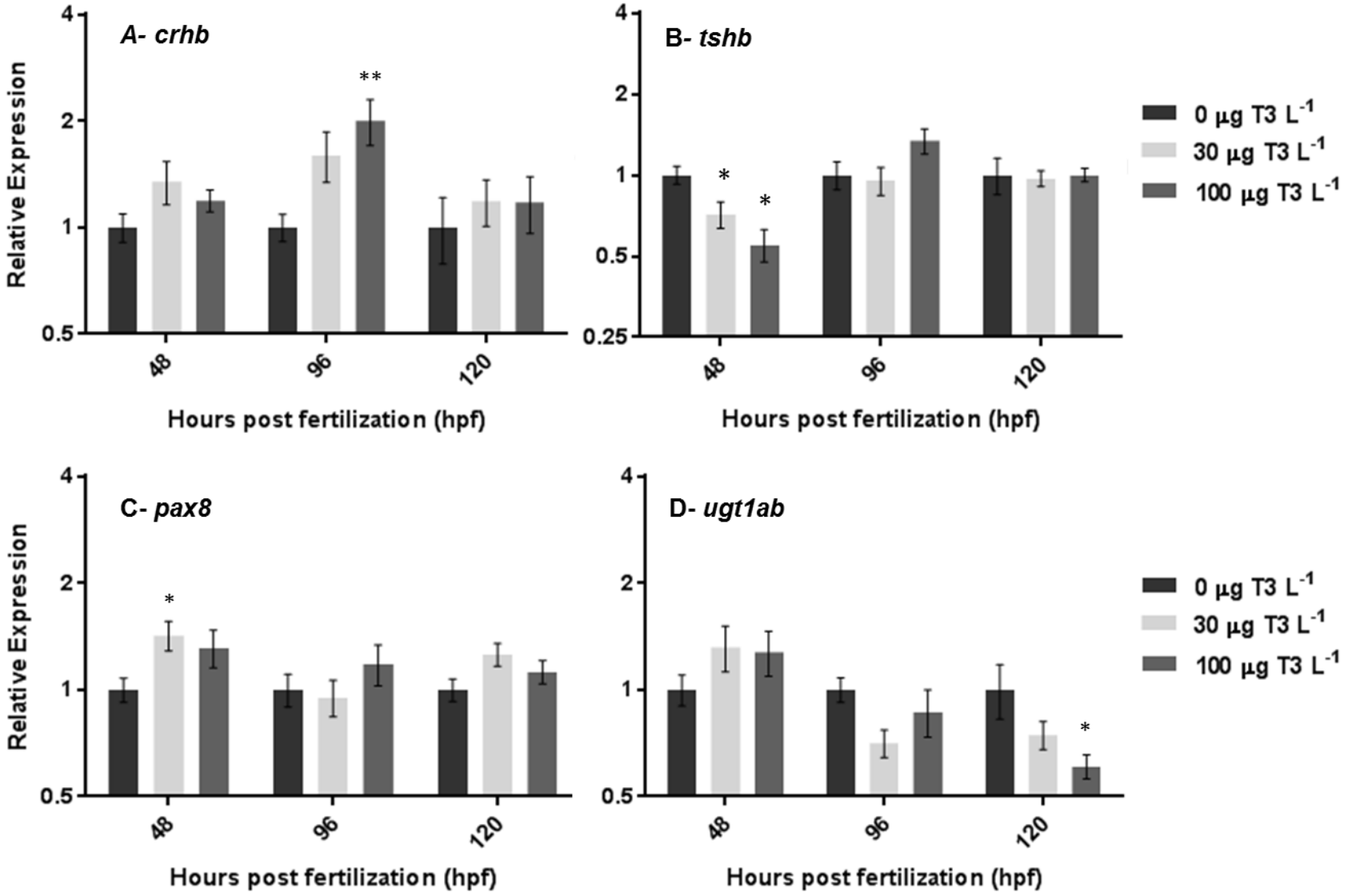
A- crhb
B- tshb
C- pax8
D- ugt1ab
Relative Expression
Hours post fertilization (hpf)
48
96
120
0 µg T3 L-1
30 µg T3 L-1
100 µg T3 L-1

Figure 15. Transcript profile of A) corticotropin-releasing hormone (*crhb),* B) thyroid-stimulating hormone (*tshb)*, C) paired box 8 (*pax8)* and D) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* in whole zebrafish following exposure to T3 (0, 30 and 100 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf), determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised pooled samples per treatment group. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

Chapter 2

Supplementary Material

Expression dynamics and tissue localisation of genes in the hypothalamic-pituitary-thyroid (HPT) cascade and their responses to 3,5,3′-tri-iodothyronine in zebrafish *(Danio rerio*) embryo-larvae

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Supplementary Tables

Table S1. Vectors, restriction enzymes and promoters used in plasmid digestion and RNA probe synthesis for whole mount *in situ* hybridisation assays for the following genes in the HPT axis: thyroid receptors (*thraa* and *thrb*), thyroid-stimulating hormone (*tshb*), deiodinases (*dio1, dio2* and *dio3b*) and transthyretin (*ttr*).

Gene	Accession Number	Vector	Restriction Enzyme	Promotor
thraa	AI497070	pSport1	EcoR1	Sp6
thrb	BC163106	PCR4_TOPO	Spe1	T7
tshb	BC163605	PCR4_TOPO	Spe1	T7
dio1	BF717782.1	pSport1	EcoR1	Sp6
dio2	BG737671.1	pBK_CMV	BamH1	T7
dio3b	NM_001177935.2	pGEM-T	-	T3
ttr	NM_001005598.2	pME18S-FL3	-	T3

Table S2: Target gene staining times (hours) in non-exposed zebrafish embryo-larvae examined in whole mount *in situ* hybridisation assays. Embryo-larvae were sampled at various developmental stages; 24, 48, 72, 96 and 120 hours post fertilisation (hpf). Staining times are shown for the following genes: thyroid receptors (*thraa* and *thrb*), thyroid-stimulating hormone (*tshb*), deiodinases (*dio1, dio2* and *dio3b*) and transthyretin (*ttr*).

Gene	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf
thraa	8.5	7.5	7.5	7.5	7.5
thrb	8.5	8.5	8.5	8.5	8.5
tshb	8.5	8.5	8.5	8.5	8.5
dio1	8.5	8.5	8.5	7.5	7.5
dio2	8.5	5	7	8.5	8.5
dio3b	5	5	6.5	5	3.5
ttr	6.5	6.5	5	5	5

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

Gene	48 hpf	96 hpf	120 hpf
thraa	2	-	3
thrb	3.5	-	3.5
dio1	4.5	10	-
dio2	3.5	10	-
dio3b	2	2	-
ttr	4.5	3.5	-

Table S4. 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	Primer Sequence	Product Size (bp)	Ta (°C)	PCR Efficiency (%)	Accession Number
thraa	F: GGC GTC CTG TAA CTG CTG R: GGT TGT GCT CCT GCT CTG	142	61	101	NM_131396.1
thrb	F: TGG GTG TCT CGC TGT CCTC R: ACA ACG CTC TAT CCG CTC AAC	119	60	93	NM_131340.1
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
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
dio2	F: TCT GGA GGA GAG GAT GTT TGC R: CTC GTA GGA CAC ACC GTA GG	124	59.5	105	NM_212789.3
dio3b	F: AGG GCT CCG CAG GTG TG R: AGG AAG TCC AGC AGG CAG AG	106	63	98	NM_001177935.2
ttr	F: CGC ACA CCT TTC CAC CAG R: TTG ACG ACC ACA GCA GTT G	122	60.5	109	NM_001005598.2
crhb	F: TTC GGG AAG TAA CCA CAA GC R: CTG CAC TCT ATT CGC CTT CC	163	59.5	110	NM_001007379.1
ugt1ab	F: CCA CCA AGT CTT TCC GTG TT R: GCA GTC CTT CAC AGG CTT TC	168	62.5	105	NM_213422.2
pax8	F: CCG TCA CTC CTC CTG AAT CTC R: GCT CTC CTG GTC ACT GTC ATC	128	62.5	106	AF072549.1
rpl8	F: CCG AGA CCA AGA AAT CCA GAG R: CCA GCA ACA ACA CCA ACA AC	91	59.5	102	NM_200713.1

Table S5: General linear models for the relationship between developmental stage (24-120 hours post fertilisation) and transcript profiles measured in zebrafish embryo larvae. Minimum adequate models (F-value) and degrees of freedom (df) for the relative expression are shown for the genes analysed: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), thyroid-stimulating hormone (*tshb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). Significance codes: *p<0.05, **p<0.01, ***p<0.001.

Gene	df	Minimum Adequate Model
thraa	36	33.91***
thrb	38	22.04***
dio1	38	94.55***
dio2	38	152.4***
dio3b	38	52.58***
ttr	38	105.7***
crhb	39	131.3***
tshb	36	144.1***
pax8	38	9.94***
ugt1ab	38	105.2***

Table S6: Generalised linear models for the relationship between T3 and zebrafish larvae mortality and deformities after a 96 hour exposure (0, 10, 30, 100 and 300 µg T3 L^{-1}). Minimum adequate models (F-value) and degrees of freedom (df) are shown. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

	df	Minimum Adequate Model
Mortality	40	NS
Deformity	40	37.07***

Table S7: General linear models for the relationship between T3 exposure and transcript profiles measured in zebrafish embryo-larvae exposed to T3 (0, 30 or 100 µg L^{-1}) for 48, 96 or 120 hours post fertilisation (hpf). Minimum adequate models (F-value) and degrees of freedom (df) for the relative expression are shown for the genes analysed: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), thyroid-stimulating hormone (*tshb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). Significance codes: *p<0.05, **p<0.01, ***p<0.001.

Gene	hpf	df	Minimum Adequate Model	Gene	hpf	df	Minimum Adequate Model
thraa	48	23	25.04***	***ttr***	48	13	NS
	96	23	24.82***		96	23	NS
	120	24	42.40***		120	23	NS
thrb	48	23	5.10*	***crhb***	48	23	NS
	96	20	186.98***		96	20	4.30*
	120	24	53.77***		120	23	NS
dio1	48	21	NS	***tshb***	48	23	8.34**
	96	22	14.80***		96	23	NS
	120	23	13.90***		120	21	NS
dio2	48	22	NS	***pax8***	48	24	3.22*
	96	23	6.88**		96	24	NS
	120	23	NS		120	21	NS
dio3b	48	23	81.02***	***ugt1ab***	48	23	NS
	96	22	38.57***		96	22	NS
	120	22	83.32***		120	21	3.96*

Table S8: General linear models for the relationship between thyroid receptor (*thraa* and *thrb*) transcript profiles between 24-120 hours post fertilisation (hpf) measured in zebrafish embryo larvae. Minimum adequate models (F-value) and degrees of freedom (df) for the relative expression are shown. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

hpf	df	Minimum Adequate Model
24	16	106.28***
48	15	15.60**
72	16	61.32***
96	15	106.59***
120	12	71.93**

Table S9: General linear models for the relationship between deiodinase (*dio1, dio2* and *dio3b*) transcript profiles between 24-120 hours post fertilisation (hpf) measured in zebrafish embryo larvae. Minimum adequate models (F-value) and degrees of freedom (df) for the relative expression are shown. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

hpf	df	Minimum Adequate Model
24	24	514.75***
48	23	440.83 ***
72	24	163.29***
96	22	318.00***
120	24	231.82***

Supplementary Figures

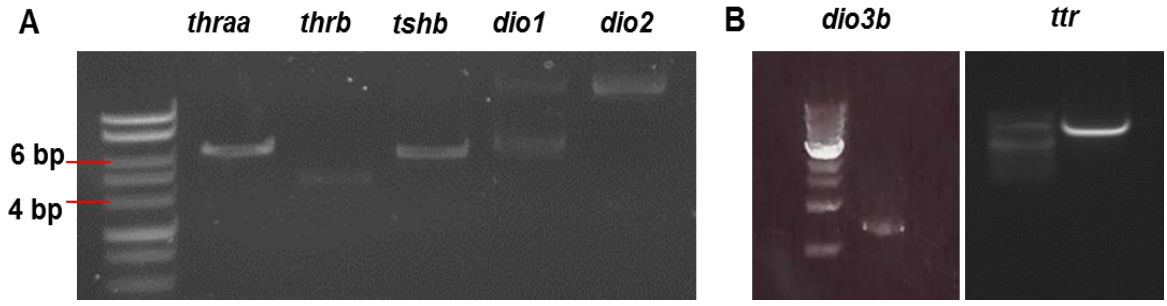

Figure S1. Image of agarose gel showing (A) *thraa, thrb, tshb, dio1* and *dio2* following plasmid digestion by EcoR*I*, Spe*I*, Spe*I*, EcoR*I* and BamH*I,* respectively. Lane 1; 1 kb ladder, Lanes 2-5; individual genes. (B) *dio3b* and *ttr* DNA following PCR amflification. Lane 1; 1 kb ladder, Lanes 2-3; individual genes.

```
CLUSTAL O(1.2.1) multiple sequence alignment


gi|319803079:36-485      ATGGCGAAAGAAGTGATTTGTGTGCTTCTCGCATCTCTGTTTGCCCTCTGTAGATCTGCT
TTR_ap_Consensus         ---------------------------------NNNNNCNNNTGNCTCTGTAGATCTGCT
                                                           **************

gi|319803079:36-485      CCAGTGGCTTTTCATGGTGGTTCAGATGCTCACTGTCCTCTGACGGTAAAAATCCTGGAT
TTR_ap_Consensus         CCNGTGGCTTTTCATGGTGGTTCAGATGCTCACTGTCCTCTGACGGTAAAAATCCTGGAT
                         ** ************************************************************

gi|319803079:36-485      GCTGTCAAAGGGACGCCTGCTGGAAATATAGCTCTGGATCTGTTTCGTCAAGATCAAGGT
TTR_ap_Consensus         GCTGTTAAAGGGACGCCTGCTGGAAATATAGCTCTGGATCTGTTTCGTCAAGATCAAGGT
                         ***** ************************************************************

gi|319803079:36-485      GGGACATGGGAAAAGATTGCCAGTGGGAAAGTGGACATGACTGGTGAAGTGCACAACTTG
TTR_ap_Consensus         GGGACATGGGAAAAGATTGCCAGTGGGAAAGTGGACATGACTGGTGAAGTGCACAACTTG
                         ************************************************************

gi|319803079:36-485      ATCACGGAGCAGGAGTTCACTCCTGGTGTGTATCGGGTGGAGTTTGACACTTTAACCTAC
TTR_ap_Consensus         ATCGCTGAGCAGGAGTTCACTCCTGGTGTGTATCGGGTGGAGTTTGACACTTTAACCTAC
                         *** * ********************************************************

gi|319803079:36-485      TGGAAGACAGAGGGTCGCACACCTTTCCACCAGCTGGCTGATGTGGTGTTTGAAGCTCAT
TTR_ap_Consensus         TGGAAGACAGAGGGTCGCACACCTTTCCACCAGCTGGCTGATGTGGTGTTTGAAGCTCAT
                         ************************************************************

gi|319803079:36-485      GCAGAGGGACATCGTCATTACACACTGGCTCTCCTTCTGAGCCCTTTCTCGTACACCACA
TTR_ap_Consensus         GCAGAGGGACATCGTCATTACACACTGGCTCTCCTTCTGAGCCCTTTCTCGTACACCACA
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gi|319803079:36-485      ACTGCTGTGGTCGTCAAGGCACATGACTGA
TTR_ap_Consensus         ACTGCTGTGGTCGTCAAGGCACATGACTGA
                         ******************************
```

Figure S2: Nucleotide sequence alignment of PCR amplified *ttr* sequence with known sequence (NM_001005598.2).

```
d3_consensus            NTGNAGATGNCTGCAGGGNTCCGCAGGTGTGCAGTNTGCGCTGAAGAACGCGGCGGTGTG
gi|364502957:94-903     -ATGGAGATGCTGCAGGGCTCCGCAGGTGTGCAGTCTGCGCTGAAGAACGCGGCGGTGTG
                                ******** *************** **********************

d3_consensus            TGTGCTGCTGCTCCCGCGCTTTCTACTCGCCGCGCTGATGCTCTGCCTGCTGGACTTCCT
gi|364502957:94-903     TGTGCTGCTGCTCCCGCGCTTTCTGCTCGCCGCGCTGATGCTCTGCCTGCTGGACTTCCT
                        ********************** ***********************************

d3_consensus            GTGCATCCGCCGGAAGCTGCTGCTGAAGATGCAGGAGGGCGCGTTCAGCAGCCCCGACGA
gi|364502957:94-903     GTGCATCCGCCGGAAGCTGCTGCTGAAGATGCAGGAGGGCGCGTTCAGCAGCCCCGACGA
                        ************************************************************

d3_consensus            CCCTCCGCTGCGCGTGTCCGACAGCAACAAGATGTTCACGCTGGAGTCGCTGCGCGCGGT
gi|364502957:94-903     CCCTCCGCTGCGCGTGTCCGACAGCAACAAGATGTTCACGCTGGAGTCGCTGCGCGCGGT
                        ************************************************************

d3_consensus            CTGGTACGGCCAGAAGCTGGACTTCTTCAAGAGCGCGCGGCTCGGCGGAGCGGCGCCCAA
gi|364502957:94-903     CTGGTACGGCCAGAAGCTGGACTTCTTCAAGAGCGCGCGGCTCGGCGGAGCGGCGCCCAA
                        ************************************************************

d3_consensus            CACCGAGGTGTTCCCGCTGGACGGAGACGCGCGCGCGGCAGAGCGCATCCTGGATTACGC
gi|364502957:94-903     CACCGAGGTGTTCCCGCTGGACGGAGACGCGCGCGCGGCAGAGCGCATCCTGGATTACGC
                        ************************************************************

d3_consensus            GCGCGGCCGGAGACCGCTGATCCTCAACTTCGGCAGCTGCTCCTGACCGCCCTTCATGAC
gi|364502957:94-903     GCGCGGCCGGAGACCGCTGATCCTCAACTTCGGCAGCTGCTCCTGACCGCCCTTCATGAC
                        ************************************************************

d3_consensus            CCGTCTGAGCGCCTTCCAGCGCGTCGCGCGGCAGTACGCGGACATCGCGGACTCTCTGCT
gi|364502957:94-903     CCGTCTGAGCGCCTTCCAGCGCGTCGCGCGGCAGTACGCGGACATCGCGGACTCTCTGCT
                        ************************************************************

d3_consensus            GGTGTACATCGAGGAGGCGCACCCGTCCGACGGCTGGGTGAGCTCCGACGCGCCCGTGCA
gi|364502957:94-903     GGTGTACATCGAGGAGGCGCACCCGTCCGACGGCTGGGTGAGCTCCGACGCGCCCGTGCA
                        ************************************************************

d3_consensus            GATCCCGCGGCACCGCTGCCTGGAGGACCGGCTCCGCGCCGCGCAGATGCTGCACCGGGA
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                        ************************************************************

d3_consensus            GGCGGCGGGCAGCGCGGTGGTCGTGGACTCCATGCAGAACTCCTGCAACGCGGCGTACGG
gi|364502957:94-903     GGCGGCGGGCAGCGCGGTGGTCGTGGACTCCATGCAGAACTCCTGCAACGCGGCGTACGG
                        ************************************************************

d3_consensus            CGCGTATTTCGAGCGGCTGTACATCGTAAAGGACGCGACGGTGGTGTACCAAGGCGGCCG
gi|364502957:94-903     CGCGTATTTCGAGCGGCTGTACATCGTAAAGGACGCGACGGTGGTGTACCAAGGCGGCCG
                        ************************************************************

d3_consensus            CGGACCCGAGGGGTACCGGATCGCGGAGCTCAGAGACTGGCTGGAGCGCTACAGGAGCGG
gi|364502957:94-903     CGGACCCGAGGGGTACCGGATCGCGGAGCTCAGAGACTGGCTGGAGCGCTACAGGAGCGG
                        ************************************************************

d3_consensus            CCT----------------------------
gi|364502957:94-903     CCTGCAGGAGTCCGCGGTGCTGCACGTCTAG
                        ***
```

Figure S3: Nucleotide sequence alignment of PCR amplified *dio3b* sequence with known sequence (NM_001177935.2)

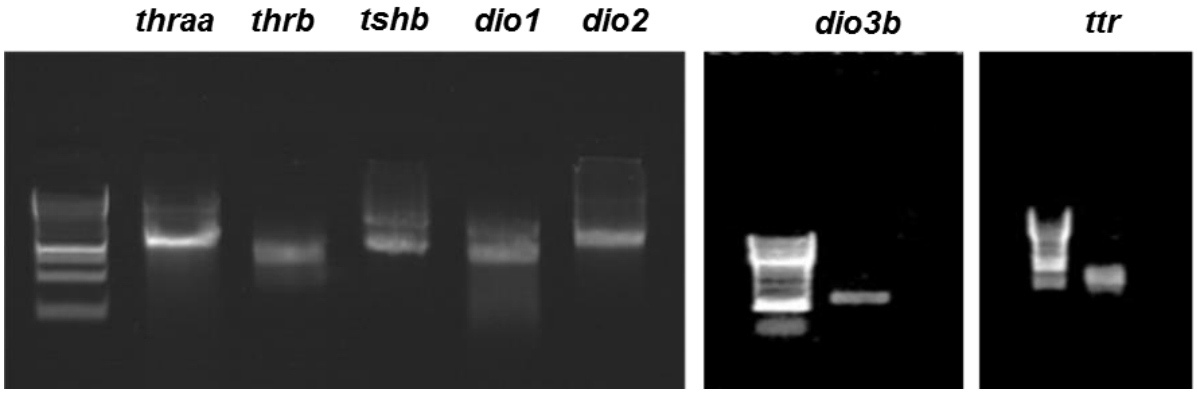

Figure S4. Image of agarose gel showing *thraa, thrb, tshb, dio1, dio2, dio3b* and *ttr* digoxigenin anti-sense RNA probes.

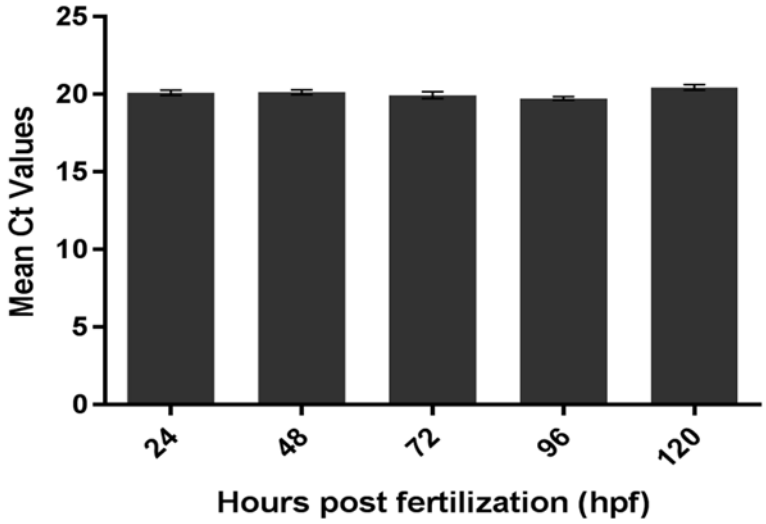

Figure S5: Mean *rpl8* mRNA levels expressed as Ct values (threshold cycle) in non-exposed zebrafish at 24, 48, 72, 96 and 120 hours port fertilisation. There was no significant difference in Ct values between treatment groups (GLM; R). n=9. Error bars represent standard error.

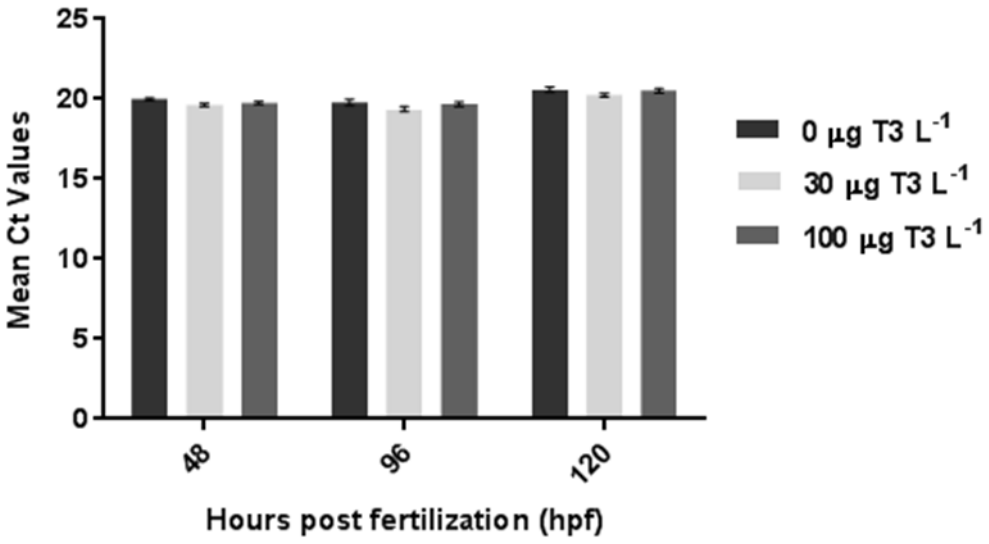

Figure S6: Mean *rpl8* mRNA levels expressed as Ct values (threshold cycle) in zebrafish exposed to T3 (0, 30 and 100 µg L^{-1}) at 48, 96 and 120 hours post fertilisation (hpf). There was no significant difference in Ct values between treatment groups and developmental stages (GLM; R). n=9. Error bars represent standard error.

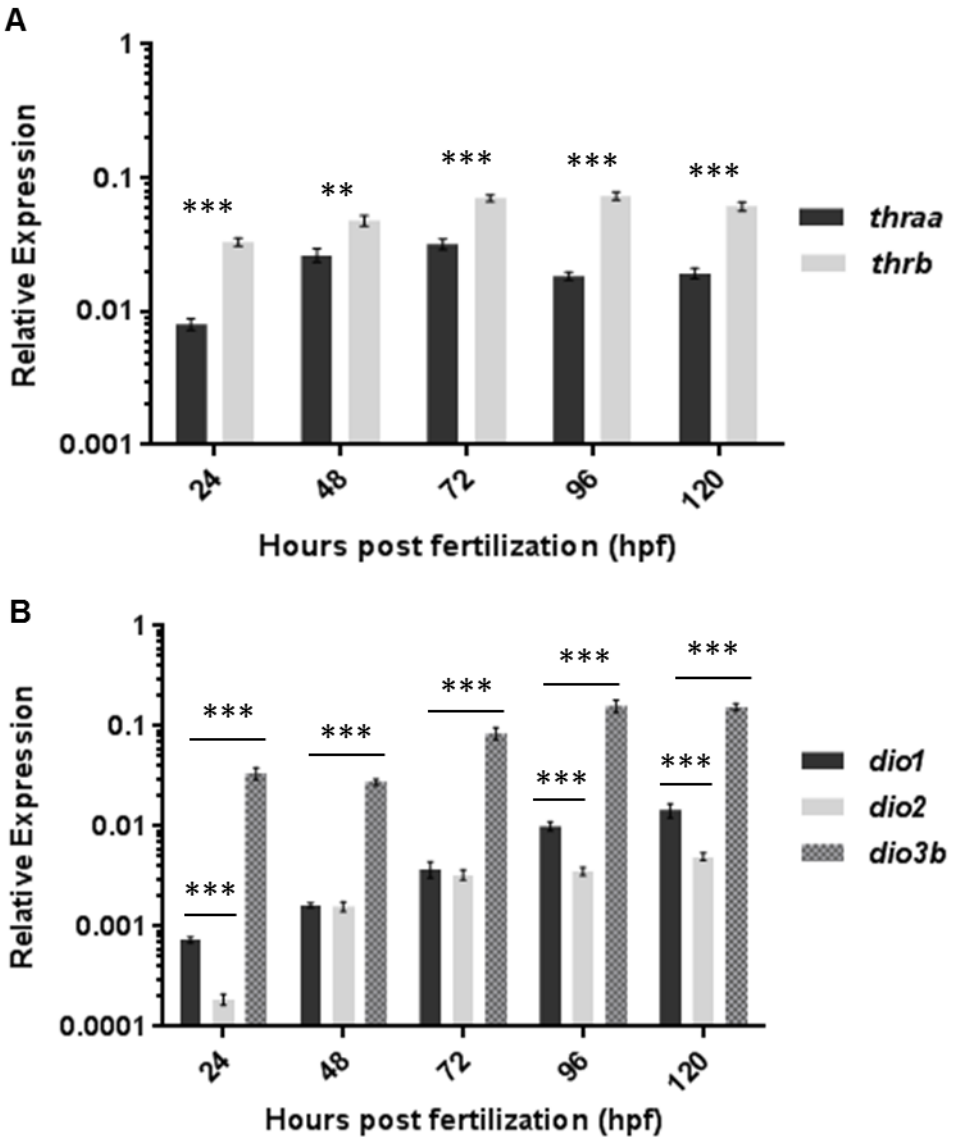

Figure S7: Comparative transcript profiles of (A) *thraa* and *thrb* and (B) *dio1*, *dio2* and *dio3b* during early zebrafish developmental stages (24-120 hours post fertilisation) as determined by qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S8 and S9. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a

replication of n= 7-9 homogenised sample per developmental stage. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

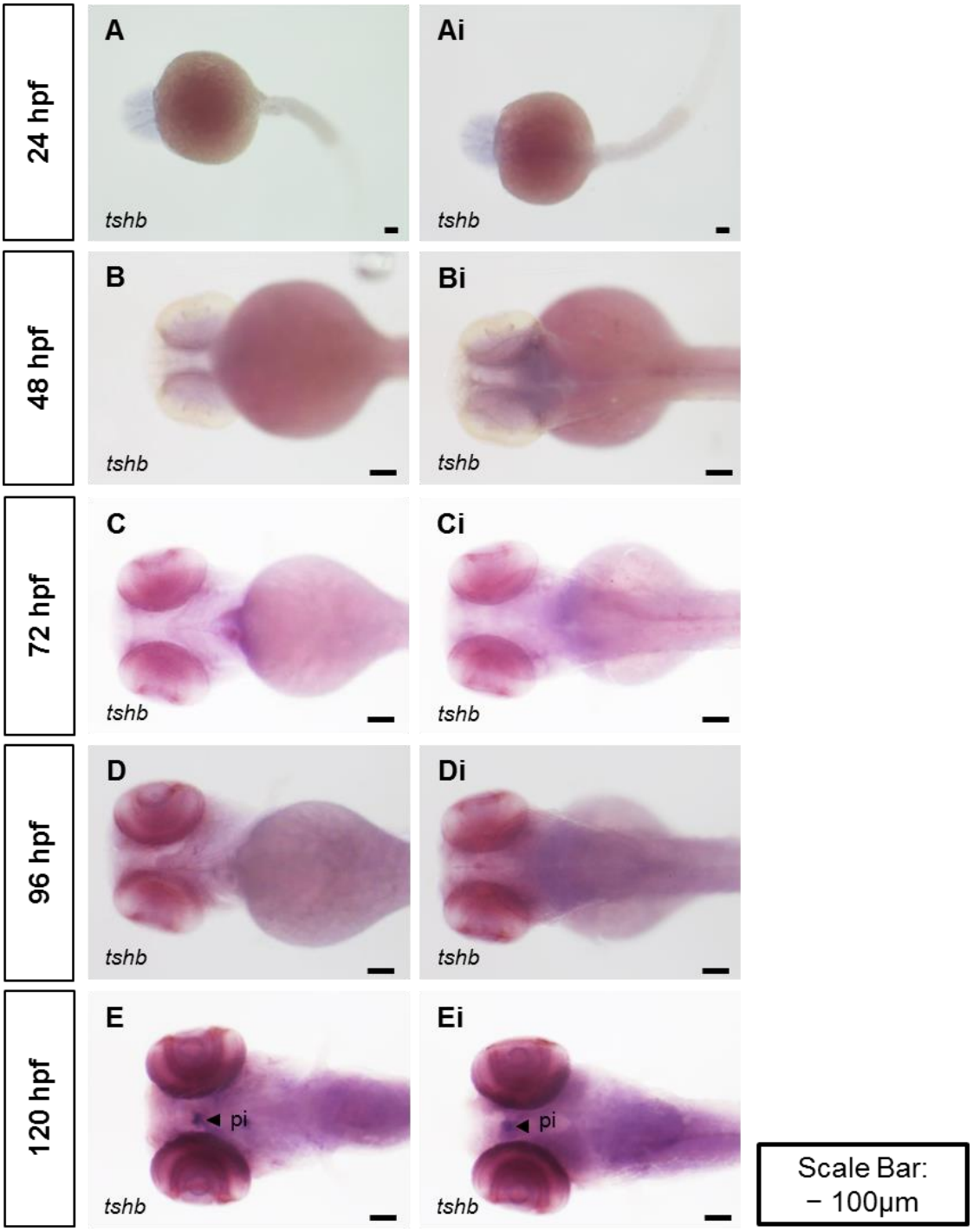

Figure S8. (A-E) Tissue expression pattern of thyroid-stimulating hormone beta (*tshb)* mRNA in zebrafish embryo-larvae between 24-120 hours post fertilisation (hpf) (B, n=31; C, n=30; D, n=28; E, n=30; F, n=30). Ventral (B-F) and dorsal (Bi-Fi) views of whole embryo-larvae are shown with anterior to the left and focal areas of

expression are indicated by black arrowheads. pi=pituitary gland. Scale bar=100 µm.

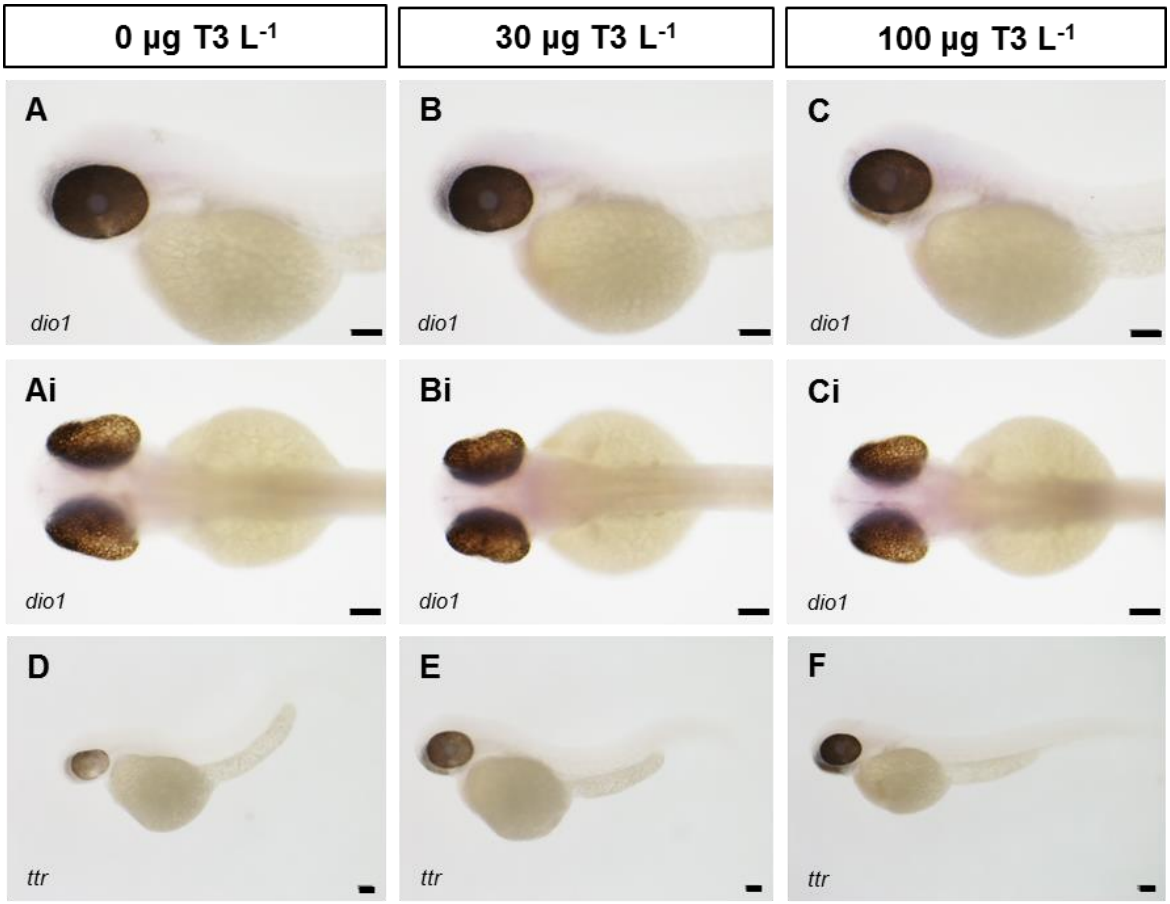

Figure S9. No change in the tissue expression pattern of (A-C) deiodinase type I (*dio1*) and (D-F) transthyretin (*ttr*) was observed in zebrafish embryos at 48 hours post fertilisation (hpf) following treatment with T3 (0, 30 and 100 µg L^{-1}). Images are representative of three experiments (A, n=29; B, n=31; C, n=30; D, n=32; E, n=32; F, n=27). Lateral (A-F) and dorsal (Ai-Ci) views of whole embryos are shown with anterior to the left. Scale bar=100 µm.

Chapter 3

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

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3.1 Abstract

Brominated flame retardants are known to disrupt thyroid hormone (TH) homeostasis in several vertebrate species, but the molecular mechanisms underlying this process and their effects on TH-sensitive tissues during the stages of early development have not yet been clarified. In this study, we exposed zebrafish (*Danio rerio*) embryo-larvae to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and tetrabromobisphenol A (TBBPA) via the water for 96 hours from fertilisation and examined the effect on survival and development. For zebrafish embryo-larvae, the 96 hour lethal median concentration for TBBPA (96-LC-50) was 638 µg L^{-1} and mortality was preceded by retardation of development (smaller animals) and morphological deformities (oedemas in the pericardial region and tail, small heads, swollen yolk sac extension). Exposure to BDE-47 did not affect zebrafish embryo-larvae survival at any of the concentrations tested (0.0564-5.64 mg L^{-1}) but resulted in yolk sac and craniofacial deformities as well as curved spines and short tails at the highest concentration. Further sub-lethal exposures were then carried out and the effect of TBBPA and BDE-47 on gene transcription in the hypothalamic-pituitary-thyroid (HPT) axis was analysed. Tissue-specific responses were measured via whole mount *in situ* hybridisation (WISH) and responses in the whole organism were assessed quantitatively using real time quantitative PCR (qRT-PCR) on whole body extracts. Exposure to TBBPA resulted in higher levels of mRNAs for genes encoding the thyroid receptor β (*thrb*), deiodinase enzymes (*dio1*), transport proteins (*ttr*), thyroid follicle synthesis proteins (*pax8*) and gluronidation enzymes (*ugt1ab*) and lower levels of *dio3b* mRNAs in whole body extracts, in patterns that varied with developmental stage. Exposure to BDE-47 resulted in higher levels of *thrb, dio1, dio2, pax8* and *ugt1ab* mRNAs and lower levels of *ttr* mRNAs in whole body extracts.

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

3.2 Introduction

Over the last two decades, there has been growing concern over the levels of brominated flame retardants (BFRs) in the environment as a result of their ability to disrupt the thyroid hormone (TH) system. THs play a key role in a wide range of physiological functions during both early development and in adult life stages of vertebrates. Developmental roles include influencing the maturation of many tissues including bone (reviewed in [1]), gonads (reviewed in [2]), intestine [3] and the central nervous system (reviewed in [4]) as well as mediating the metamorphic transition from larval to adult stages in fish and amphibians [5]. In adults they modulate growth [6], energy homeostasis [7], cardiac rhythm [8], osmoregulation [9] and the behaviours/physiology associated with rheotaxis and migration in fish [10,

11]. Given the detrimental effects that can result from subtle changes in TH status, particularly during crucial developmental windows [12], even relatively low environmental levels of BFRs pose a potential risk to the health of humans and wildlife.

BFRs have been commercially important high production compounds since their introduction to the global markets in the 1970s and are used routinely in industrial and consumer products in an effort to reduce fire-related injury and property damage [13]. Polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA) are amongst the most extensively used BFRs worldwide [13]. PBDEs are a family of 209 possible congeners that can be divided into 10 congener groups (mono- to decabromodiphenyl ethers). Commercial PBDE mixtures are made up of congeners with varying numbers of bromine atoms on their two phenyl rings and are classified according to their average bromine content; penta-, octa-, and deca-BDEs [13]. Penta-BDE mixtures, traded under brand names such as DE-71 and Bromkal70-5DE, were used as additive BFRs in textiles, carpets, electronics and polyurethane foams found in upholstered furniture, mattresses and seat cushions [14]. Approximately 7,500 metric tonnes of penta-BDE mixtures were produced globally in 2001, with the largest market in North America. The congener BDE-47 (2,2′,4,4′-tetrabromodiphenyl ether) was one of the main components of the commercial penta-BDE mixtures, accounting for approximately 40% weight per weight (w/w) [15].

Since PBDEs are blended physically rather than bonded chemically to the polymer, they subsequently migrate into the environment and over the past four decades they have become ubiquitous environmental contaminants [16]. Some of the highest

concentrations of PBDEs in the environment have been observed in matrices such as suspended solids [up to 4,600 μg/kg dry weight (dw)] [17], sediment (up to 3,250 mg/kg dw) [18], sewage sludge (up to 97,400 μg/kg dw) [18] and raw sewage (up to 2,496 ng/L) [19] from rivers, estuaries and coastal regions in urban areas of Europe, China and the US. While BDE-209 is frequently the predominant congener detected in sediments and the particulate phases of water samples [18, 20, 21], BDE-47 is often the most commonly detected congener in the aqueous phase of environmental water samples [20]. BDE-47 also often dominates the PBDE profiles of human samples collected from Europe and the USA [22-25], marine mammals [26, 27], birds and birds eggs [28-30], invertebrates [31] and fish [32, 33]. The production and usage of penta-BDE commercial mixtures were prohibited globally in 2004 and officially labelled as Persistent Organic Pollutants (POPs) by the Stockholm Convention in 2009 [34]. Controls on the use of the penta-BDE products are only just beginning to yield declines in the lower brominated congener concentrations in environmental samples (reviewed in [35]). Nonetheless, global contamination occurs today as a result of the continued use of older products, manufactured prior to the restrictions, which still contain PBDEs. These products therefore are continuing to act as a source of environmental contamination including after their disposal and at electronic waste (e-waste) recycling sites [36-42]. In 2016, over a decade since the introduction of restrictions, there is a continued presence of penta-BDE constituents in indoor air and dust sampled from homes in the UK, US and China, with emissions from older products suggested to be the primary source of these banned compounds in the indoor environments [41, 43, 44]. Furthermore, PBDE are highly persistent in biotic matrices and even today the constituents of penta-BDE technical mixtures, such as BDE-47, BDE-99 and BDE-100, are still the dominant PBDEs detected in

humans and wildlife samples [45-48]. Finally, there is growing evidence which suggests that the release and body burdens of PBDEs is increasing in non-industrialised developing countries, where in the past they have been undocumented or relatively low [37, 49, 50].

Over 120,000 and 170,000 tonnes of TBBPA were produced globally in 2001 and 2004, respectively [51, 52], making TBBPA the most widely used BFR (representing approximately 60% of the total BFR market) [53]. The primary application (90%) of TBBPA is as a reactive flame retardant covalently bound to the epoxy and polycarbonate resins of electronic circuit boards. It is also used to a lesser extent (10%) as an additive flame retardant in acrylonitrile-butadiene-styrene (ABS) resin and high-impact polystyrene, used in automotive parts, pipes, refrigerators and television and computer housings [13]. Despite the primary use of TBBPA as a reactive flame retardant, it has been identified in occupational, household and environmental dust samples [54-56], in sewage sludge [57], river sediments [58] and the water phase of lakes and rivers [59, 60]. TBBPA has a relatively short half-life (ranging between <1 and <5 days in fish, shellfish and mammals) [61-64], indicating little potential for bioaccumulation. Despite this, TBBPA has been detected in various biotic samples including human breast milk and plasma (up to 37.3 µg/kg lw) [65], the eggs of predatory birds (up to 13 ng/kg ww) [66], marine mammals (up to 418 µg/kg lw) [53, 64], fish (up to 245 µg/kg lw) [64] and invertebrates (up to 100 µg/kg lw) [67]. The fact that various studies have detected TBBPA in a suite of organisms from around the world suggests their exposure to this chemical has been both recent and continuous.

Since several BFRs are structurally similar to thyroxine (T4), the precursor of the biologically active TH 3,3′,5-triiodo-L-thyronine (T3), concerns have been raised about their effect on the thyroid system of both mammalian and non-mammalian vertebrates [16, 68]. Indeed, numerous *in vivo* studies have shown that both TBBPA and PBDEs (as individual congeners and commercial mixtures) can alter the circulating levels of both T4 and T3 in a range of vertebrate species, including fish [69-76]. The thyroidal system is centrally driven by the hypothalamic-pituitary-thyroid (HPT) axis and under peripheral control in all vertebrates. The HPT axis is regulated as a negative feedback mechanism in which the level of thyroid-stimulating hormone (TSH) secreted by the pituitary controls the productions and release of T4 by the thyroid follicles. In the peripheral system, TH activity is tightly regulated by the metabolizing iodothyronine deiodinase enzymes, type I, II and III (D1, D2 and D3), which can modulate TH signalling in individual tissues as well as controlling serum TH concentrations [77, 78]. D2 catalyses the outer ring deiodination (ORD) of T4 to produce the bioactive T3 [79]. In contrast, D3 catalyses the inner ring deiodination (IRD) of T4 and T3 producing the inactive metabolites reverse T3 (rT3) and 3, 3′-diiodo-L-thyronine (T2), respectively. D1 is a kinetically inefficient enzyme that is capable of catalysing both IRD and ORD [80].

Until recently, evaluating thyroid disruption by environmental chemicals has mainly relied on measures of circulating TH levels, thyroid size or histopathology. It is however important to note that the thyroid system can maintain normal physiological functions in response to TH level perturbations by changing the production and/or metabolism of THs by the thyroid gland itself, in target tissues or in the liver [81, 82]. Thyroid-disrupting chemicals (TDCs) may not cause obvious changes to TH levels,

but may nonetheless alter TH homeostasis. Consequently, evaluating thyroid disruption using the aforementioned endpoints is problematic. In recent years, analysing gene expression across the entire HPT axis of small model organism fish species has been viewed as a sensitive endpoint and an effective means of directing mechanistic understanding on the thyroid disrupting capabilities of PBDEs (reviewed in [83]). Information on the mechanisms associated with TBBPA's ability to disrupt the HPT axis of fish is relatively limited with only one study to date carried out into the molecular effect mechanisms [84]. Studies have largely analysed gene expression changes in pooled samples of whole animals and therefore provide no information on the tissue-specific gene expression changes. Identifying tissue-specific responses to environmental contaminants is fundamental to building our understanding on the effect mechanisms of BFRs. Several studies have demonstrated that BFRs can elicit localised effects on the expression of thyroid related genes in adult fish species, such as adult fathead minnows (*Pimephales promelas*) and Chinese rare minnows (*Gobiocypris rarus)* [72, 85, 86], however isolating organs and/or tissues of interest from embryos and larvae for studies on gene expression is more challenging. One approach that has been applied successfully to study localised gene expression in embryos/larvae in small fish species such as the zebrafish (*Danio rerio*) is whole mount *in situ* hybridisation (WISH). WISH involves the synthesis of antisense RNA probes for particular genes of interest which are then labelled with digoxigenin-linked nucleotides. These probes allow the sites of expression of these genes to be detected in whole mount zebrafish embryo-larvae. A recent study by Dong *et al.,* (2013) used WISH to examine the effect of the hydroxylated metabolite 6-OH-BDE-47 on the tissue-specific expression pattern of the deiodinase encoding genes in zebrafish [87]. They found that exposure

resulted in a significant up-regulation of *dio1* mRNA expression in the periventricular region of the brain and *dio3b* mRNA expression in the pronephric ducts of embryos [87]. Though the importance of tissue-specific transcriptional responses is increasingly recognised in environmental toxicology research, developmental stage specific transcriptional responses remain largely understudied.

The aim of this study was to assess the effect of two important BFR compounds, TBBPA and BDE-47, on the expression of a suite of genes in the HPT axis of zebrafish during early development, with the goal of highlighting potential effect mechanisms and target tissues of thyroid disruption. Given the plasticity of the TH system, we further undertook to examine the effects of TBBPA across different developmental life stages. We used a combination of real time quantitative PCR (qRT-PCR) assays to quantify changes in gene transcript levels in whole body extracts and WISH to assess changes in tissue gene expression patterns.

3.3 Materials and Methods

3.3.1 Materials and reagents

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

3.3.2 Zebrafish maintenance

Adult zebrafish [*casper* (*mitfa^w2; roy^a9*) mutant strain that lack skin pigmentation] were obtained from breeding stocks at University of Exeter. The *casper* mutant zebrafish was employed in this work because they lack all melanocytes and iridophores, facilitating the visualisation of gene expression via WISH. Fish were maintained at 28 ± 1°C in a 12:12 hour light: dark cycle in a closed flow-through system. Embryos were collected approximately 1 hour post fertilisation (hpf) from breeding colonies, washed twice with culture water with the addition of methylene blue to prevent fungal growth as described in Nusslein-Volhard, 1996 [88]. No known effects of methylene blue on thyroid signalling in fish are reported in the literature to the best of our knowledge. Eggs were incubated in culture water without methylene blue. Eggs were then incubated in culture water without methylene blue. Culture water was aerated artificial freshwater made according to the ISO-7346/3 guideline (ISO water diluted 1:5, pH 6.5-7.5, air saturation 95-100%) [89]. Embryos were examined under a stereomicroscope and only those fertilised were selected for subsequent experimental work.

3.3.3 Acute toxicity of TBBPA and BDE-47

TBBPA exposures were carried out at 0, 100, 250, 500, 750 and 1500 µg L^{-1} (DMSO at 0.01% v/v of the culture medium). BDE-47 exposures were carried out at 0, 0.564, 2.82, 5.64, 28.2 and 56.4 mg L^{-1} (with DMSO at 0.1% of the culture medium). In the TBBPA and BDE-47 exposures, control groups were incubated in DMSO at 0.01% and 0.1% respectively. Twenty fertilised embryos were included in each treatment group and half of the exposure solutions were replaced every 24 hours with freshly prepared solutions. Exposures were conducted for 96 hours from fertilisation. The

number of dead embryo-larvae and phenotypic deformities were recorded every 24 hours. Experiments were carried out in triplicate and repeated three times (n=9).

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

TBBPA exposures were carried out at concentrations of 0, 25, 100 and 250 µg L^{-1} (with DMSO at 0.01% v/v of the culture medium) and BDE-47 exposures were carried out at concentrations of 0, 0.0564, 0.564 and 5.64 mg L^{-1} (with DMSO at 0.1% v/v of the culture medium). These concentrations were chosen based on range-finding tests to determine treatments that were sub-lethal and are lower than or similar to the concentrations examined by other authors in previous studies reported (e.g. 110, 270, 550 and 820 µg TBBPA L^{-1} in [84]; and 0, 100, 200, 300 and 400 µg TBBPA L^{-1} in [90]; 2.03, 5.08, 10.2 and 15.2 mg BDE-47 L^{-1} in [84]). In TBBPA and BDE-47 exposures, control groups were incubated in DMSO at 0.01% and 0.1% v/v, respectively. Fifty fertilised embryos were selected for each treatment group. Half of the exposure solutions were replaced every 24 hours with freshly prepared solutions. TBBPA exposures were conducted for 48, 96 and 120 hours from fertilisation. BDE-47 exposures were conducted for 96 hours from fertilisation.

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

3.3.5 Effect of TBBPA on T3 regulation of gene transcripts in HPT axis

To investigate whether there was an interaction between TBBPA and T3 in terms of their effect on the transcription of genes in the HPT axis, zebrafish embryos were exposed to T3, TBBPA and/or a combination of each chemical from 0-96 hpf. Dosing groups were as follows: T3 (1 µg L^{-1}), T3 (3 µg L^{-1}), T3 (10 µg L^{-1}), TBBPA (25 µg L^{-1}), TBBPA (250 µg L^{-1}), T3 (1 µg L^{-1}) + TBBPA (25 µg L^{-1}), T3 (1 µg L^{-1}) + TBBPA (250 µg L^{-1}), T3 (3 µg L^{-1}) + TBBPA (25 µg L^{-1}), T3 (3 µg L^{-1}) + TBBPA (250 µg L^{-1}), T3 (10 µg L^{-1}) + TBBPA (25 µg L^{-1}) and T3 (10 µg L^{-1}) + TBBPA (25 µg L^{-1}) with DMSO at 0.02% v/v of the culture medium. The control group were dosed with 0.02% DMSO. Range-finding tests were conducted to determine the appropriate T3 exposure concentrations. Half of the exposure solutions were replaced every 24 hours with freshly prepared solutions. At 96 hpf, 10 embryo-larvae from each treatment group were pooled, frozen in liquid nitrogen and stored at -80°C for qRT-PCR analyses. Experiments were carried out in triplicate.

3.3.6 WISH

WISH was used to examine tissue-specific changes in gene expression for several genes of interest in the HPT axis including: thyroid receptors (*thraa and thrb),* deiodinase enzymes *(dio1, dio2 and dio3b)* and transthyretin *(ttr).* DNA was obtained via PCR or plasmid vectors (Supplementary Material) and used to synthesis digoxigenin (DIG) antisense RNA probes using DIG RNA labelling mix (Roche), transcription buffer x5 (Fermentas), RNAse inhibitor (Roche), water and the appropriate polymerase (T7, T3 or SP6) and incubated at 37°C for 5 hours. Probes were then treated with DNAse at 37°C for 1 hour and purified by lithium chloride precipitation. RNA probe integrity was confirmed by agarose gel electrophoresis and

an equal volume of hyper buffer was added and the probes stored at -20°C with subsequent use at 1/100 dilution.

Fixed embryos were rehydrated through as a series of methanol dilutions— 75%, 50%, and 25% in PBS before being washed with PBS + 0.1% Tween20 wash (PBTw) for 10 minutes. Embryo-larvae were subsequently treated with protinease K: 48 hpf stage for 22min (1x strength), 96 hpf and 120 hpf stages for 40 mins (3x and 4x strength, respectively). Embryos were then washed repeatedly with PBTw to stop the digestion process, fixed for 1 hour in PFA and rinsed again in PBTw. A pre-hybridisation step was performed in which embryos were placed in hybridisation buffer for 3 hours and subsequently incubated with the appropriate probe (diluted 1:100) overnight at 65ºC. The embryos were then washed in 50% formamide 2xSSC, 0.1%Tween20 wash for 30-60 minutes at 65ºC, followed by a 2xSSC 0.1%Tween20 wash at 65ºC for 30 minutes and then 2x 30 minutes wash at 65ºC with 0.2xSSC 0.1%Tween 20. Non-specific binding was reduced with the addition of blocking solution (Blocking solution+5%NGS) for 3 hours. Embryos were then incubated in anti-DIG antibody (Roche; x5000, diluted 1/100 with blocking solution) for 3 hours at room temperature. A series of 4x 30minutes of PBTw washes were conducted before a 10 minute wash with AP buffer (Tris 0.1M pH 9.5, NaCl 0.1M, $MgCl_2$ 50mM, Tween20 0.1%). Embryos were transferred to a 24-well plate and placed in BM-Purple AP Substrate (Roche). Staining reactions were carried out at room temperature until signal or background staining became visible. Staining times varied depending on the probe and developmental stage tested (Table S2). Staining reactions were terminated by rinsing embryos in PBTw and fixing in fresh PFA.

Embryos were observed and photographed using Nikon SMZ1500 microscope equipped with a digital camera.

3.3.7 Transcript profiling

qRT-PCR was used to quantify the transcript profiles, in whole zebrafish samples, of a number of target genes including: thyroid receptors α and β (*thraa* and *thrb*), thyroid-stimulating hormone (*tshb*), deiodinase enzymes type I, II and III (*dio1*, *dio2*, *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). Total RNA was isolated from whole homogenised zebrafish samples using Tri-Reagent following the manufacturer's instructions. The total RNA concentration was quantified using a NanoDrop spectrophotometer and purity estimated by obtaining an absorbance ratios (A260/A280 = 1.8-2.0 and A260/A230 = 2.0-2.2) for each sample. To remove genomic DNA contamination, RNA (1 µg/µl) was treated with 1µg RQ1 RNase-Free DNase (Promega, UK) and subsequently reverse transcribed to complementary DNA (cDNA) using random hexamers (Eurofins MWG Operon, Germany) and M-MLV reverse transcriptase (Promega), according to the manufacturer's instructions. Following reverse transcription, synthesised first strand cDNA was diluted 1:2 in high performance liquid chromatography (HPLC) water and stored at -20°C until used for real-time qPCR.

Specific oligonucleotide primers for each of the selected genes in the HPT axis were designed according to the full length sequences published in GenBank and using Beacon Designer software (Table S3). Primers were purchased from Eurofins and diluted to working solutions of 10uM. BioRad CFX96 Manager was used to optimise

annealing temperatures (Ta) for selected primer pairs. Specificity was confirmed by melt curve analysis and the detection range, linearity and amplification efficiency (E) was established using serial dilutions of zebrafish larvae cDNA. In all experiments, cDNA samples were amplified in triplicate. A no-template control was included in every assay to screen for contamination of reagents. The mRNA levels of each target gene were calculated by the comparative CT method and normalised with an endogenous reference gene, ribosomal protein L8 (*rpl8*) [91]. The formula was as follows:

$$RE = (E\ Ref)^{Ct\ Ref} / (E\ Target)^{Ct\ Target}$$

where RE is relative gene expression, ref is the housekeeping gene, target is the gene of interest, and E is PCR amplification efficiency for that particular gene. Results are expressed as fold changes relative to the control. In order to assess the stability of the housekeeping gene, mean *rpl8* CT values for TBBPA and BDE-47 treatments were compared with control values (Fig. S1A and B).

3.3.8 Statistical analyses

Statistical analyses were conducted in R. The differences between the proportion of mortalities and deformities following exposure to TBBPA and BDE-47 were assessed using generalised linear models with binomial error structures. Minimum adequate models were derived by model simplification using Chisq tests based on analysis of deviance. The effect of TBBPA and BDE-47 exposures on gene transcripts in the HPT axis were analysed using general linear models with Gaussian error structures. Gene expression data was first scrutinised by Chauvenet's criterion to detect outliers for each gene and these were subsequently removed [92]. Minimum adequate models were derived by model simplification using F tests based on analysis of

deviance. A similar approach of model simplification of general linear models was used to examine the effect of co-exposure on gene transcripts following exposure to TBBPA, T3 and their interaction. When a significant effect was identified, pairwise comparisons to determine which groups differed were conducted using Tukey's honest significant difference (HSD) post hoc test. F and Chisq tests refer to the significance of removing terms from the models. All statistical models were checked for homoscedasticity and normality of residuals and data was log-transformed where necessary to ensure linearity. For all statistical analyses, differences were considered significant at $p<0.05$.

3.4 Results

3.4.1 Acute toxicity of TBBPA and BDE-47

The 96-LC-50 value of TBBPA for zebrafish embryo-larvae based on mortality was 638 μg L^{-1}. Embryos exposed to the highest concentrations of TBBPA (750 and 1,500 μg L^{-1}) had mean mortality rates of 100% after 96 hours ($p<0.01$: Fig. 1Ai). In these groups, mortality was preceded by retardation of development (smaller animals) and morphological deformities (oedemas in the pericardial region and tail, small heads, swollen yolk sac extension; Fig. 1B). Deformity data was however not taken in these two groups at 96 hpf since 100% of larvae were dead. Mean mortality rates amongst embryo-larvae exposed to 500 μg TBBPA L^{-1} were 54 ± 16% (SEM; Fig. 1Ai). At 96 hpf, 80 ± 16% (SEM) of surviving embryo-larvae exposed to 500 μg TBBPA L^{-1} for 96 hpf had presenting deformities (Fig. 1Aii), including oedema, short body, curved spine, swollen yolk sac, small eyes and craniofacial deformities (Fig.

1C). The lowest-observable-effect concentration (LOEC) in terms of mortalities and deformities in zebrafish embryo-larvae after 96 hours was 500 µg TBBPA L^{-1}.

For BDE-47, no significant effect on mortality rates was observed in the whole dose range (0.0564-5.64 mg L^{-1}) compared with the controls (Fig. 2Ai), therefore no 96-LC-50 value was calculated. The number of zebrafish larvae presenting morphological deformities increased significantly in those exposed to BDE-47 at 5.64 mg L^{-1} (41.48 ± 10.87%), 28.2 mg L^{-1} (50.99 ± 9.74%) and 56.4 mg L^{-1} (68.97 ± 11.99%) and a dose-response relationship was observed ($p<0.05$; Fig. 2Aii). At these higher BDE-47 concentrations, zebrafish embryos at 24 hpf presented deformities such as oedema, short tail and head deformities (Fig. 2B). At 96 hpf, deformities additionally included curved spines, yolk sac and craniofacial deformities (Fig. 2C). The 96-EC-50 and LOEC values for BDE-47 based on deformity rates were 4.51 mg L^{-1} and 5.64 mg L^{-1}, respectively.

3.4.2 Effect of TBBPA on gene transcripts in HPT axis

The effect of TBBPA (pools of n=10 larvae) on the transcription of genes in the HPT axis were quantified at 48, 96 and 120 hpf via qRT-PCR and also analysed by WISH at specific developmental stages (*thraa* at 48 hpf and 120 hpf, *thrb, dio1*, *dio2*, *dio3b* and *ttr* at 48 hpf and 96 hpf). TBBPA had no effect on *thraa* mRNA levels in whole body extracts (PCR analysis) at any of the developmental stages examined (48, 96 and 120 hpf) relative to controls (Fig. 3A). Via *in situ* hybridisation analysis however, after a 48 hour exposure to TBBPA *thraa* expression appeared to be higher in the brain (25, 100 and 250 µg L^{-1}) and the branchial arches (100 and 250 µg TBBPA L^{-1}) compared with controls (Fig. 3B-E). After 120 hours, no apparent effect of TBBPA

(25, 100 and 250 µg L^{-1}) on the expression pattern of *thraa* was observed compared with the controls (Fig. S3A-D).

TBBPA had no effect on the level of whole body *thrb* relative to controls after 48 and 120 hour exposures (Fig. 4A). After 96 hours, however, there was a 1.3- fold higher whole body *thrb* transcript level in TBBPA treated animals compared with controls ($p<0.05$, Fig. 4A). TBBPA had no effect of the expression pattern of *thrb* in the brain and otic vesicles in 48 hpf embryos compared with controls (Fig. S3E-H). In 96 hpf larvae, TBBPA (100 and 250 µg L^{-1}) exposure resulted in a strong (and concentration related) elevation in *thrb* expression in the liver, brain, jaw cartilage and swim bladder. There was also an enhanced expression in the otic vesicles in TBBPA (250 µg L^{-1}) treated animals compared with the controls (Fig. 4F-I). *Thrb* expression in the pronephric ducts appeared to be reduced in TBBPA treated individuals (Fig. 4Fi-Ii) with 56% of control larvae exhibiting *thrb* expression in the pronephric ducts compared to 15% of larvae exposed to 250 µg TBBPA L^{-1}.

There was no effect of TBBPA on the expression of *dio1* at 48 hpf and 120 hpf in whole body samples as determned by qRT-PCR (Fig. 5A). After 96 hours, however, exposure to 100 µg TBBPA L^{-1} resulted in a 1.5- fold higher level of *dio1* mRNA in whole body samples compared with controls ($p<0.01$; Fig. 5A). WISH analysis revealed that *dio1* expression was enhanced in the brain of TBBPA exposed (25, 100 and 250 µg L^{-1}) animals compared with controls at 48 hpf (Fig. 5B-E). At 96 hpf, *dio1* expression was enhanced in the liver and intestinal bulb in TBBPA exposed larvae (25, 100 and 250 µg L^{-1}).

TBBPA had no significant effect on the transcription of *dio2* in whole body samples at any of the developmental stages tested (Fig. 6A). However, a greater proportion of embryos exposed to TBBPA displayed a detectable *dio2* expression in the pituitary gland at 48 hpf; 52% in control versus 74% of embryos treated with 250 µg L^{-1} TBBPA (Fig. 6Bi-Ei). After 96 hours, TBBPA exposure (25, 100 and 250 µg L^{-1}) resulted in enhanced *dio2* signalling in the liver, brain and intestinal bulb compared with control larvae (Fig. 6E-I). At 96 hpf, enhanced *dio2* signalling was also observed in the pituitary gland of TBBPA treated larvae; 17% in control versus 34% of larvae treated with 250 µg TBBPA L^{-1} (Fig. 6Eii-Iii).

After 48 and 120 hour exposures, TBBPA (25, 100 and 250 µg L^{-1}) had no significant effect on *dio3b* whole body transcript levels (Fig. 7A). After 96 hours, however, there was a concentration-dependent suppression of TBBPA on *dio3b* transcription ($p<0.05$), with *dio3b* mRNA levels 1.7- , 2.8- and 3.2- fold lower in 25, 100 and 250 µg TBBPA L^{-1} treatment groups compared to control groups, respectively (Fig. 7A). Via *in situ*, no effect was seen on the expression pattern of *dio3b* in 48 hpf embryos (Fig. S3I-L) but in 96 hpf larvae treated with TBBPA *dio3b* signalling was reduced in the liver (250 µg L^{-1}; Fig. 7F-I).

TBBPA (250 µg L^{-1}) caused a significant elevation in the transcription of *ttr* in whole body samples after 48h exposure, with mRNA levels 2- fold higher compared to the controls ($p<0.05$; Fig. 8A), but there were no effects thereafter (96 and 120 hour exposures). Similarly, TBBPA had no effect on the expression pattern of *ttr* observed in the liver of 96 hpf larvae (Fig. 8B-E).

There were no effects of TBBPA exposure on the transcription of *crhb* or *tshb* in whole body samples at any of the developmental stages examined (Fig. 9A and B). For *pax8,* transcript levels were higher (1.5- fold) in whole body samples after 48 hours exposure to TBBPA (25, 100 and 250 µg L^{-1}) compared with the control ($p<0.05$; Fig. 9C), but this was not the case after 96 and 120 hour exposures (Fig. 9C). *Ugt1ab* transcription was significantly enhanced, in a concentration-dependent manner, following 48 and 96 hour exposures to TBBPA ($p<0.05$; Fig. 9D). After 48 hours, *ugt1ab* mRNA levels were 1.7- and 5.1- fold higher in the 100 and 250 µg L^{-1} TBBPA treatment groups respectively, compared to controls and after 96 hours, 2-fold higher in the 100 and 250 µg TBBPA L^{-1} treatment groups compared with controls. No significant effect of TBBPA exposure on *ugt1ab* transcription was detected after 120 hours (Fig. 9D).

3.4.3 Effect of TBBPA on T3 regulation of gene transcripts in HPT axis

T3 exposure (1, 3 and 10 µg L^{-1}) caused a significant increase in *thraa* mRNA levels in 96 hpf zebrafish larvae compared to controls ($p<0.001$), while no significant effect was observed in TBBPA treated larvae. Exposure to T3 (3 µg L^{-1}) and TBBPA (250 µg L^{-1}) together significantly elevated *thraa* mRNA levels compared to the observed induction by T3 alone (3 µg L^{-1}; $p<0.05$; Fig. 10A). Exposure to T3 (10 µg L^{-1}) and TBBPA (25 µg L^{-1}) significantly suppressed *thraa* transcript levels compared to the observed induction by T3 alone (10 µg L^{-1}; $p<0.05$; Fig.10A). T3 exposure (1, 3 and 10 µg L^{-1}) caused a significant increase in *thrb* mRNA levels in zebrafish larvae compared to controls ($p<0.001$), while no significant effect was observed in TBBPA treated larvae. Exposure to T3 (10 µg L^{-1}) and TBBPA (25 and 250 µg L^{-1}) together significantly increased *thrb* transcription compared to the induction observed by T3

alone (10 µg L^{-1}; $p<0.05$; Fig. 10B). Exposures to T3 and TBBPA alone had no significant effect on *dio2* transcription at any of the tested concentrations. Exposure to T3 (10 µg L^{-1}) and TBBPA (25 µg L^{-1}) together significantly increased *dio2* transcript levels ($p<0.05$; Fig. 10C). TBBPA (250 µg L^{-1}) significantly reduced *dio3b* transcript levels in larvae compared to controls, while T3 (1, 3 and 10 µg L^{-1}) significantly increased *dio3b* transcript levels. Exposure to T3 (10 µg L^{-1}) and TBBPA (25 µg L^{-1}) together significantly elevated *dio3b* mRNA levels compared to the increase observed by T3 alone (10 µg L^{-1}; $p<0.05$; Fig. 10D). There was no significant interaction between T3 and TBBPA on the expression of the other examined genes in the HPT axis (*dio1, ugt1ab, crhb, tshb, pax8* and *ttr*; Fig. S3).

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

BDE-47 exposure had no effect on *thraa* transcription in whole body samples as detected by qRT-PCR experiments (Fig. 11A). However, enhanced *thraa* expression was observed in the brain, liver, pronephric ducts and branchial arches of larvae exposed to BDE-47 (0.564 mg L^{-1} and 5.64 mg L^{-1}) as measured via *in situ* hybridisation (Fig. 11B-E). Following a 96- hour exposure to BDE-47 *thrb* transcription was significantly elevated in whole body samples, with mRNA levels 1.5- and 1.9- fold higher in the 0.564 and 5.64 mg L^{-1} treatment groups respectively compared with controls ($p<0.05$; Fig. 12A). In larvae exposed to BDE-47 (0.564 and 5.64 mg L^{-1}) *thrb* expression was seen to be supressed in the brain, intestinal bulb and liver, while expression in the pronephric ducts was enhanced (Fig. 12B-E). In whole body samples, *dio1* transcript levels were1.6- fold higher in BDE-47 exposed larvae (0.564 mg L^{-1}; $p<0.01$; Fig. 13A). *In situ* analysis revealed *dio1* expression was enhanced in the pronephric ducts and liver of BDE-47 exposed larvae compared

to controls (0.564 and 5.64 mg L^{-1}; Fig. 13B-E). BDE-47 had no significant effect on d*io3b* mRNA levels as observed in whole larvae samples (Fig. 14A). Via *in situ*, a greater proportion of larvae exposed to BDE-47 displayed *dio3b* expression in the pronephric ducts compared to controls; 28% and 7% of exposed larvae (0.564 and 5.64 mg L^{-1} respectively) versus 0% in control larvae (Fig. 14B-E). BDE-47 (5.64 mg L^{-1}), significantly suppressed whole body transcript levels of *ttr,* with mRNA levels reduced by 1.4- fold compared with controls ($p<0.05$; Fig. 15A). *Ttr* expression was suppressed in the liver of BDE-47 treated larvae compared to controls, while expression in the pronephric ducts appeared enhanced (0.564 and 5.64 mg L^{-1}; Fig. 15B-D). In the 0.564 mg L^{-1} BDE-47 exposure group, *dio2* transcription was 1.4- fold higher compared with controls ($p<0.05$; Fig. 16A). There was no effect of BDE-47 on the transcription of *crhb* and *tshb* as detected by qRT-PCR (Fig. 116B and 16C, respectively). Exposure to BDE-47 (0.564 and 5.64 mg L^{-1}) resulted in a significant increase in *pax8* transcription, with whole body mRNA levels 2.3- and 2.2- fold higher than controls, respectively ($p<0.01$; Fig. 16D). There was a significant increase (2- fold) in whole body *ugt1ab* transcript levels in the 0.564 mg L^{-1} BDE-47 treatment group compared with the control group ($p<0.01$; Fig. 16E).

3.5 Discussion

This study adopted zebrafish embryo-larvae as a biological model to evaluate TBBPA and BDE-47 induced developmental toxicity and gene expression changes in the HPT axis. TBBPA (≥ 500 µg L^{-1}) caused lethal effects on zebrafish larvae, slowed development and induced abnormalities such pericardial and tail oedemas, small heads and swollen yolk sac extensions. Our results are in line with those observed by previous studies and morphological abnormalities may be a result of

apoptosis and/or oxidative stress [93, 94]. BDE-47 was not lethal to zebrafish larvae after 96 hour exposures, but caused deformities such as short tails, oedemas, curved spines and craniofacial deformities (≥ 5.64 mg L^{-1}). Liu *et al*., (2015) observed similar defects in zebrafish larvae exposure to BDE-47 for a slightly longer exposure period (0-120 hpf), at a lower concentration (2.5 μM/ 1.41 mg L^{-1}) [95]. Chan and Chan (2012) report higher 96-EC-50 values for TBBPA and BDE-47 (1.09 and 20.30 mg L^{-1}, respectively), but these were based on zebrafish hatching rates, not observed morphological abnormalities [84]. In the case of TBBPA, the EC-50 values they obtained corresponded well with the lethal concentrations observed in this study.

Effects of TBBPA and BDE-47 exposures on TR expression

Thyroid receptors (TRs) are essential elements of the vertebrate TH system, mediating the thyroid system's genomic control over several key developmental processes, including growth [96], auditory [97], eye [98, 99], brain [100, 101] and skeletal [102] development. TRs act as ligand-dependent transcription factors by inducing or repressing the transcription of genes containing thyroid response elements (TREs) [103]. We found that both TBBPA and BDE-47 had no effect on *thraa* mRNA levels in whole body extracts during early zebrafish development. Similarly, zebrafish whole body TRα transcript levels were unaffected by TBBPA, BDE-47 and the commercial mixture DE-71 when exposed from the point of fertilisation [76, 84], with the exception of elevated *thraa* mRNA levels in the highest TBBPA treatment group (820 μg L^{-1}) [84]. In contrast, increased *thraa* transcript levels were observed in zebrafish larvae exposed to TBBPA and BDE-47 later in

development (from the point of hatching) [84], consistent with the hypothesis that TR regulation by endocrine disrupting chemicals may vary with life-stages [104].

BFR-induced tissue-specific TR transcriptional changes in embryo-larvae have received little attention and most studies have focused on changes in transcripts in adults (on isolated tissues) [85]. No effects were observed on *thraa* levels in whole body extracts, but in WISH analyses we found tissue-specific alterations in *thraa* expression for both TBBPA and BDE-47. *Thraa* was higher in the brain and branchial arches of TBBPA exposed embryos (48 hpf), while *thraa* expression was enhanced in the liver, brain, branchial arches and pronephric ducts of larvae (96 hpf) treated with BDE-47. These results are consistent with the findings of increased TRα transcript levels in the brain of adult fathead minnows following dietary exposure to BDE-47 and BDE-209 for 21 and 28 days, respectively [72, 85].

We found that exposure to both TBBPA and BDE-47 resulted in higher levels of *thrb* mRNA in whole zebrafish larvae exposed for 96 hpf. This contrasts with previous studies, which found that various BFRs (BDE-47, TBBPA or DE-71) either had no effect or decreased levels of TRβ mRNA transcripts in whole body zebrafish larvae samples, depending on the exposure period [76, 84]. Similarly, in adult fathead minnows, dietary exposure to BDE-47 and BDE-209 was found to lower TRβ mRNA levels in the brain and livers, respectively [85, 105]. To our knowledge no previous study has examined the tissue-specific effects of these compounds during early fish development. We found that *thrb* expression was enhanced in the liver, brain, jaw cartilage, otic vesicle and swim bladder of larvae following a 96 hour exposure to TBBPA. Meanwhile *thrb* expression was suppressed in the liver, brain, intestinal bulb

and otic vesicle of BDE-47 exposed larvae but induced in the pronephric ducts. Taking these results together, it appears that TBBPA and BDE-47 are capable of altering *thrb* expression and these changes may be age-related, influenced by congener-specific differences in PBDEs and/or depend on the stability of housekeeping genes used to normalise expression data.

Whether the BFR-induced changes in TR transcripts observed here translate to altered TH-dependant developmental processes remains unclear. As far as we are aware Essner *et al*., (1999) is the only study to examine the effect of overexpression of TRα1 in zebrafish and report disrupted hindbrain patterning in embryos [106]. Based on the observed transcriptional changes of TRs, our results suggest that TH signalling in the brain, as well as the liver and jaw cartilage are sensitive to TBBPA and BDE-47 during early zebrafish development. Several studies have shown that BFRs can negatively impact limb and vertebrae ossification as well as neural functions and behaviours in fish, rodents and humans [107-113], though the exact mechanisms involved in these effects are not fully understood. Given that hypothyroidism has previously been associated with impaired bone and neural development [114, 115], it is hypothesised that altered TH-signalling may play a critical role. It is also interesting to note that abnormal expression and/or mutations in TR genes in mammals have been associated with carcinogenesis [116].

The mechanisms by which TBBPA and BDE-47 altered both TR transcripts in this study are not fully understood. Changes may be as a result of BFR-induced alterations in circulating and/or local TH levels or a result of a direct interaction with TRs or their co-repressors/activators (since the genes encoding TRs themselves

contain TREs). Since several i*n vitro* studies have demonstrated that BFRs have limited agonistic effects on TR-mediated gene transcription [117-119] and the observed increase in *dio2* expression in the brain of zebrafish larvae, it seems plausible that the TBBPA and BDE-47 induced increases in *thraa* and *thrb* transcription were a result of increased local T3 levels.

Effects of TBBPA and BDE-47 exposures on deiodinase expression

We also examined the effect of TBBPA and BDE-47 exposure on the transcription of genes encoding the deiodinase enzymes (D1, D2 and D3). The deiodinase enzymes play a key role in regulating both the systemic and peripheral levels of THs in vertebrates by selectively removing iodide from T4 and its derivatives, thus activating or inactivating these hormones [78]. As far as we are aware no previous studies have examined the effect of TBBPA on deiodinase genes in fish or any other vertebrate species. D3 is the major inactivating pathway, catalysing the metabolism of both T3 and T4 by IRD. Thyroidal status often parallels hepatic D3 activity and *dio3b* transcript levels in fish species, increasing during hyperthyroidism and decreasing during hypothyroidism (reviewed in [78]). We found that TBBPA (25, 100 and 250 µg L^{-1}) exposure resulted in a reduced *dio3b* mRNA level in whole larval samples, and in the liver of *in situ* samples (250 µg L^{-1}). Given that *dio3b* mRNA expression changes are often reflected in the level of protein product [120], our results suggest a systemic decrease in D3 activity and reduced deactivation of THs in TBBPA exposed larvae. It was not possible to measure T3 and T4 levels in this study, however, this is likely a compensatory mechanism in response to decreased TH levels. Interestingly, we found that BDE-47 had no effect on *dio3b* transcript levels in whole body samples or in the liver (*in situ* samples) but there were higher

levels in the pronephric ducts. Increased renal D3 activity as a result of increased *dio3b* expression in the pronephric ducts may be a reflection of increased local T3 production (*dio1* expression also increased in pronephric ducts).

D2 is the major TH-activating enzyme catalysing the conversion of T4 to the bioactive T3 by ORD. Thyroidal status is the main factor controlling D2 activity both at the pre-transcriptional and post-transcriptional levels, with hyperthyroidism in general suppressing D2 activity and *dio2* mRNA levels and hypothyroidism increasing them [121, 122]. While D1 is capable of both activating and deactivating THs by ORD and IRD respectively [77], increased hepatic D1 activity and increased *dio1* transcription have been shown to be indicative of hypothyroid conditions in Nile tilapia (*Oreochromis niloticus*) [120]. This suggests that hepatic D1 functions to activate THs in fish, in contrast to the regulation and function of D1 observed in mammals where the opposite has been shown [123]. In this study, TBBPA exposure resulted in a higher transcript level of *dio1* in whole larval samples (96 hpf), in the brain of embryos (48 hpf), and the liver and intestinal bulb of larvae (96 hpf). We also found that exposure to TBBPA, while having no effect on *dio2* expression in whole zebrafish embryo-larvae at any stage examined, resulted in higher levels of *dio2* expression in the pituitary of embryos (48 hpf) and in the brain, pituitary and intestinal bulb of larvae (96 hpf). The higher level of *dio1* transcripts in the liver of TBBPA treated larvae is strong evidence of an induced systemic hypothyroid condition [120]. Meanwhile, the elevated *dio1* and *dio2* mRNA levels in the brain and intestinal bulb are likely associated with reduced local T3 levels and may be compensatory responses to maintain local TH homeostasis during the development of neurological and gastrointestinal tissues. It is well known that even minor

fluctuations in TH availability can have profound effects on brain development [124], therefore any delayed onset of this compensatory increase in T3 production may have a detrimental effect on motor and cognitive functions. The increased expression of *dio2* in the pituitary of TBBPA exposed embryo-larvae is not fully understood. In fish under normal physiological regulations, reduced circulating TH levels leads to increased stimulation and secretion of TSH from the pituitary through the negative feedback system of the HPT axis [82]. An increased conversion of T4 into T3 in the pituitary (catalysed by increased D2 levels) may deplete local T4 levels, inducing the negative feedback loop and increasing the production of THs by the thyroid follicles. Similar to that for TBBPA, we found that BDE-47 exposure resulted in higher levels of *dio1* and *dio2* transcripts in zebrafish larvae exposed for 96 hours, again indicative of induced hypothyroidism. Depending on the life stage examined, exposure period and compound tested, there appears to be varying effects on *dio1* and *dio2* transcript levels by various BFRs in fish. The increased *dio1* and *dio2* transcripts observed here is in agreement with results observed in adult fathead minnows exposed to BDE-209 [85] and zebrafish larvae/adults exposed to DE-71 [76], BDE-209 [125] and BDE-47 [126]. However, alternative effects were observed in zebrafish juveniles exposed to BDE-47 [127] and zebrafish adults and offspring exposed to DE-71 [75]. Our results suggest that altering the expression of deiodinase genes could be a potential mechanism by which TBBPA and BDE-47 disrupts/corrects TH homeostasis.

The induction of uridine diphosphate glucoronosyltransferases (UDPGTs) has been implicated in disrupting circulating TH levels [128]. UDPGTs catalyse the conjugation of THs with glucuronic acid in the liver and this process is an important metabolic

prerequisite for their excretion into bile [129]. In our study, elevated *ugt1ab* mRNA levels were detected in whole zebrafish samples exposed to TBBPA (at 48 hpf and 96 hpf) and BDE-47 (at 96 hpf). Provided mRNA expression changes are reflected in the level of protein product, increased *ugt1ab* expression might lead to the increased elimination of THs and is a plausible explanation for the altered TH signalling observed throughout the HPT axis. This is consistent with previous findings in mammals and fish which report reduced circulating levels of T4 combined with increased UDPGT activity and/or transcription following exposure to BFRs [69, 76, 130]. It should be noted that glucuronidation is also an important detoxifying mechanism involved in the metabolism and elimination of various xenobiotic compounds in fish [131], which may also explain the increased *ugt1ab* expression observed here.

Pax8 proteins are essential for the late differentiation of the thyroid follicular cells during development [132]. Elevated levels of *pax8* transcripts were observed here in zebrafish embryo-larvae following exposures to TBBPA (at 48 hpf) and BDE-47 (at 96 hpf). These results suggest the promotion of thyroid primordium growth and possibly a compensation mechanism for reduced TH levels. Similar findings have been reported in zebrafish embryos/larvae exposed to various other pollutants [76, 133, 134], though no studies to our knowledge have yet examined the effect of TBBPA on *pax8* transcription.

TTR is proposed to be the major TH carrier protein in fish [135] and plays a key role in maintaining extra-thyroidal stores of TH and regulating its supply to various target tissues [136]. Here we found that TBBPA significantly increased *ttr* transcripts levels

in 48 hpf embryos while BDE-47 significantly decreased *ttr* transcript levels in 96 hpf larvae. Our results are consistent with previous studies which found increased and decreased *ttr* mRNA levels in zebrafish larvae following exposure to TBBPA [84] and BDE-47 respectively [84, 126]. Similarly, exposure to BDE-209 resulted in reduced levels of *ttr* at the transcriptional and translational levels in zebrafish larvae [125], while DE-71 suppressed expression of *ttr* in the liver of zebrafish adults after long-term exposures [76]. *In vitro* studies have demonstrated that TBBPA potently competes with T4 for binding sites on human TTR proteins [137] and with T3 on sea bream TTR proteins [138]. Higher TTR protein levels increases the substrate available for TBBPA binding, making the displaced and unbound THs in the plasma more susceptible to hepatic metabolism. This could lead to increased THs clearance rates and therefore suppress circulating levels of T4/T3 [139]. While PBDEs have been shown to compete for human T4-TTR only after metabolic conversion by liver microsomes [137], BDE-47 was found to be potent inhibitor of sea bream T3-TTR binding [138]. Reduced concentrations of circulating TTR proteins may limit T4-TTR binding and, combined with a potential increase in displaced T3, may increase hepatic metabolism and hinder T4 transport to target organs. Both of these outcomes are supported by the aforementioned observed increase in *ugt1ab* transcript levels. The altered transthyretin mRNA expression by TBBPA and BDE-47, suggest that while induction of UGTs may be partly responsible for disrupting systemic TH levels, other mechanisms may also be involved.

Corticotropin-releasing hormone (CRH) and thyroid-stimulating hormone (TSH) regulate THs synthesis and secretion in fish through a negative feedback mechanism within the HPT axis, triggered via alteration in circulating T4 levels [140].

It is therefore believed that measurements of their gene expression are good indicators of thyroid dysfunction. Interestingly, in the present study, both TBBPA and BDE-47 had no effect on *tshb* and *crhb* transcript levels in zebrafish embryo-larvae. Similarly no effect on *tshb* mRNA levels was observed in juvenile zebrafish exposed to BDE-47 for 40 days [127]. However the expression of *crhb* and *tshb* was elevated in fathead minnows exposed to BDE-47 [105] and in zebrafish exposed to DE-71 [76], BDE-209 [125], TBBPA [84] and BDE-47 [126]. Increased *crhb* and *tshb* expression in these studies were accompanied by reduced T4 levels. Interestingly, recent studies have reported lower *crhb* and/or *tshb* transcript levels in zebrafish adults, embryos and larvae exposed to TBBPA [84] and BDE-47 [75, 84], associated with increased T4 and T3 levels [75]. From these studies, it appears evident that BFRs have varying effects on the expression of CRH and TSH genes in fish, highlighting the importance of examining the effect of TDC on multiple levels of the HPT axis.

Given the wide range of tissues influenced by THs during early development and the complex nature involved in regulating the TH system, it is important to assess the effect of TDCs on the tissue-specific expression patterns of thyroid gene mRNAs. To date, assessing transcriptional changes in fish embryo-larvae following exposure to BFRs have largely relied on qRT-PCR, while tissue-specific effects have been limited to adult studies where isolating organs/tissues of interest was relatively straightforward. In the present study, we examined the effects of TBBPA and BDE-47 on transcription of several thyroid gene of interest simultaneously using WISH and qRT-PCR. While qRT-PCR provides quantitative data on gene expression and is sensitive at detecting even small changes in mRNA levels, when carried out on

pooled homogenates of whole embryo-larvae, transcriptional changes in small and/or localised tissues may not be detected if diluted by non-responsive organ/tissues of the whole animal. Here, the transcription of several genes in the HPT axis appeared unaffected by BFR exposures when examined using qRT-PCR but tissue-specific effects were observed using WISH. These included *thraa* (at 48 hpf; TBBPA and at 96 hpf; BDE-47) *dio1* (at 48 hpf; TBBPA), *dio2* (at 48 and 96 hpf; TBBPA) and *dio3b* (at 96 hpf; BDE-47). While WISH has only recently been used in ecotoxicology studies, here demonstrates its values as an approach for understanding the effects of TDCs at the tissue level.

We also examined the effect of TBBPA exposure on thyroid gene transcripts at three early developmental stages (48, 96 and 120 hpf) and found that effects were developmental stage specific. TBBPA-induced alterations to *thrb*, *dio1* and *dio3b* transcription were observed in 96 hpf larvae while *ttr* and *pax8* mRNAs were altered in 48 hpf embryos only. Finally, *ugt1ab* expression was altered at 48 and 96 hpf. TBBPA had no effect on any of the genes examined following 120 hour exposures. This study is one of only a few that examined the effect of BFRs on thyroid mRNA transcription at different life stages. Similar to our findings, several genes in the HPT axis were found to be transiently altered in the liver and/or brain of adult male fathead minnows exposed to BDE-209 [85]. The TH system is regulated by sophisticated compensatory mechanisms both centrally (negative feedback loop in HPT axis) and peripherally (by deiodinase enzymes), which if adequate can normalise both serum and peripheral TH concentrations [141]. These compensatory mechanisms may explain why no transcriptional effects were detected here in 120 hpf larvae. It is also important to note that increased endogenous T3 levels are

associated with the embryo-larval transition of teleost fish, and this natural increase may intensify the effects of BFRs on the thyroid system at this life stage. We observed that zebrafish at 96 hpf, a period coinciding with increased TH levels, were particularly sensitive to TBBPA exposure.

In general, a chemical is believed to have TH agonistic activity if it promotes TH-responsive gene expression. On the contrary, it is suggested to have TH antagonistic activity if it inhibits spontaneous or TH-induced expression of TH-responsive genes. With this in mind and based on our previous study which examined T3-responsive genes (Chapter 2), the effects of TBBPA on gene transcription observed here could be classified as having T3 agonistic effects, T3 antagonistic effects, or having no significant effects. Therefore we hypothesised that exposure to TBBPA, in the presence of T3, might have a stimulatory effect on the transcription of a number of TH-regulated genes (*thraa, thrb* and *pax8*) and an antagonistic effect on others (*dio1*, *dio3b* and *ugt1ab*). As expected we found that TBBPA clearly enhanced T3-induced up-regulation of *thrb* transcription in zebrafish larvae at 96 hpf, while TBBPA had both stimulatory and suppressive effects on T3-induced up-regulation of *thraa* transcription. Interestingly, TBBPA and T3 co-exposure had no effect on *pax8, dio1* or *ugt1ab* transcript levels as was predicted (along with other genes such as *ttr, tshb* and *crhb*). Unexpectedly, TBBPA had a stimulatory effect on *dio2* and *dio3b* transcription when exposed with T3. As far as we are aware, no previous studies have examined the effect of TBBPA on TH-responsive genes in fish. As opposed to the observed stimulatory effect of TBBPA on TH-responsive genes observed in the present study, Jagnytsch *et al.,* (2006) found that a 21- day exposure to TBBPA induced the expression of TRβ mRNAs

(along with basic region leucin zipper transcription factor, b/Zip) in the head tissues of tadpole *Xenopus laevis*, but inhibited their T3-induced elevation in co-exposed groups [142]. Similarly, several *in vitro* reporter gene systems found that TBBPA acted as an agonist on TH action in the absence of TH but as an antagonist in the presence of TH induction [143, 144]. A study by Zhang *et al.,* (2014) found that TBBPA alone had no effect on the expression of TRβ and Dio2 mRNAs (along with genes encoding basic transcription element binding protein and matrix metalloproteinase 2) in the intestine and hindlimbs of *X. laevis* tadpoles after a 6-day exposure, but inhibited T3-induced up-regulation of these genes. The same study also reported that lower concentrations of TBBPA promoted T3-induced stromelysin-3 expression (involved in metamorphic development), but higher concentrations had inhibitory effects [145]. It is apparent that the effect of TBBPA on TH signalling is extremely complex and warrants further study. Given that the effect of TBBPA on TH signalling has been shown to be dependent on endogenous TH levels [145], a more detailed examination of TBBPAs effect on TH-responsive genes across various zebrafish developmental stages, as well as tissues, is required in order to increase our understanding of TBBPAs mechanisms of toxicity and potential health effects.

Summary

In summary, the present study demonstrates that the brominated flame retardants, TBBPA and BDE-47, can disrupt the thyroid axis of zebrafish embryo-larvae at multiple levels by altering mRNA transcripts of genes encoding thyroid receptors, deiodinase enzymes, thyroid synthesis proteins and glucuronidation enzymes. We have also shown that both compounds induced tissue-specific transcriptional

changes for several genes in the HPT axis, with TH signalling in the brain, liver, pronephric duct and craniofacial tissues appearing particularly sensitive to TBBPA and BDE-47 exposures. Furthermore, TH disruption by TBBPA appeared more pronounced in larvae at 96 hpf, compared to 48 and 120 hpf. These results demonstrate the effects of BFRs on the thyroid system should not be generalised across tissues or developmental stages in fish species. Future work should focus on understanding the consequences of chronic low level exposure to BFRs and examine whether the BFR-induced changes in TR transcript levels in the brain and skeleton as observed here translate into health consequences for wild fish populations and humans.

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Figures

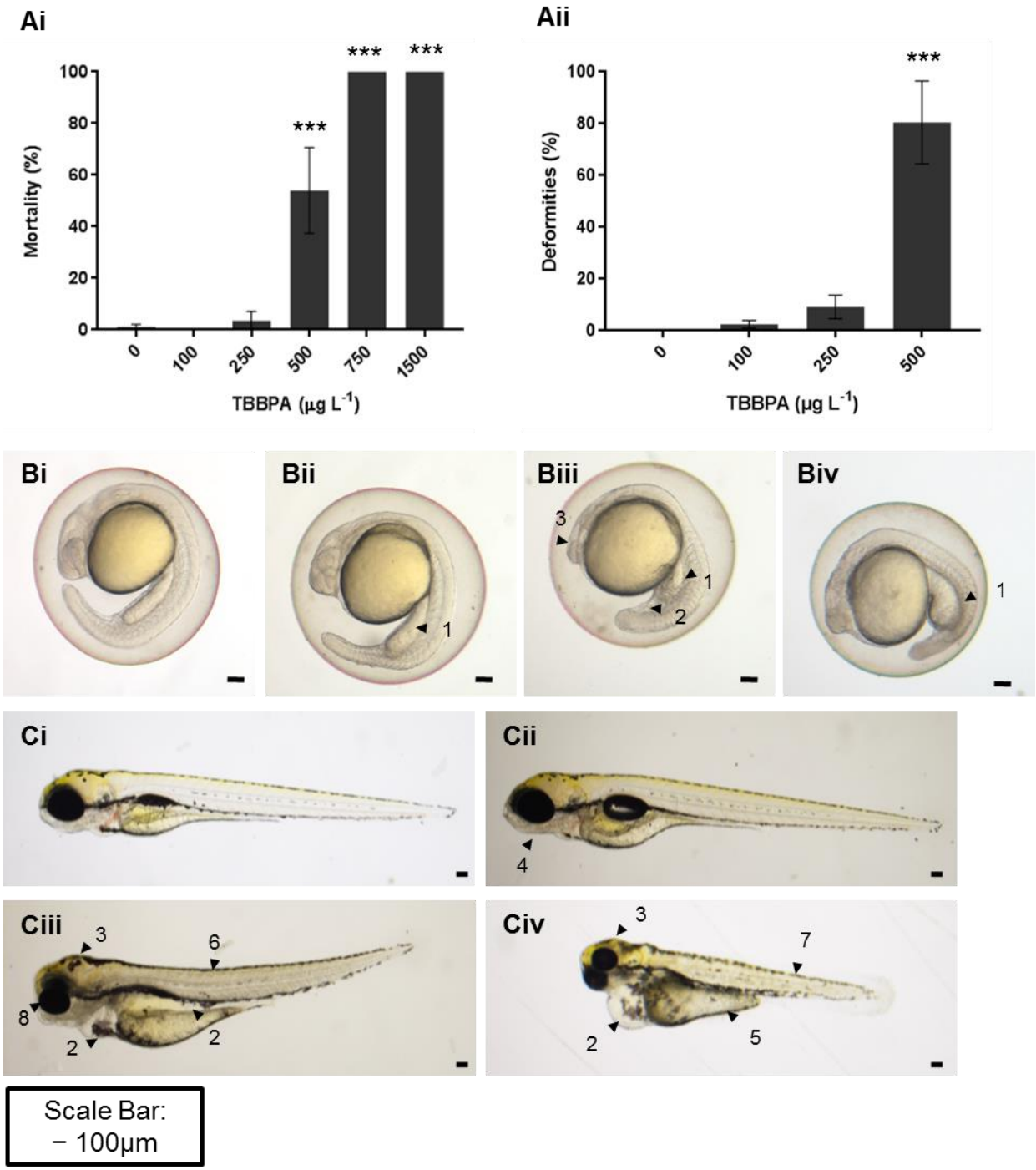


Figure 1. (A) The effect of TBBPA on (i) percentage mortality and (ii) percentage of morphological deformities in zebrafish larvae following exposure for 96 hours post fertilisation (hpf). The results are represented as means ± SEM. Asterisks represent significant differences between treatment groups compared to the control group

(***p<0.001; GLM). Statistics were carried out using generalised linear models in R, with model details reported in Table S4. (B) Images of zebrafish embryos at 24 hpf exposed to (i) 0 µg TBBPA L^{-1}, (ii) 100 µg TBBPA L^{-1}, (iii) 750 µg TBBPA L^{-1} and (iv) 1500 µg TBBPA L^{-1}. (C) Images of zebrafish larvae at 96 hpf exposed to TBBPA at (i) 0 µg L^{-1}, (ii) 100 µg L^{-1}, (iii) 250 µg L^{-1} and (iv) 500 µg L^{-1} showing- 1. Swollen yolk sac extension, 2. Oedema, 3. Head deformity, 4. Lower jaw deformity, 5. Yolk sac deformity, 6. Bent spine, 7. Short Tail and 8. Small Eyes. Scale bar=100 µm.

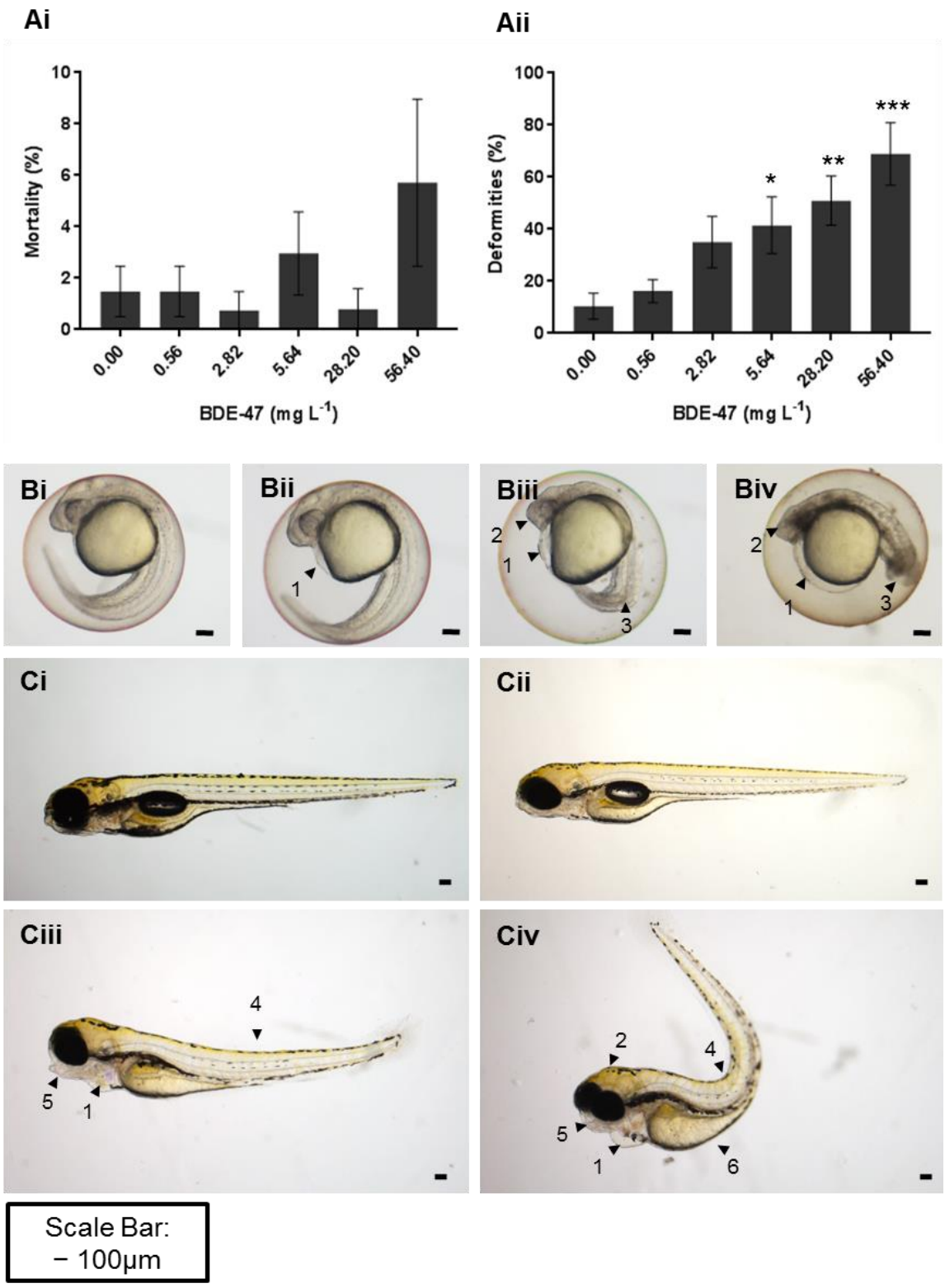
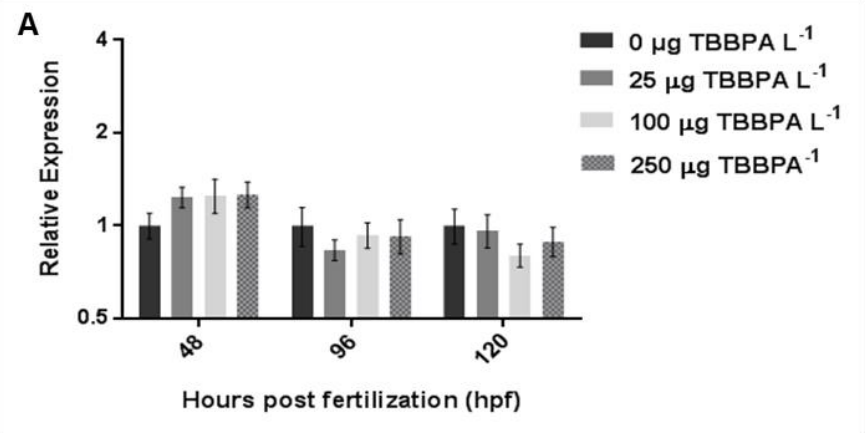


Figure 2. (A) The effect of BDE-47 on (i) percentage mortality and (ii) percentage of morphological deformities in zebrafish larvae following exposure for 96 hours post fertilisation (hpf). The results are represented as means ± SEM. Asterisks represent

significant differences between treatment groups compared to the control group (*p<0.05, **p<0.01 and ***p<0.001; GLM). Statistics were carried out using generalised linear models in R, with model details reported in Table S4. Images of zebrafish embryo-larvae at (B) 24 hpf and (C) 96 hpf following exposure to BDE-47 at (i) 0 µg L^{-1} (ii) 0.564 µg L^{-1}, (iii) 5.64 µg L^{-1} and (iv) 56.4 µg L^{-1} and showing— 1. Oedema, 2. Head deformity, 3. Short tail, 4. Bent spine, 5. Lower jaw deformity and 6. Yolk sac deformity. Scale bar=100 µm.



F

TBBPA Treatment	n	B.A	Brain
0 µg L^{-1}	14	86%	100%
25 µg L^{-1}	12	93%	100%
100 µg L^{-1}	17	81%	100%
250 µg L^{-1}	14	50%	100%

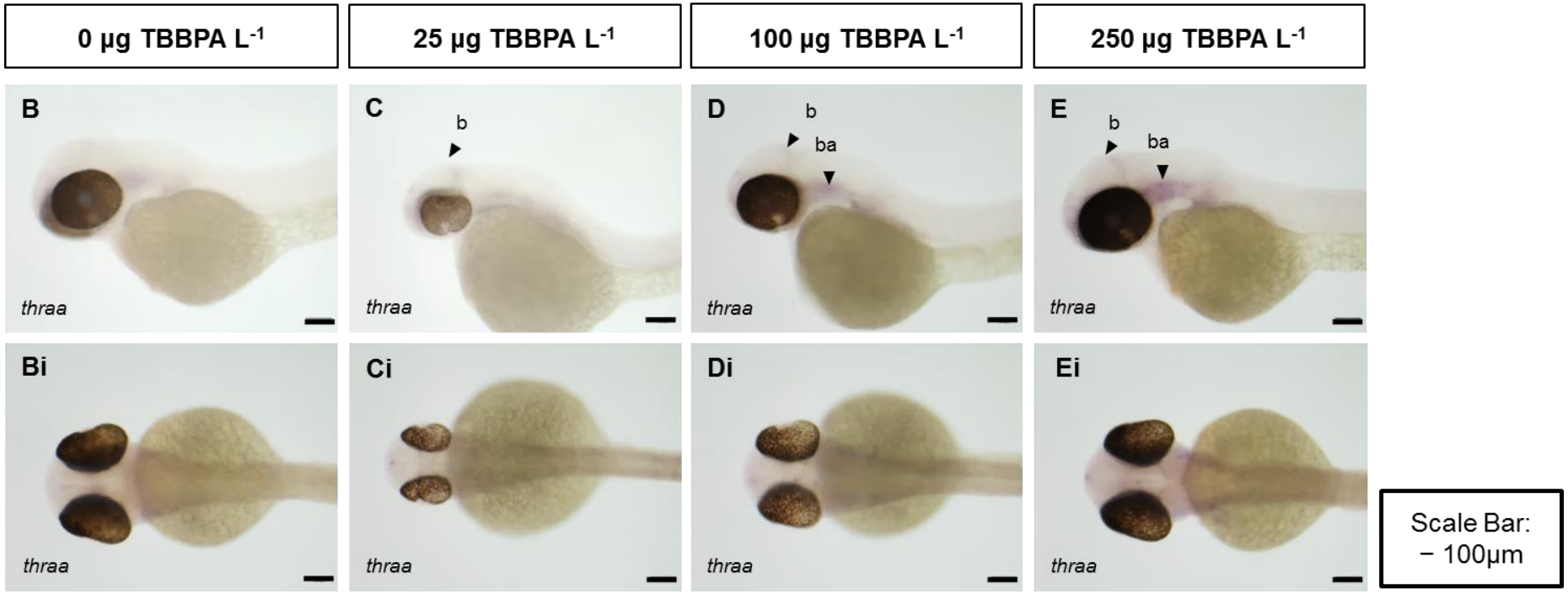
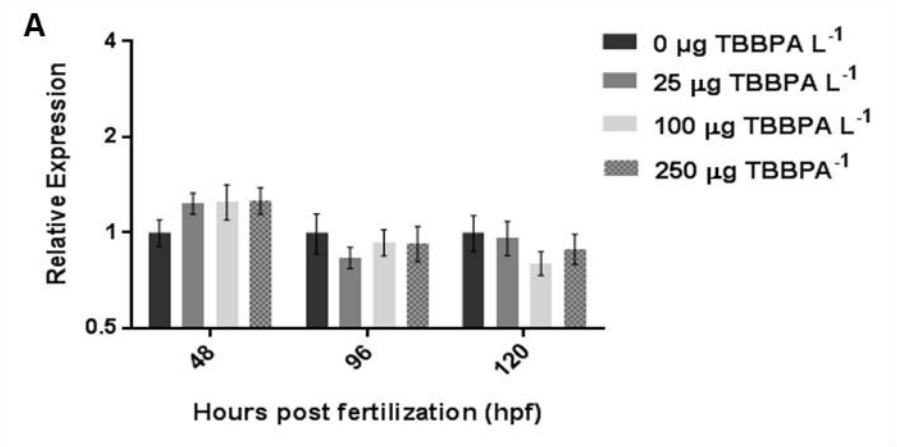


Figure 3. (A) Transcript profile of thyroid receptor alpha (*thraa*) in whole zebrafish following exposure to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised sample per treatment group. There was no significant difference between TBBPA treatment groups compared to the control group at all stages examined. (B-E) Representative images of *thraa* mRNA expression patterns in zebrafish embryos at 48 hpf treated with TBBPA (0, 25, 100 and 250 µg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole embryos are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, ba=branchial arches. Scale bar=100 µm. (F) Summary of the variability in *thraa* mRNA expression in different zebrafish tissues [branchial arches (B.A) and brain] following exposure to TBBPA for 48 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.



F

TBBPA Treatment	n	B.A	Brain
0 µg L^{-1}	14	86%	100%
25 µg L^{-1}	12	93%	100%
100 µg L^{-1}	17	81%	100%
250 µg L^{-1}	14	50%	100%

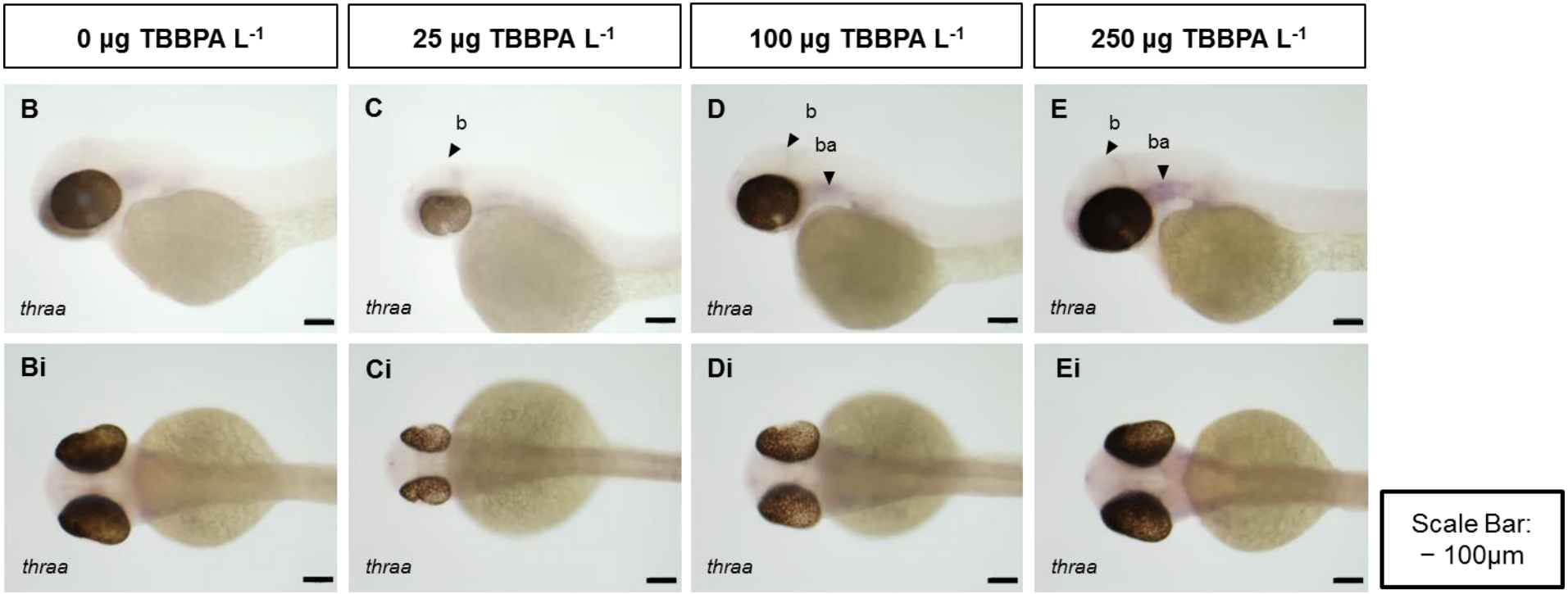
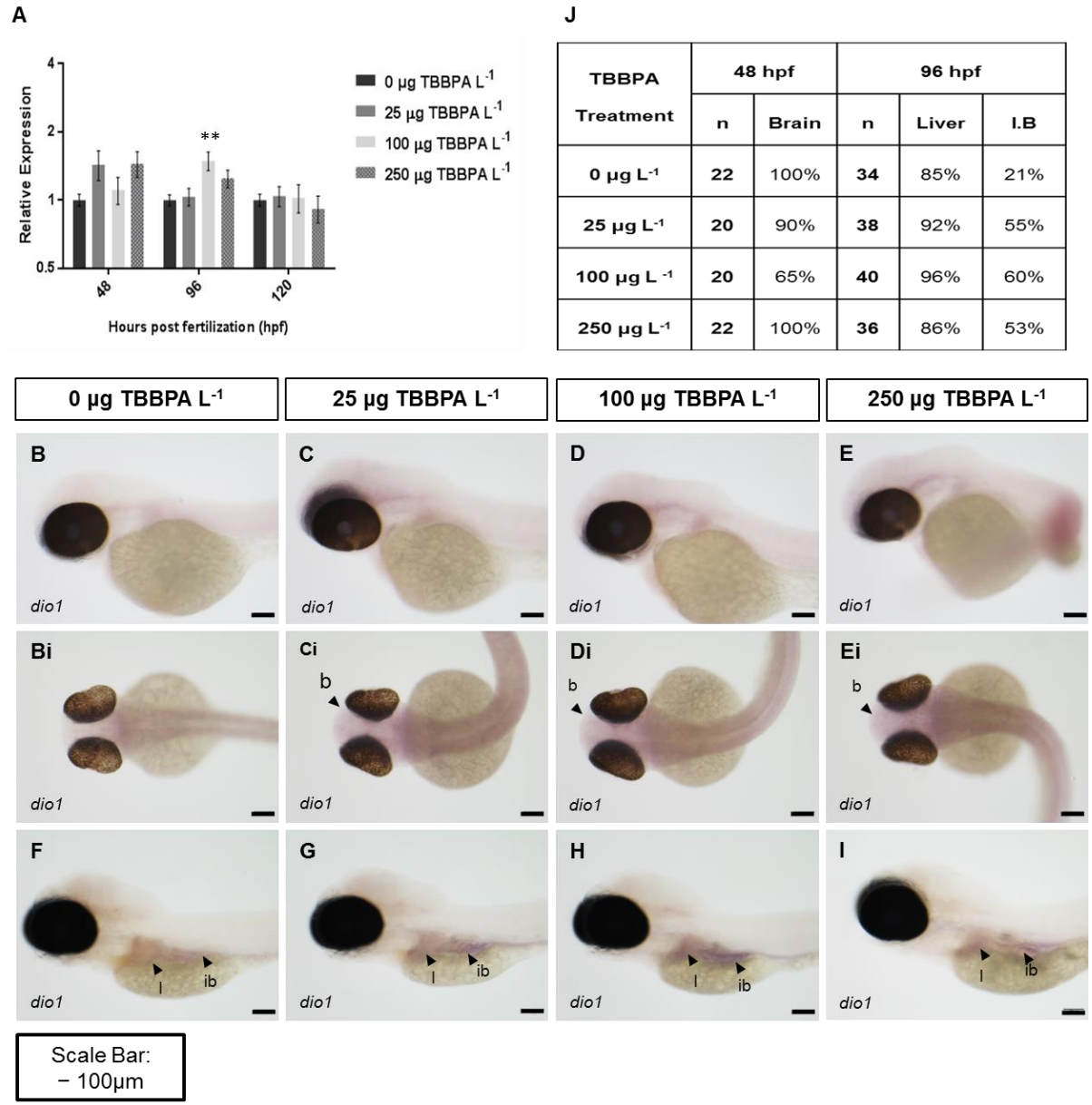


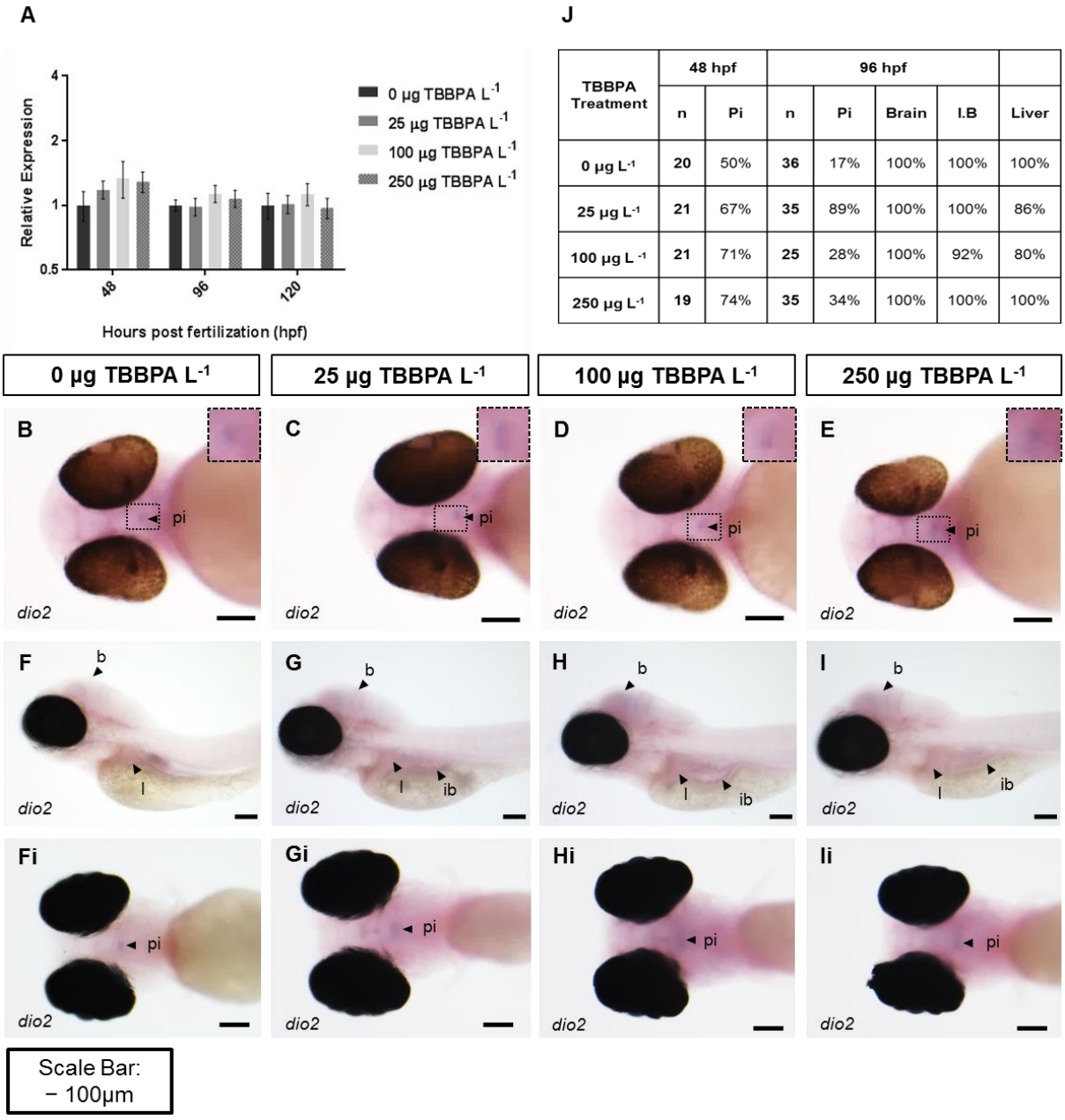
Figure 4. (A) Transcript profile of thyroid receptor beta (*thrb*) in whole zebrafish embryo-larvae following exposure to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised sample per treatment group. Exposure to TBBPA for 96 hpf resulted in a significant increase in *thrb* mRNA levels at all concentrations tested (25, 100 and 250 µg L^{-1}) compared to controls. There was no significant difference between TBBPA treatment groups compared to the control group at 48 and 120 hpf. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-E) Representative images of *thrb* mRNA expression patterns in zebrafish larvae at 96 hpf treated with TBBPA (0, 25, 100 and 250 µg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, ov=otic vesicle, l=liver, sb=swim bladder, pd=pronephric duct, jc=jaw cartilage. Scale bar=100 µm. (F) Summary of the variability in *thrb* mRNA expression in different zebrafish tissues [pronephric ducts (P.D), swim bladder (S.B), otic vesicle (O.V), jaw cartilage (JC), brain and liver] following exposure to TBBPA for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.



TBBPA Treatment	48 hpf		96 hpf		
	n	Brain	n	Liver	I.B
0 µg L^{-1}	22	100%	34	85%	21%
25 µg L^{-1}	20	90%	38	92%	55%
100 µg L^{-1}	20	65%	40	96%	60%
250 µg L^{-1}	22	100%	36	86%	53%

Figure 5. (A) Transcript profile of deiodinase type I (*dio1*) in whole zebrafish following exposure to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised sample per treatment group.

Exposure to TBBPA (100 µg L^{-1}) for 96 hpf resulted in a significant increase in *dio1* mRNA levels compared to controls. There was no significant difference between TBBPA treatment groups compared to the control group at 48 and 120 hpf. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-I) Representative images of *dio1* mRNA expression patterns in zebrafish embryo-larvae at 48 hpf (B-E) and 96 hpf (F-I) treated with TBBPA (0, 25, 100 and 250 µg L^{-1}. Lateral (B-I) and dorsal (Bi-Ei) views of whole embryo-larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, l=liver, ib=intestinal bulb. Scale bar=100 µm. (J) Summary of the variability in *dio1* mRNA expression in different zebrafish tissues [brain, liver and intestinal bulb (I.B)] following exposure to TBBPA for 48 and 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

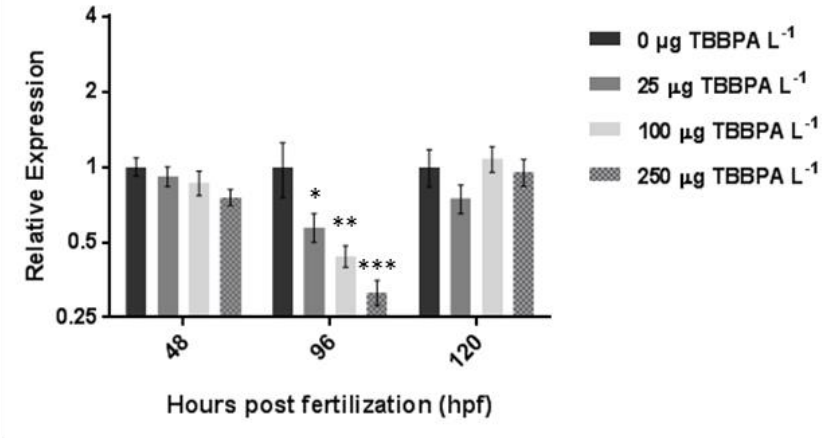


TBBPA Treatment	48 hpf		96 hpf				
	n	Pi	n	Pi	Brain	I.B	Liver
0 µg L^{-1}	20	50%	36	17%	100%	100%	100%
25 µg L^{-1}	21	67%	35	89%	100%	100%	86%
100 µg L^{-1}	21	71%	25	28%	100%	92%	80%
250 µg L^{-1}	19	74%	35	34%	100%	100%	100%

Figure 6. (A) Transcript profile of deiodinase type II (*dio2*) in whole zebrafish embryo-larvae following exposure to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from

the analysis, resulting in a replication of n=6-9 homogenised sample per treatment group. There were no significant differences between TBBPA treatment groups compared to the control group at all stages examined. (B-I) Representative images of *dio2* mRNA expression patterns in zebrafish embryo-larvae at 48 hpf (B-E) and 96 hpf (F-I) treated with TBBPA (0, 25, 100 and 250 µg L^{-1}). Lateral (F-I) and ventral (B-E; Fi-Ii) views of whole embryo-larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. l=liver, ib=intestinal bulb, b=brain, pi=pituitary. Scale bar=100 µm. (J) Summary of the variability in *dio2* mRNA expression in different zebrafish tissues [intestinal bulb (I.B), pituitary (Pi) brain and liver] following exposure to TBBPA for 48 and 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

A



F

TBBPA Treatment	n	Liver
0 µg L-1	42	81%
25 µg L-1	33	82%
100 µg L -1	44	98%
250 µg L-1	36	92%

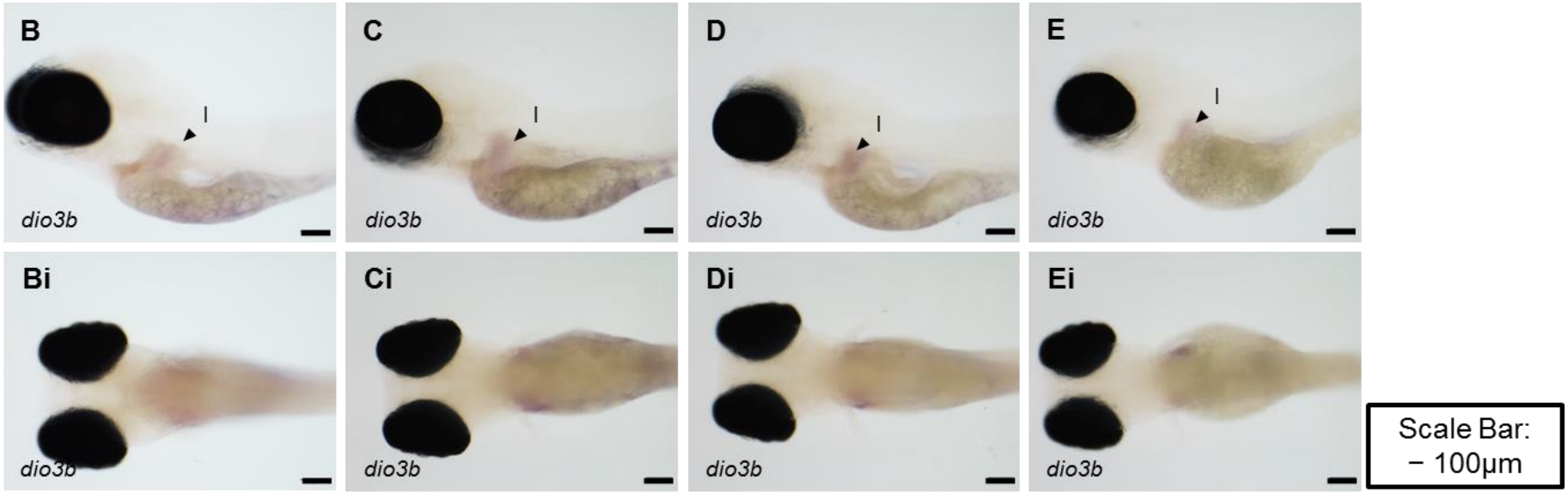
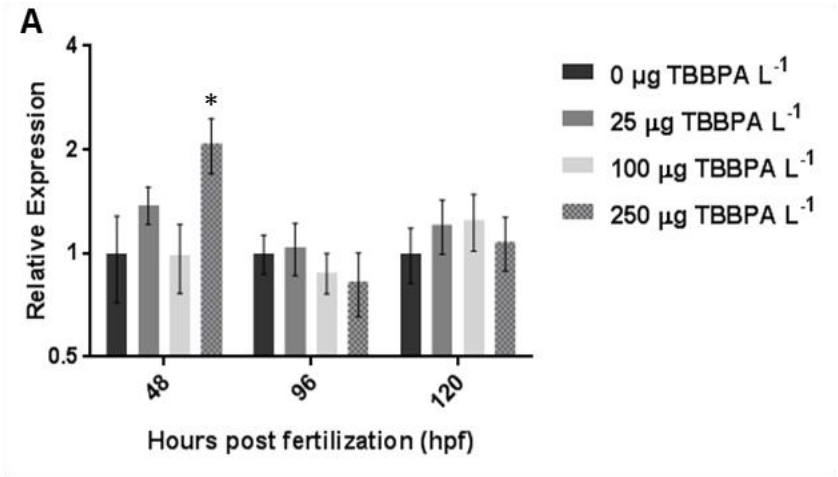


Figure 7. (A) Transcript profile of deiodinase type III (*dio3b*) in whole zebrafish embryo-larvae following exposure to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised sample per treatment group. Exposure to TBBPA (25, 100 and 250 µg L^{-1}) for 96 hpf resulted in a significant decrease in *dio3b* mRNA levels. There was no significant difference between TBBPA treatment groups compared to the control group at 48 and 120 hpf. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-I) Representative images of whole mount expression patterns of *dio3b* mRNA in zebrafish larvae at 96 hpf (B-E) treated with TBBPA (0, 25, 100 and 250 µg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. l=liver. Scale bar=100 µm. (F) Summary of the variability in *dio3b* mRNA expression in different zebrafish liver following exposure to TBBPA for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.



F

TBBPA Treatment	n	Liver
0 µg L-1	30	67%
25 µg L-1	31	94%
100 µg L -1	32	100%
250 µg L-1	30	97%

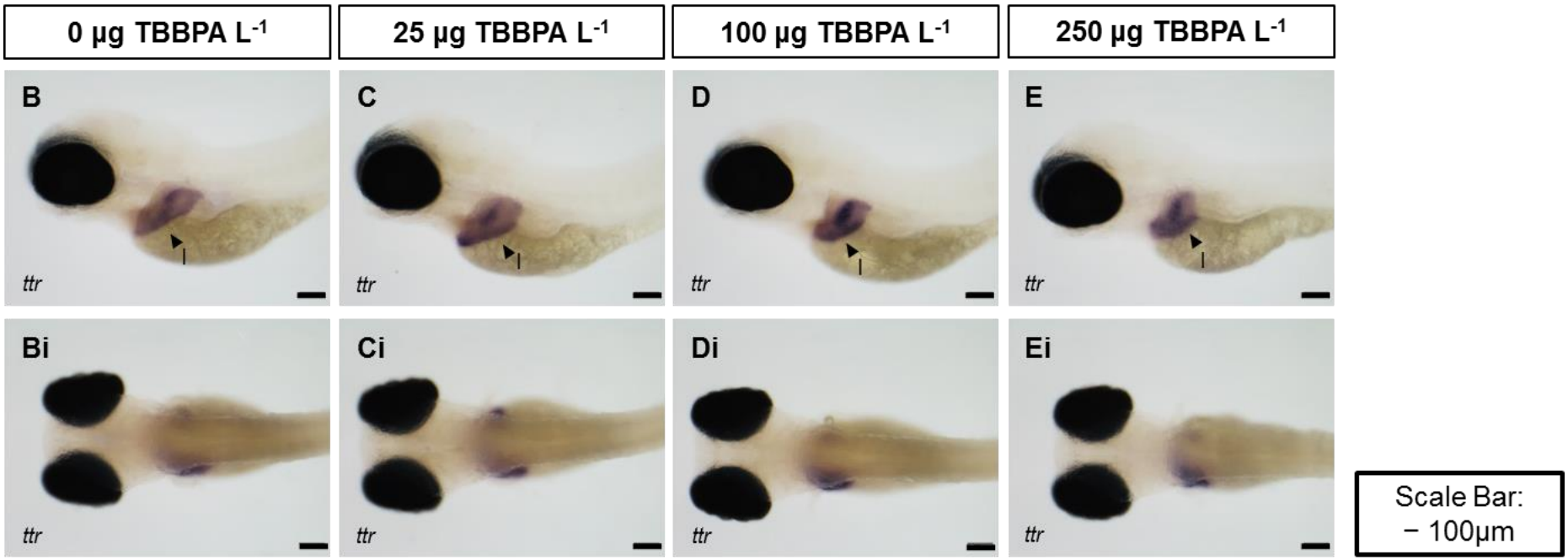


Figure 8. (A) Transcript profile of transthyretin (*ttr*) in whole zebrafish embryo-larvae following exposure to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised sample per treatment group. Exposure to TBBPA (250 µg L^{-1}) for 48 hpf resulted in a significant increase in *ttr* mRNA levels. There was no significant difference between TBBPA treatment groups compared to the control group at 96 and 120 hpf. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-E) Representative images of *ttr* mRNA expression patterns in zebrafish larvae at 96 hpf treated with TBBPA (0, 25, 100 and 250 µg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. l=liver. Scale bar=100 µm. (F) Summary of the variability in *ttr* mRNA expression in different zebrafish tissues liver following exposure to TBBPA for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

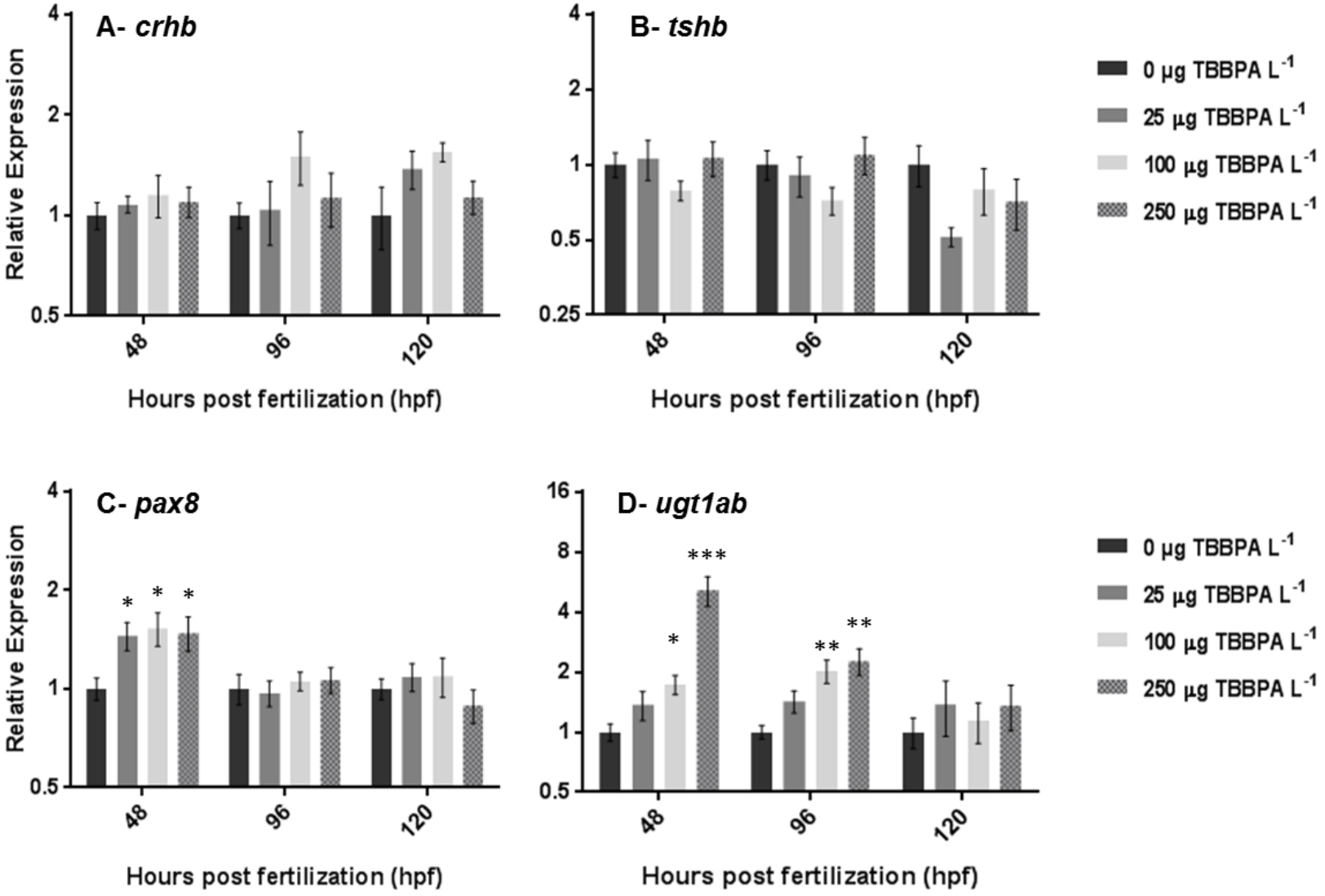

A- crhb
B- tshb
C- pax8
D- ugt1ab
Relative Expression
Hours post fertilization (hpf)
48
96
120
0 µg TBBPA L-1
25 µg TBBPA L-1
100 µg TBBPA L-1
250 µg TBBPA L-1

Figure 9. Transcript profiles of A) corticotropin-releasing hormone (*crhb),* B) thyroid-stimulating hormone (*tshb)*, C) paired box 8 (*pax8)* and D) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* in whole zebrafish following exposure to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8)* ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 6-9 homogenised sample per treatment group. For *crhb* and *tshb*, there was no significant difference between TBBPA treatment groups compared to the control group at any developmental stage examined. For *pax8*, exposure to TBBPA (25, 100 and 250 µg L^{-1}) for 48 hpf resulted in a significant increase in mRNA levels. For *ugt1ab*, exposures to TBBPA (100 and 250 µg L^{-1}) for 48 hpf and 96 hpf resulted in a significant increases in mRNA levels. Significance codes: *p<0.05, **p<0.01, ***p<0.001.

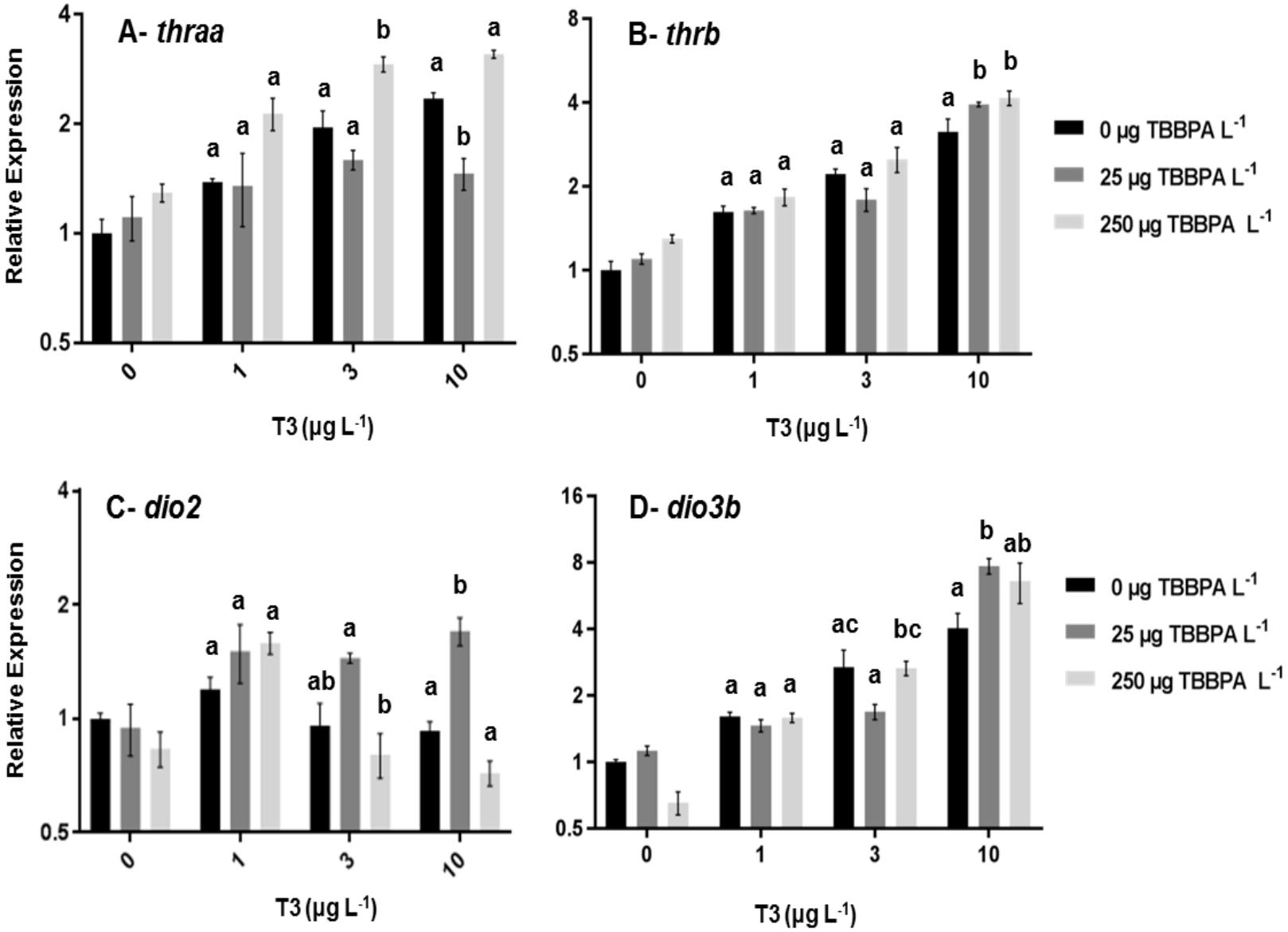
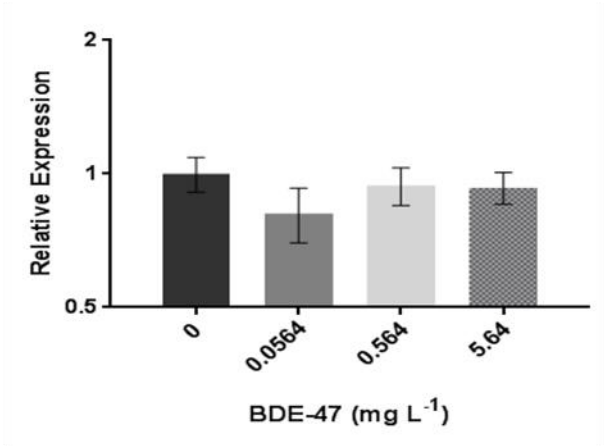

A- thraa
B- thrb
C- dio2
D- dio3b
Relative Expression
T3 (µg L-1)
0 µg TBBPA L-1
25 µg TBBPA L-1
250 µg TBBPA L-1

Figure 10. Transcript profiles of A) thyroid receptor alpha (*thraa*), B) thyroid receptor beta (*thrb*), C) deiodinase type II (*dio2*) and D) deiodinase type III (*dio3b*) in whole zebrafish larvae at 96 hours post fertilisation (hpf) following exposure to TBBPA under different doses of T3, as determined by qRT-PCR. Embryos were exposed to 0, 25, 100 or 250 µg TBBPA L^{-1} for 96 hours along with 0, 1, 3 or 10 µg T3 L^{-1}. The results are represented as means ± SEM. Statistics were carried out using accepted minimum adequate models (GLM; R; Table S6). Letters above each bar indicate significant differences within each treatment group when there was a significant interaction from the model (Tukey's HSD post hoc test; $p<0.05$).

A



F

BDE-47 Treatment	n	P.D	Brain	Liver	B.A
0 mg L^{-1}	31	100%	100%	100%	100%
0.0564 mg L^{-1}	37	100%	100%	100%	100%
0.564 mg L^{-1}	37	35%	100%	100%	100%
5.64 mg L^{-1}	38	61%	100%	100%	100%

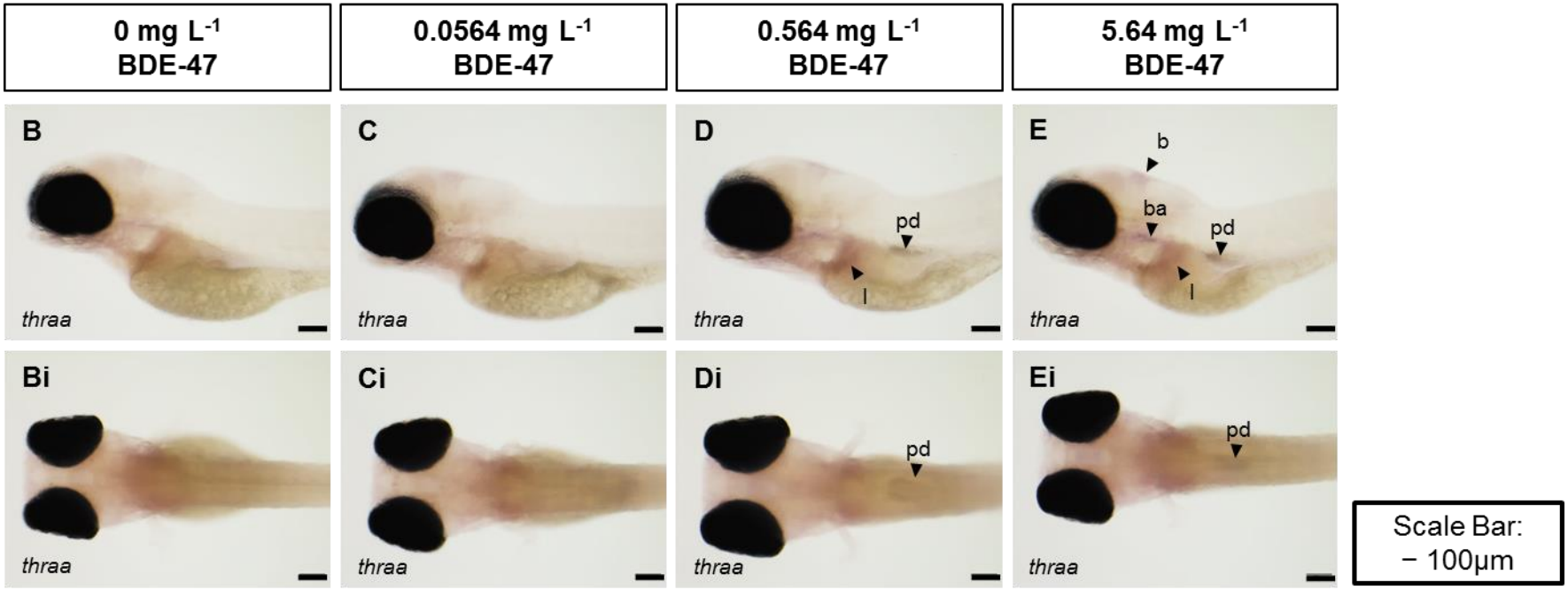
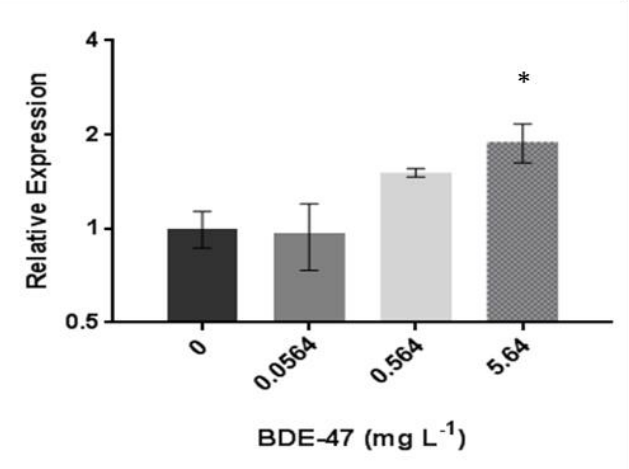


Figure 11. (A) Transcript profile of thyroid receptor alpha (*thraa*) in whole zebrafish larvae following exposure to BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}) for 96 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 5-6 homogenised sample per treatment group. There were no significant differences between treatment groups compared to the control group. (B-E) Representative images of *thraa* mRNA expression patterns in zebrafish larvae at 96 hpf treated with BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, ba=branchial arches, pd=pronephric ducts, l=liver. Scale bar=100 µm. (F) Summary of the variability in *thraa* mRNA expression in different zebrafish tissues [pronephric ducts (P.D), branchial arches (BA), brain and liver] following exposure to BDE-47 for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

A



F

BDE-47 Treatment	n	P.D	Liver	O.V	Brain	I.B
0 mg L^{-1}	29	93%	100%	100%	100%	93%
0.0564 mg L^{-1}	35	100%	100%	100%	100%	91%
0.564 mg L^{-1}	37	41%	100%	100%	100%	95%
5.64 mg L^{-1}	34	71%	100%	100%	100%	85%

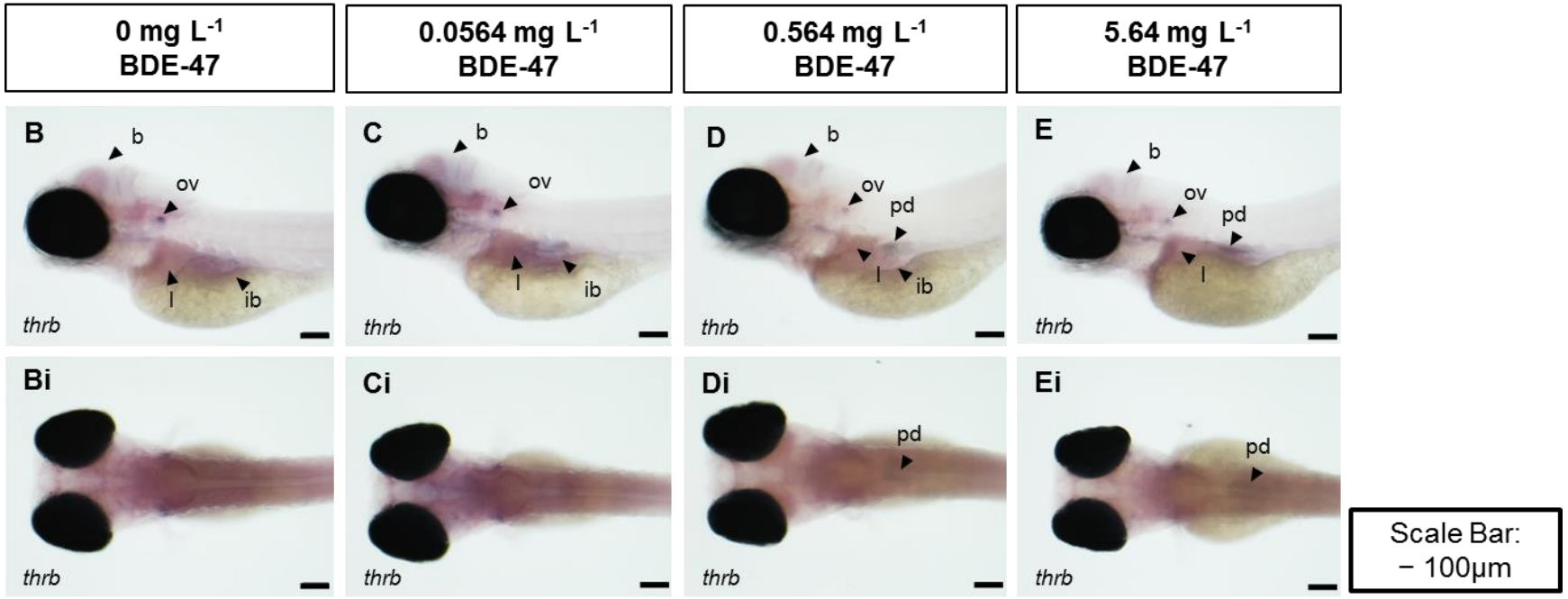
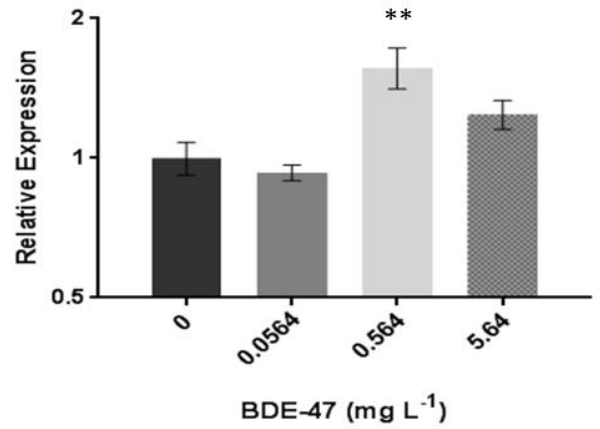


Figure 12. (A) Transcript profile of thyroid receptor beta (*thrb*) in whole zebrafish larvae following exposure to BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}) for 96 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 5-6 homogenised sample per treatment group. BDE-47 (5.64 mg L^{-1}) significantly increased *thrb* expression compared to controls. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-E) Representative images of *thrb* mRNA expression patterns in zebrafish larvae at 96 hpf treated with BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior positioned to the left. Focal areas of expression are indicated by black arrowheads. b=brain, pd=pronephric ducts, l=liver, ov=otic vesicle, ib=intestinal bulb. Scale bar=100 μm. (F) Summary of the variability in *thrb* mRNA expression in different zebrafish tissues [pronephric ducts (P.D), otic vesicle (O.V), intestinal bulb (I.B), brain and liver] following exposure to BDE-47 for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

A



F

BDE-47 Treatment	n	P.D	Liver
0 mg L^{-1}	29	0%	10%
0.0564 mg L^{-1}	32	0%	16%
0.564 mg L^{-1}	33	49%	27%
5.64 mg L^{-1}	35	63%	29%

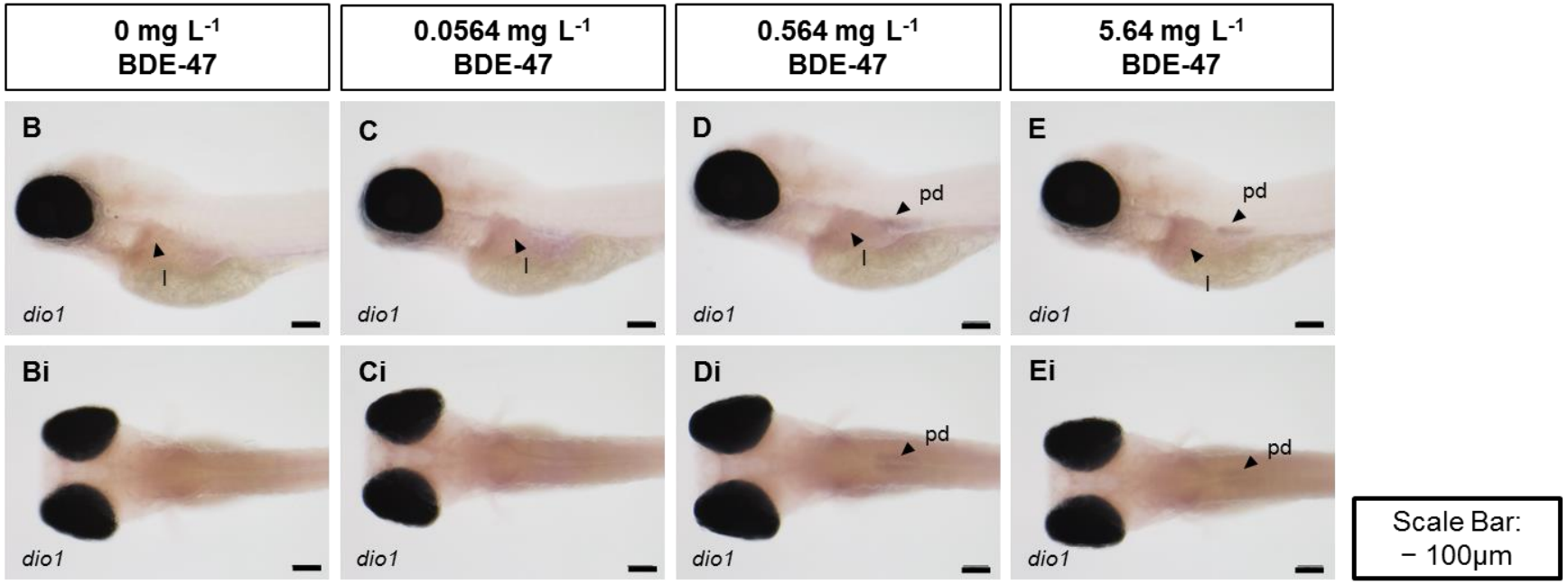
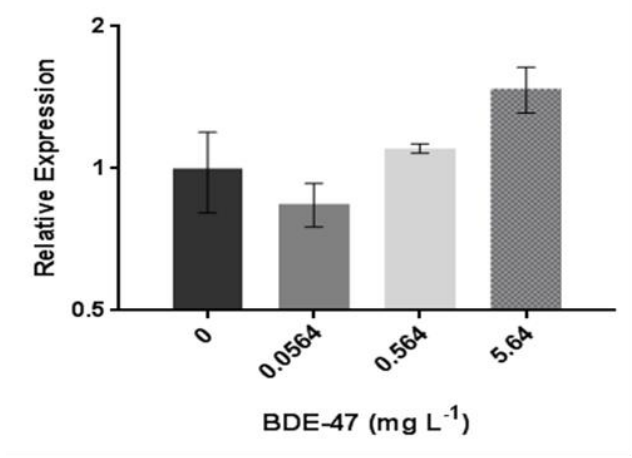


Figure 13. (A) Transcript profile of deiodinase type I (*dio1*) in whole zebrafish larvae following exposure to BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}) for 96 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 5-6 homogenised sample per treatment group. Exposure to BDE-47 (0.564 mg L^{-1}) significantly increased *dio2* mRNA levels in treated larvae compared to controls. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-E) Representative images of *dio1* mRNA expression patterns in zebrafish larvae at 96 hpf treated with BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. b=brain, pd=pronephric ducts and l=liver. Scale bar=100 µm. (F) Summary of the variability in *dio1* mRNA expression in different zebrafish tissues [pronephric ducts (P.D) and liver] following exposure to BDE-47 for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

A



F

BDE-47 Treatment	n	Liver	P. D
0 mg L^{-1}	37	97%	100%
0.0564 mg L^{-1}	30	97%	100%
0.564 mg L^{-1}	29	100%	7%
5.64 mg L^{-1}	36	92%	28%

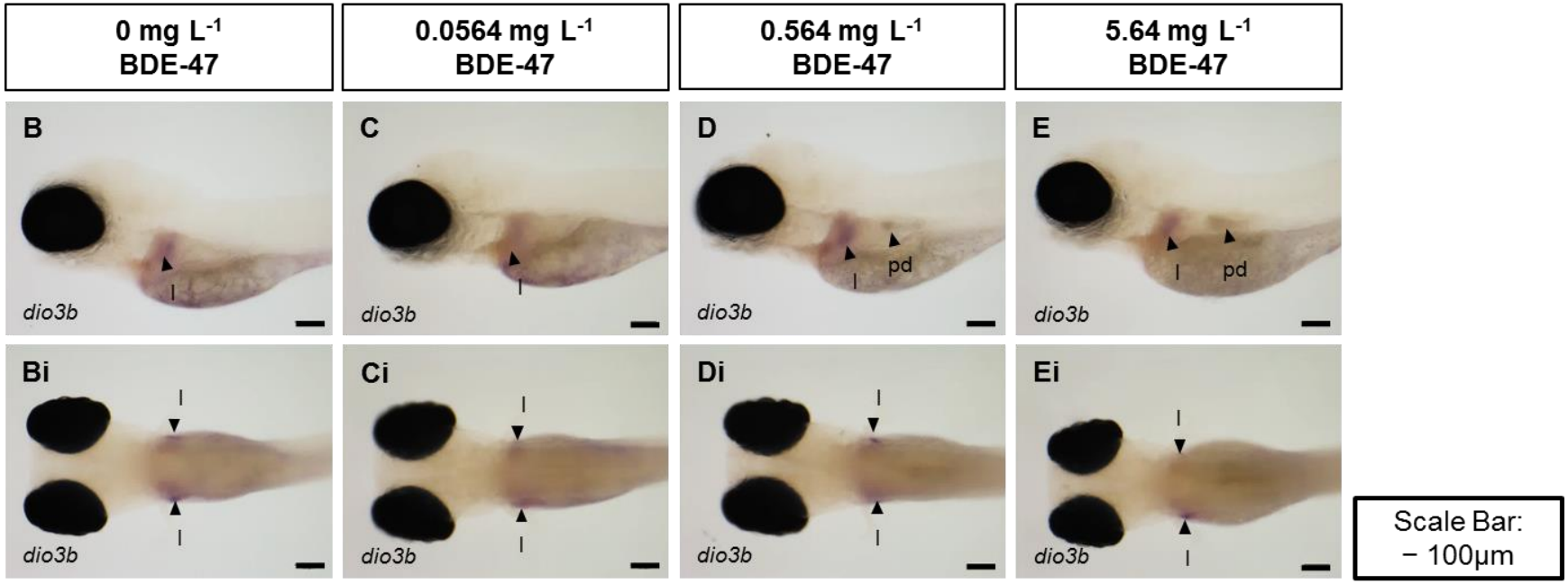
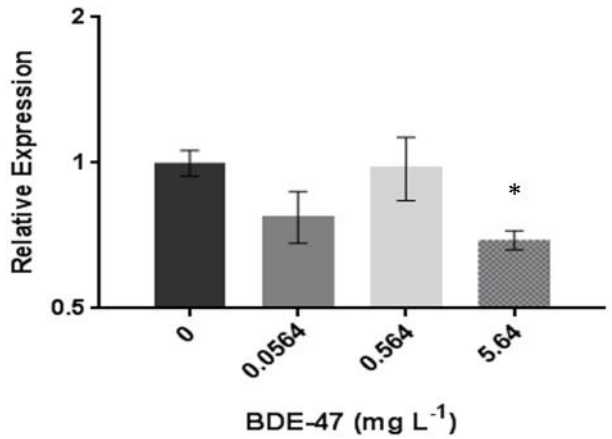


Figure 14. (A) Transcript profile of deiodinase type III (*dio3b*) in whole zebrafish larvae following exposure to BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}) for 96 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 5-6 homogenised sample per treatment group. There was no significant effect of BDE-47 treatment on *dio3b* mRNA levels. (B-E) Representative images of *dio3b* mRNA expression patterns in zebrafish larvae at 96 hpf treated with BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. pd=pronephric ducts, l=liver. Scale bar=100 µm. (F) Summary of the variability in *dio3b* mRNA expression in different zebrafish tissues [pronephric ducts (P.D) and liver] following exposure to BDE-47 for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

A



F

BDE-47 Treatment	n	Liver	P.D
0 mg L^{-1}	35	100%	100%
0.0564 mg L^{-1}	30	87%	100%
0.564 mg L^{-1}	42	100%	21%
5.64 mg L^{-1}	34	94%	27%

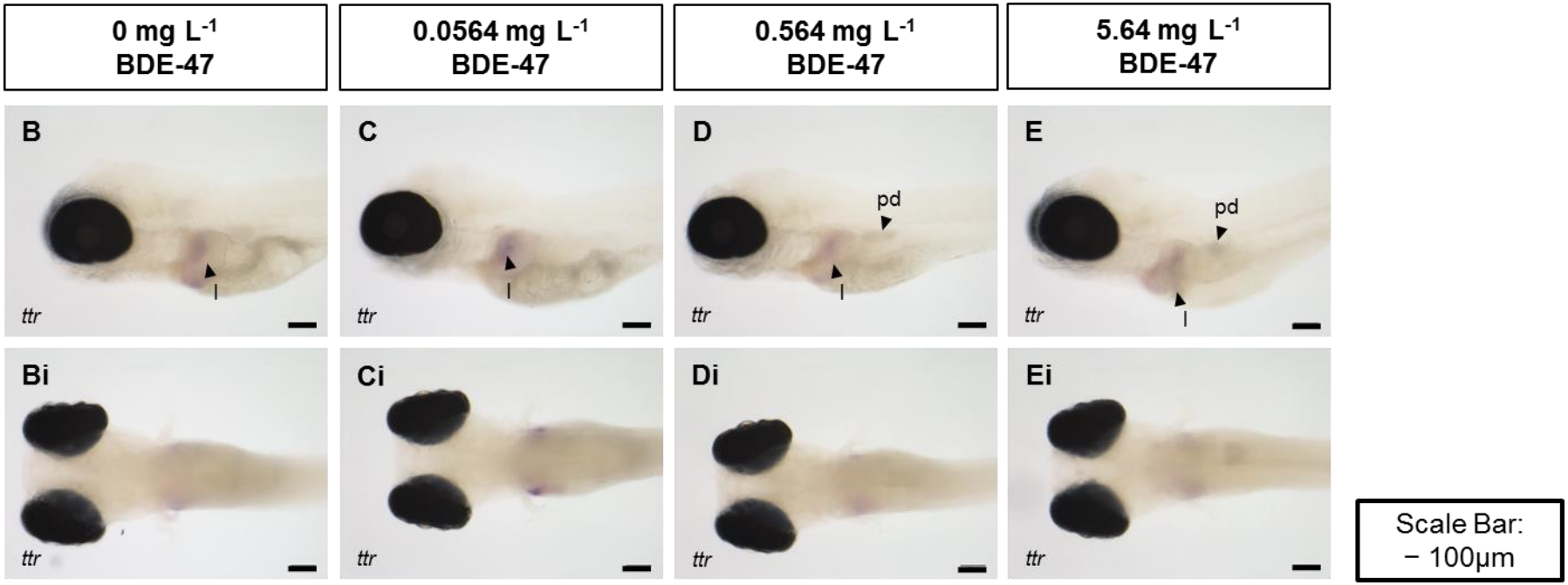


Figure 15. (A) Transcript profile of transthyretin (*ttr*) in whole zebrafish larvae following exposure to BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}) for 96 hours post fertilisation (hpf). Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S7. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n= 5-6 homogenised sample per treatment group. Exposure to BDE-47 (5.64 mg L^{-1}) significantly reduced *ttr* mRNA levels compared to controls. Significance codes: *p<0.05, **p<0.01, ***p<0.001. (B-E) Representative images of *ttr* mRNA in zebrafish larvae at 96 hpf treated with BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}). Lateral (B-E) and dorsal (Bi-Ei) views of whole larvae are shown with anterior to the left and focal areas of expression are indicated by black arrowheads. l=liver and pd= pronephric ducts. Scale bar=100 µm. (F) Summary of the variability in *ttr* mRNA expression in different zebrafish tissues [pronephric ducts (P.D) and liver] following exposure to BDE-47 for 96 hours. The % given is the proportion of individuals with the staining pattern shown in the images provided.

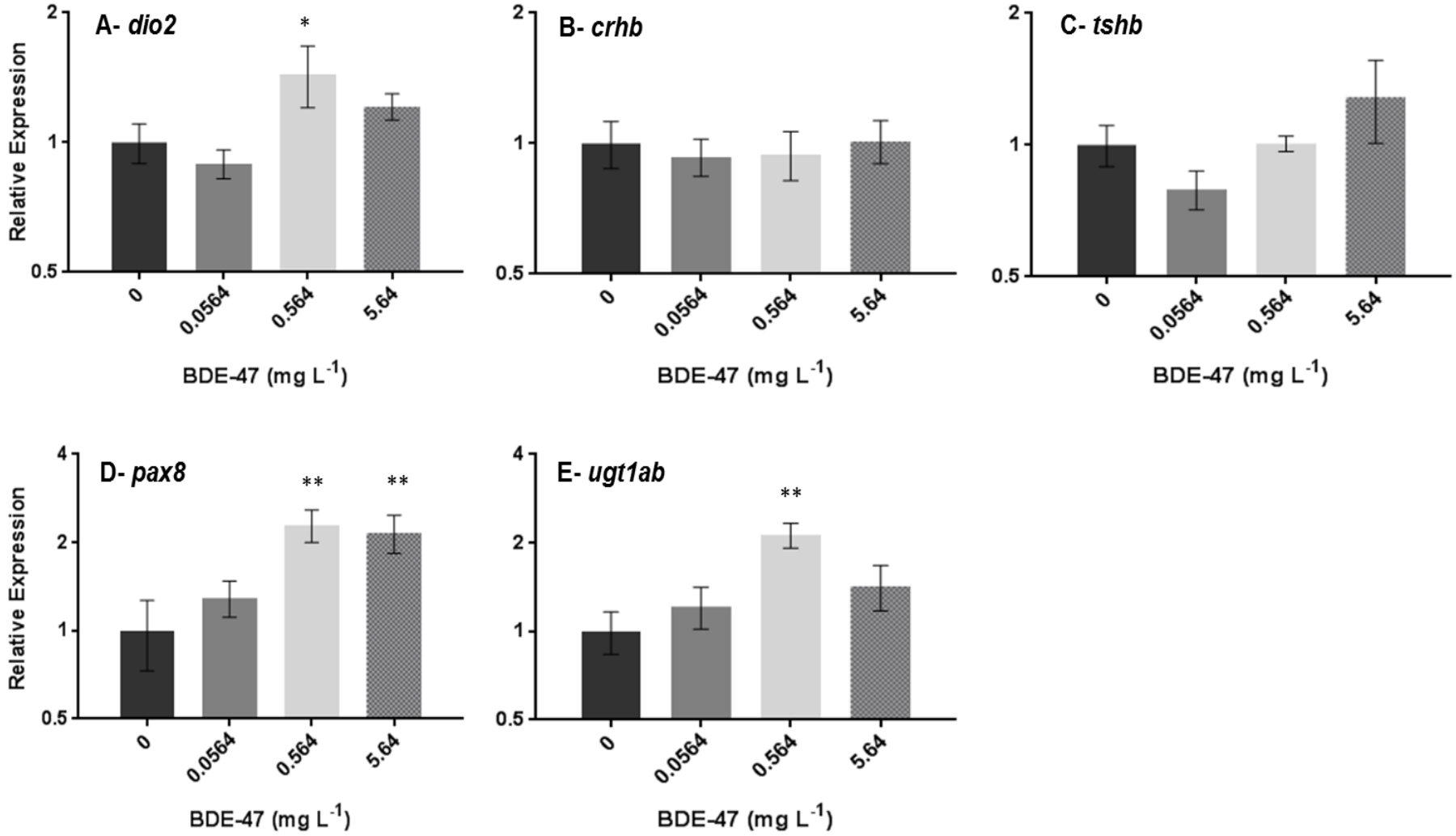

A- dio2
B- crhb
C- tshb
D- pax8
E- ugt1ab
Relative Expression
BDE-47 (mg L-1)
0
0.0564
0.564
5.64
*
**

Figure 16. Transcript profiles of A) deiodinase type II (*dio2),* B) corticotropin-releasing hormone (*chrb*) C) thyroid-stimulating hormone (*tshb)*, D) paired box 8 (*pax8)* and E) uridine diphosphate-glucuronosyltransferase (*ugt1ab)* in whole zebrafish following exposure to BDE-47 (0, 0.0564, 0.564 and 5.64 mg L^{-1}) for 96 hpf. Transcript profiles were determined using qRT-PCR. Plotted data are presented as average relative expression (normalised against the expression of the control gene *rpl8*) ± SEM. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S5. Individual data points classified as outliers, identified by Chauvenet's criterion, were excluded from the analysis, resulting in a replication of n=5-6 homogenised sample per treatment group. Exposure to BDE-47 significantly increased *dio2, pax8* and *ugt1ab* mRNA levels compared to controls, but had no effect on *crhb* and *tshb* mRNA levels. Significance codes: *p<0.05, **p<0.01, ***p<0.001

Chapter 3

Supplementary Material

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

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Materials and Methods

RNA probe synthesis for WISH

DNA-containing plasmids were purchased from Source Bioscience (for *ttr, tshb, thraa* and *thrb* genes) or donated from Dr. Alain Lescure, Université de Strasbourg (*dio1* and *dio2*) and Professor Heather Stapleton, Duke University (*dio3b*). Plasmids were cultured overnight in LB broth at 37°C on a shaker and cultures were purified using a Thermo Scientific GeneJet Plasmid Midiprep Kit (#K0481 Fermentas). DNA sequences were confirmed by Source Bioscience and the direction of gene insert was obtained by BLAST (NCBI). Plasmids were then digested at 37°C for 2 hours using the appropriate restriction enzymes (Table S1). DNA samples were purified by phenol chloroform extraction and recovered by standard precipitation with ethanol and the purified DNA was run on a 1% agarose TAE gel by electrophoresis to confirm probe integrity and stored at -20°C until required for WISH.

To prepare the transthyretin (*ttr*) and deiodinase type III (*dio3b*) DNA template, a PCR-based technique was used. Plasmid DNA (10 ng) was used as a template in a PCR reaction using GoTaq-Polymerase (Promega) to obtain a full coding *ttr* sequence (Genbank accession number NM_001005598.2) of 744 base pairs (bp). The following PCR conditions were employed: 95°C for 2 minutes, followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 48°C for 30 seconds and elongation at 72˚C for 1 minute and a final extension at 72˚C for 5 minutes, using the forward: ATG GCG AAA GAA GTG ATT and reverse: GGA TAG AAA TGG TGC TTT primers. Plasmid DNA (10 ng) was used as a template in a PCR reaction using DreamTaq DNA Polymerase (Thermo Scientific) to obtain the full coding *dio3b* sequence (Genbank accession number NM_001177935.3) of 809 bps. The following

PCR conditions were employed: 95°C for 2 minutes, followed by 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 69°C for 30 seconds and elongation at 72˚C for 1 minute and a final extension at 72˚C for 10 minutes using the forward: ATG GAG ATG CTG CAG GGC TCC GCA GGT GTG and reverse: ATG CTA GAC GTG CAG CAC CGC GGA primers. The downstream-primers contained an artificially introduced T3-promoter (GCA ATT AAC CCT CAC TAA AGG) at the 5`-end to enable synthesis of antisense transcripts. DNA was purified using (Thermo Scientific GeneJET PCR Purification Kit) and stored at -20°C. The integrity of *dio3b* and *ttr* DNA was confirmed on a 1% agarose TAE electrophoretic gel and DNA was stored at -20°C. DNA sequences were obtained by Source Bioscience and verified using BLAST (NCBI) and Clustal Omega alignment software (EMBL-EBI).

Supplementary Tables

Table S1. Vectors, restriction enzymes and promoters used in plasmid digestion and RNA probe synthesis for whole mount *in situ* hybridisation assays for the following genes in the HPT axis: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*) and transthyretin (*ttr*).

Gene	Accession Number	Vector	Restriction Enzyme	Promotor
thraa	AI497070	pSport1	EcoR1	Sp6
thrb	BC163106	PCR4_TOPO	Spe1	T7
dio1	BF717782.1	pSport1	EcoR1	Sp6
dio2	BG737671.1	pBK_CMV	BamH1	T7
dio3b	NM_001177935.2	pGEM-T	-	T3
ttr	NM_001005598.2	pME18S-FL3	-	T3

Table S2: Whole mount *in situ* hybridisation staining times (hours) of target genes in zebrafish embryo-larvae following exposures to TBBPA and BDE-47. Embryo-larvae were sampled at various developmental stages; 48, 96 or 120 hours post fertilisation (hpf). Staining times are shown for the following genes: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*) and transthyretin (*ttr*).

Gene	TBBPA			BDE-47
	48 hpf	96 hpf	120 hpf	96 hpf
thraa	5	4.5	8	5
thrb	8	4.5	-	8
dio1	6	4	-	5
dio2	5	8	-	-
dio3b	5	1.5	-	5
ttr	-	3	-	5

Table S3. 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	Primer Sequence	Product Size (bp)	Ta (°C)	PCR Efficiency (%)	Accession Number
thraa	F: GGC GTC CTG TAA CTG CTG R: GGT TGT GCT CCT GCT CTG	142	61	101	NM_131396.1
thrb	F: TGG GTG TCT CGC TGT CCTC R: ACA ACG CTC TAT CCG CTC AAC	119	60	93	NM_131340.1
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
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
dio2	F: TCT GGA GGA GAG GAT GTT TGC R: CTC GTA GGA CAC ACC GTA GG	124	59.5	105	NM_212789.3
dio3b	F: AGG GCT CCG CAG GTG TG R: AGG AAG TCC AGC AGG CAG AG	106	63	98	NM_001177935.2
ttr	F: CGC ACA CCT TTC CAC CAG R: TTG ACG ACC ACA GCA GTT G	122	60.5	109	NM_001005598.2
crhb	F: TTC GGG AAG TAA CCA CAA GC R: CTG CAC TCT ATT CGC CTT CC	163	59.5	110	NM_001007379.1
ugt1ab	F: CCA CCA AGT CTT TCC GTG TT R: GCA GTC CTT CAC AGG CTT TC	168	62.5	105	NM_213422.2
pax8	F: CCG TCA CTC CTC CTG AAT CTC R: GCT CTC CTG GTC ACT GTC ATC	128	62.5	106	AF072549.1
rpl8	F: CCG AGA CCA AGA AAT CCA GAG R: CCA GCA ACA ACA CCA ACA AC	91	59.5	102	NM_200713.1

Table S4: Generalised linear models for the relationship between chemical treatment and zebrafish larvae mortality and deformities after 96 hour exposures to TBBPA (0, 100, 250, 500, 750 and 1500 µg L^{-1}) and BDE-47 (0, 0.56, 2.82, 5.64, 28.2, 56.4 mg L^{-1}). Minimum adequate models (F-value) for are shown. (Significance codes: *p<0.05, **p<0.01, ***p<0.001).

	Treatment	df	Minimum Adequate Model
Mortality	**TBBPA**	46	87.99***
	BDE-47	48	NS
Deformity	**TBBPA**	28	53.94***
	BDE-47	48	5.77***

Table S5: General linear models for the relationship between TBBPA and transcript profiles measured in zebrafish larvae after 48, 96 or 120 hour exposures to TBBPA (0, 25, 100 or 250 µg L^{-1}). Minimum adequate models (F-value) for the relative expression are shown for the genes analysed: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), thyroid-stimulating hormone (*tshb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). (Significance codes: *p<0.05, **p<0.01, ***p<0.001).

Gene	hpf	df	Minimum Adequate Model	Gene	hpf	df	Minimum Adequate Model
thraa	48	31	NS	**ttr**	48	20	3.19*
	96	29	NS		96	30	NS
	120	29	NS		120	28	NS
thrb	48	30	NS	**crhb**	48	29	NS
	96	27	2.98*		96	27	NS
	120	29	NS		120	25	NS
dio1	48	28	NS	**tshb**	48	30	NS
	96	29	4.17*		96	31	NS
	120	28	NS		120	17	NS
dio2	48	32	NS	**pax8**	48	32	3.14*
	96	32	NS		96	31	NS
	120	25	NS		120	23	NS
dio3b	48	31	NS	**ugt1ab**	48	30	19.35***
	96	29	7.37***		96	30	4.88**
	120	26	NS		120	22	NS

Table S6: General linear models for the relationship between T3, TBBPA and the interaction between T3 and TBBPA on transcript profiles measured in zebrafish larvae after a 96 hour exposure to TBBPA (0, 25 or 250 µg L^{-1}) under different doses of T3 (0, 1, 3 or 10 µg L^{-1}). Minimum adequate models (F-value) for the relative expression are shown for the genes analysed: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), thyroid-stimulating hormone (*tshb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). (Significance codes: *p<0.05, **p<0.01, ***p<0.001).

Gene	df	Minimum Adequate Model		
		T3	TBBPA	T3/TBBPA Interaction
thraa	6	35.13***	41.91***	4.70**
thrb	6	138.48***	7.96**	2.82*
dio1	3	2.94*	NS	NS
dio2	6	9.13***	14.20***	4.91**
dio3b	6	136.50***	NS	6.65***
ttr	3	NS	10.23***	NS
crhb	3	NS	NS	NS
tshb	3	5.48**	NS	NS
pax8	3	3.48*	NS	NS
ugt1ab	5	2.95*	18.40***	NS

Table S7: General linear models for the relationship between BDE-47 and transcript profiles measured in zebrafish larvae after a 96 hour exposure to BDE-47 (0, 0.0564, 0.564 or 5.64 µg L^{-1}). Minimum adequate models (F-value) for the relative expression are shown for the genes analysed: thyroid receptors (*thraa* and *thrb*), deiodinases (*dio1, dio2* and *dio3b*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), thyroid-stimulating hormone (*tshb*), paired box 8 (*pax8*) and uridine diphosphate-glucuronosyltransferase (*ugt1ab*). (Significance codes: *p<0.05, **p<0.01, ***p<0.001).

Gene	df	Minimum Adequate Model	Gene	df	Minimum Adequate Model
thraa	17	NS	***ttr***	14	NS
thrb	14	4.20*	***crhb***	14	NS
dio1	13	8.56**	***tshb***	16	NS
dio2	14	4.06*	***pax8***	14	5.53*
dio3b	13	NS	***ugt1ab***	14	4.35*

Supplementary Figures

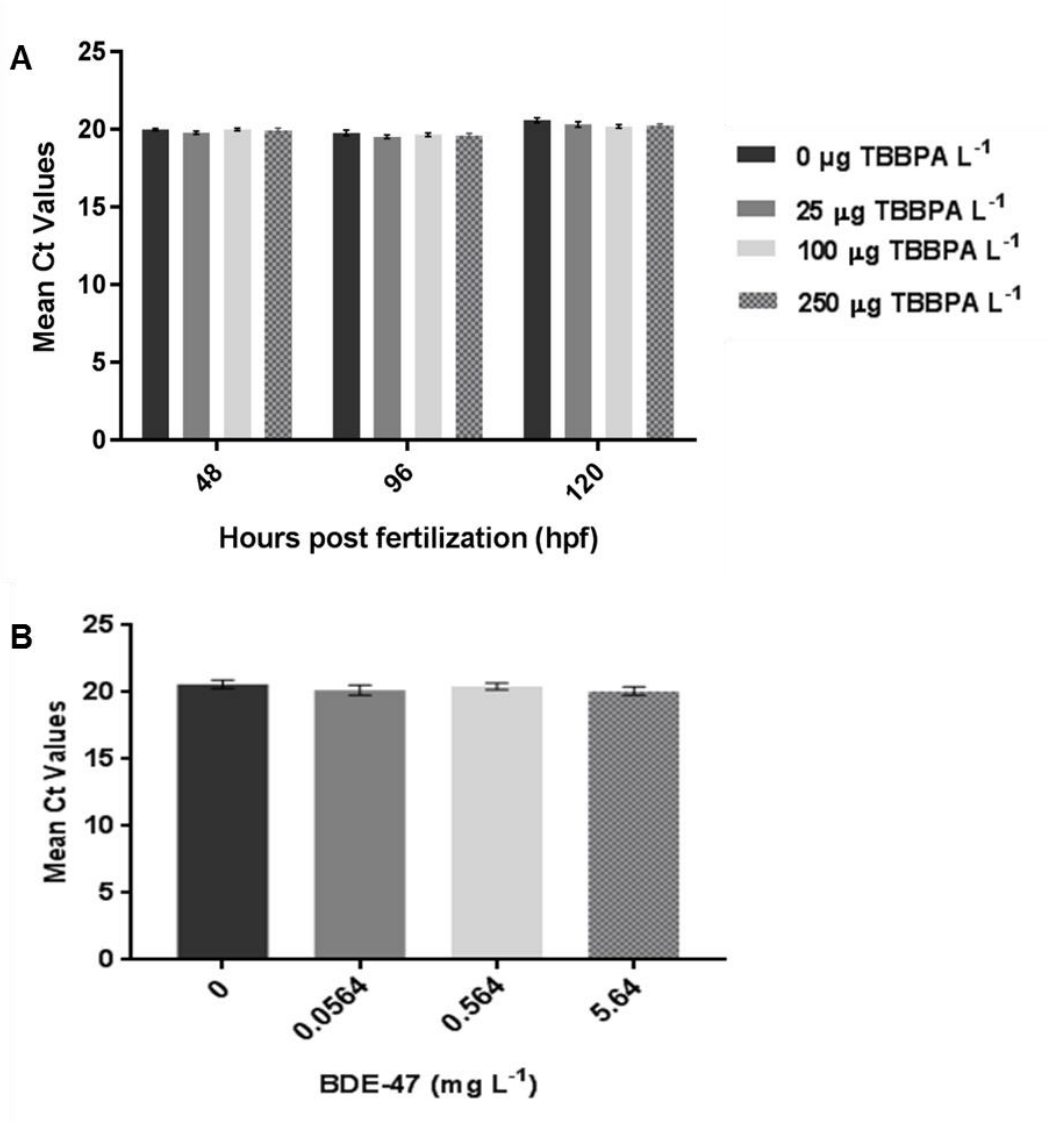

Figure S1: A) Mean *rpl8* mRNA levels expressed as Ct values (threshold cycle) in zebrafish A exposed to TBBPA (0, 25, 100 and 250 µg L^{-1}) for 48, 96 and 120 hours post fertilisation (hpf). B) Mean *rpl8* mRNA levels expressed as Ct values in zebrafish exposed to BDE-47 (0, 0.0564, 0.564 or 5.64 µg L^{-1}) for 96 hours. For both TBBPA and BDE-47 exposures, there were no significant differences in Ct values between treatment groups (GLM: R). n=9. Error bars represent standard error.

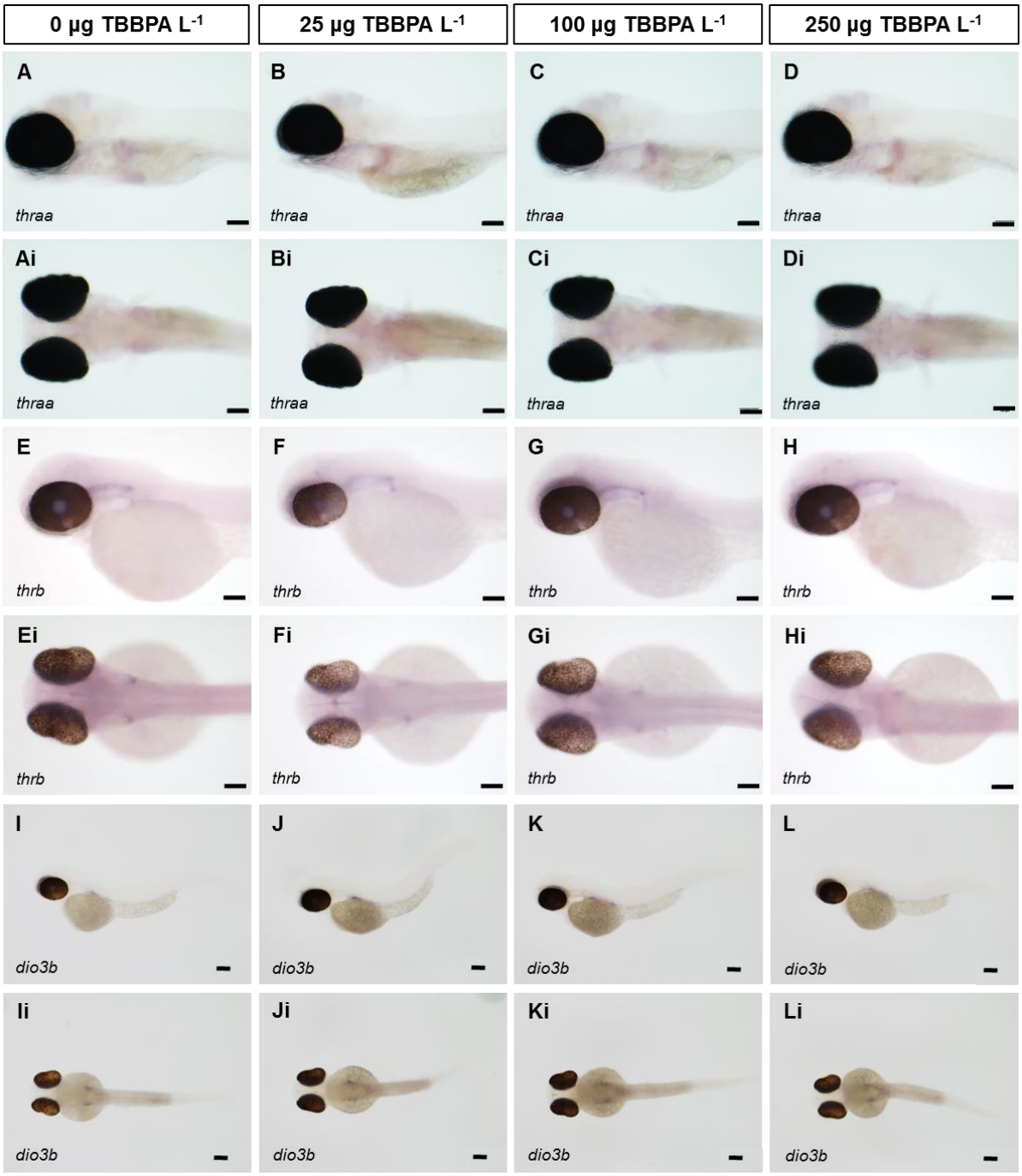

Figure S2: Representative images of mRNA expression patterns in zebrafish embryo larvae treated with TBBPA (0, 25, 100 and 250 µg L^{-1}) for the following genes; (A-D) thyroid receptor alpha (*thraa)* at 120 hours post fertilisation (hpf), (E-H) thyroid receptor beta (*thrb)* mRNA at 48 hpf and (I-L) deiodinase type III (*dio3b*) at 48 hpf. No effect of TBBPA exposure on the expression of the three genes was observed.

Lateral (A-L) and dorsal (Ai-Li) views of whole embryos are shown with anterior positioned to the left Scale bar=100 μm

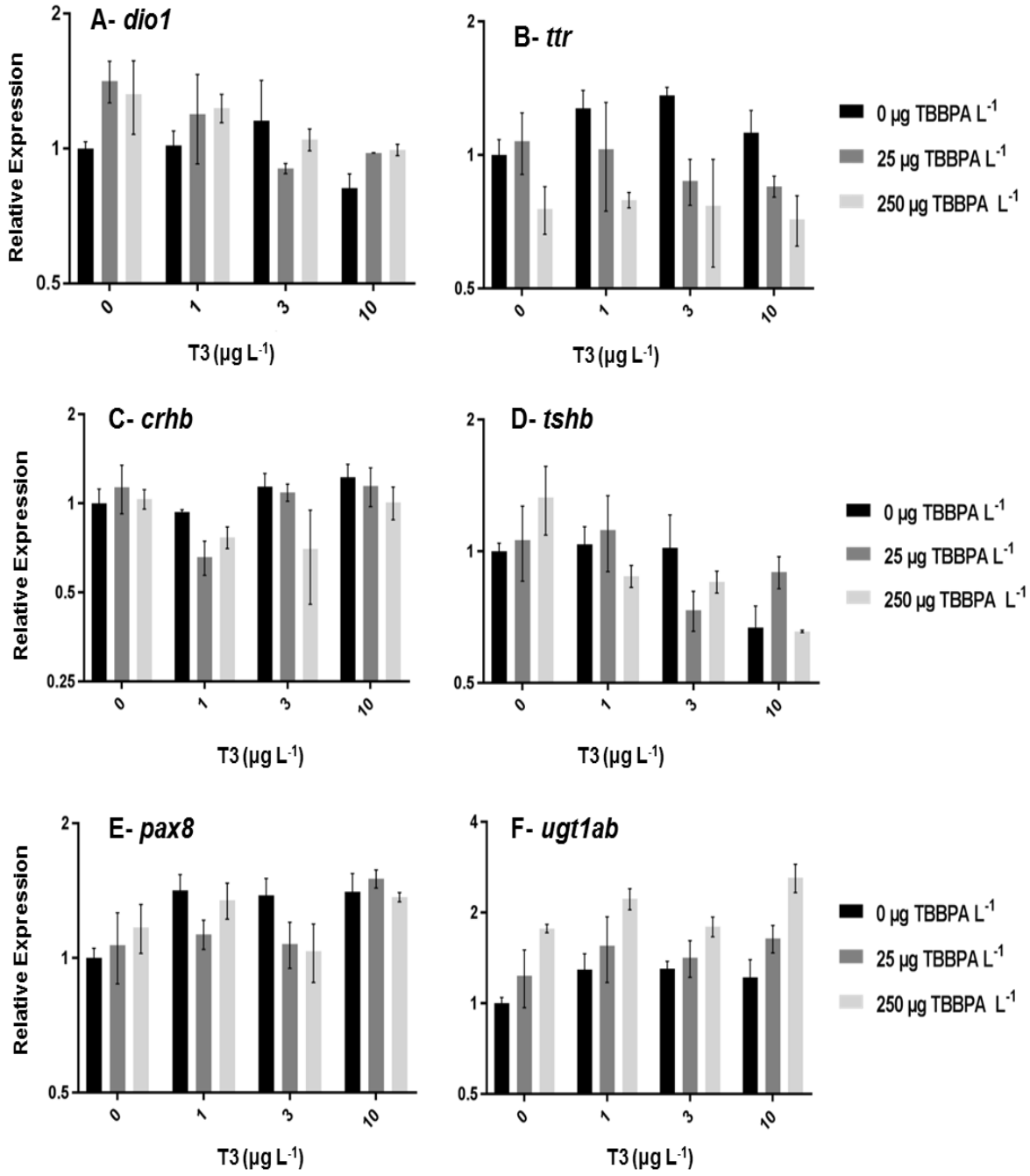

Figure S3. Transcript profiles for selected target genes in whole zebrafish following exposure to TBBPA under different doses of T3. Embryos were exposed to 0, 25, 100 or 250 µg TBBPA L^{-1} for 96 hours along with 0, 1, 3 or 10 µg T3 L^{-1}. Transcript profiles were determined using qRT-PCR. Genes analysed included: deiodinase type I (*dio1*), transthyretin (*ttr*), corticotropin-releasing hormone (*crhb*), thyroid-stimulating hormone (*tshb*), paired box 8 (*pax8*) and uridine diphosphate-

glucuronosyltransferase (*ugt1ab*). Plotted data are presented as average relative expression (normalised against the expression of the control gene rpl8) ± SEM. Statistics were carried out using accepted minimum adequate models (GLM; R; Table S6). For all of the genes analysed there was no significant interaction between T3 and TBBPA treatments. For *dio1* and *tshb* there was a significant effect of T3 treatment ($p<0.05$ and $p<0.01$, respectively). For *ttr*, there was a significant effect of TBBPA treatment ($p<0.001$). For *crhb*, there was no effect of T3 or TBBPA treatment. For *ugt1ab*, there was a significant effect of T3 and TBBPA treatment ($p<0.05$ and $p<0.001$, respectively).

Chapter 4

Development of a transactivation reporter gene assay for zebrafish (*Danio rerio*) thyroid receptors TRα and TRβ and an investigation into their interactions with brominated flame retardants.

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4.1 Abstract

Brominated flame retardants (BFR) are the second largest market group of flame retardants and are known to disrupt thyroid hormone (TH) homeostasis in humans and wildlife. In mammals, BFRs have been shown to interact with TH nuclear receptors (TRs) and disrupt their transcriptional activity, but the mechanisms of toxicity of BFRs in fish are unclear. In this study, we report on the development of reporter gene transcriptional assays for two zebrafish thyroid hormone receptors (zfTRα and zfTRβ) in human embryonic kidney cells (HEK) and investigate their interactions with selected BFRs. The assays were optimised and validated using the natural TR agonist 3,3′,5- triiodo-L-thyronine (T3) in cells transiently transfected with two reporter vector constructs, pGL4.24-PAL and pGL4.24-DR4. In both receptor (zfTRα and zfTRβ) assays, T3 induced luciferase activity in a concentration-dependent manner and there was a greater sensitivity with the pGL4.24-DR4 vector. The maximum luciferase activity induced in the zfTRα and zfTRβ assays were 5.1-fold, (± 0.26 SEM) and 15.5- fold (± 1.13 SEM) relative to the controls following exposure to 10 nM T3 (LC-50 of 1.73 $x10^{-9}$) and 100 nM T3 (LC-50 of 1.56 $x10^{-9}$) for 48 hours respectively. None of the six brominated flame retardants tested, namely, tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), 2,2′,4,4′,6-penta-bromodiphenyl ether (BDE-100), 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183) and deca-bromodiphenyl ether (BDE-209) induced luciferase activity in the zfTRα and zfTRβ assays, which may indicate an alternative mode of action of thyroid disruption for these chemicals in fish. The zebrafish-specific reporter gene assays developed provide effective screens for investigating other chemicals suspected of disrupting TR-mediated gene transcription.

4.2. Introduction

Thyroid hormones (TH) play crucial roles in a wide range of physiological processes during development and in adult life stages of vertebrates. They influence the maturation of many tissues including bone (reviewed in [1]), gonads (reviewed in [2]), intestine [3] as well as the central nervous system (reviewed in [4]). In addition, they modulate growth [5], energy homeostasis [6], cardiac rhythm [7], osmoregulation, the metamorphic transition from larval to adult stages in fish and amphibians [8] and the behaviours and underlying physiology associated with rheotaxis and migration in fish [9, 10].

Over the last two decades there has been concern over the presence of endocrine disrupting chemicals (EDCs) in the environment, with attention largely focused on compounds that interfere with hormone signalling in the reproductive system of humans and wildlife (reviewed in [11-13]). However, environmental contaminants which act as thyroid disrupting chemicals (TDCs) have also gained increasing attention in recent years [14-16]. TDCs are xenobiotics that alter the structure or function of the thyroid gland, alter thyroid hormone (TH) regulatory enzymes or change circulating or tissue concentrations of THs [17]. Lipophilic compounds such as polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), polyhalogenated aromatic hydrocarbons (PHAHs), phthalates and brominated flame retardants (BFR) are structurally similar to thyroxine (T4), the precursor of the active TH, 3,3′,5-triiodo-L-thyronine (T3), and have been shown to alter circulating levels of TH in a range of vertebrate species including fish (reviewed in [14]).

The genomic actions of THs are mediated by binding to nuclear TH receptors (TRs), which are ligand-dependent transcription factors binding T3 or, with a lower affinity, T4 [18]. The TRs share a typical nuclear receptor domain structure, consisting of an N-terminal regulatory domain (termed A/B), a central DNA-binding domain (DBD) where the receptor interacts with the DNA, a less conserved hinge region, the ligand-binding domain (LBD) where TH binds and activates the receptor and the C-terminal region [19]. In general, vertebrates have two TR genes encoding TRα and TRβ [20]. Due to ancestral gene duplication, several fish species have two TRα-encoding genes (*thraa* and *thrab*) as well as a single TRβ gene (*thrb*) [21, 22]. The zebrafish *thraa* gene, homologous to human THRA, generates two isoforms, TRαA1 and TRαA1-2, that show high similarity with TRα's from other vertebrates [23]. The *thrab* gene encodes TRαB which lacks a large portion of the N-terminal domain and the associated transactivation function [24]. The zebrafish *thrb* gene generates two TRβ1 isoforms (one of which has a 9-amino acid insert in the hinge region between the DBD and the LBD). It has been shown that TRα receptors have constitutive transactivating activity in the absence of ligand while the transactivating activity of TRβ receptors is T3-dependent and repressed in the absence of T3 [23]. TRs control gene transcription by binding as either heterodimers with retinoid X receptor (RXR), as homodimers or monomers to specific DNA acceptor sites known as thyroid response elements (TRE) [25]. TREs are either composed of direct repeats of the sequence AGGTCA spaced by four nucleotides (DR4), arranged as palindromes (PAL) or inverse palindromes (IP) [26].

BFRs have been commercially important high production compounds since their introduction to the global markets in the 1970s and are used routinely in industrial

and consumer products in an effort to reduce fire-related injury and property damage [27]. Limited information is available on the current global market volume, but approximately 311,000 metric tonnes of BFRs were used worldwide in 2005, which accounts for 21% of the total consumption of flame retardants globally [28]. Among the many BFRs used worldwide, the most extensively used compounds have been polybrominated diphenyl ethers (PBDEs), hexabromocyclododecanes (HBCDs) and tetrabromobisphenol A (TBBPA) [27]. Over the past three decades, these BFRs have become ubiquitous environmental contaminants and have accumulated in the environment up to levels that could potentially lead to negative effects on the health of human and wildlife [29]. For instance, PBDEs levels of up to 47,900 µg/kg lipid weight (lw) were recorded in fish from the Hyco River, Virginia [30], up to 51,100 µg/kg lw in humpback dolphin from Hong Kong [31] and up to 2,010 µg/kg lw in human breast milk of people living in the US [32].

PBDEs are a family of additive BFRs used in a variety of commercial and household products [27, 33]. There are 209 possible BDE congeners, with two halogenated aromatic rings connected by an ether bridge, which resembles the structure of polychlorinated biphenyls (PCBs). Commercial PBDE mixtures are made up of congeners with varying numbers of bromine atoms on their two phenyl rings and are classified according to their average bromine content; penta-BDEs, octa-BDEs, and deca-BDEs [27]. Technical penta-BDE mixtures, composed mostly of BDE-47 and BDE-99 congeners, are used in polyurethane foam and textiles [34]. Meanwhile octa-BDE technical formulations predominantly consist of both hepta- and octa- BDE congeners such as BDE-183, BDE-196, BDE-197, BDE-203 are used in thermoplastic resins and in the housings of electrical and electronic (E&E) equipment

[27, 34]. Deca-BDE mixtures, which consist mainly of the fully brominated BDE-209 congener, are a general purpose flame retardant used in nearly any type of polymer including; polycarbonates, polyester resins, polyolefins, ABS, polyamindes, polyvinyl chloride and rubber [27]. HBCDs are used as flame retardants in polystyrene foams, thermal insulation building materials, upholstery textiles and electronic products [29]. Commercial HBCD products mainly consist of α-, β-, and γ-diastereoisomers, with γ-HBCD dominating the mixture (>70%) [35]. The annual global production of HBCD was 23,000 tonnes in 2011, making it the third most used BFR globally [36].

PBDEs and HBCDs are blended physically rather than bonded chemically to the polymer and thus migrate out into the environment from the products into which they are incorporated. Their physio-chemical characteristics (long half-lives, small molecular weights, low water solubility and high lipophilicity) means they tend to persist and thus are taken up readily into human and wildlife tissues (reviewed in [29]). Accordingly, the production and usage of penta- and octa- BDE commercial mixtures was prohibited globally in 2004, and they were officially labelled as Persistent Organic Pollutants (POPs) by the Stockholm Convention in 2009 [37]. The use of deca-BDE products was banned in E&E applications in the EU in 2008, while manufacturers in the U.S. agreed to cease production from 2013 [38, 39]. The production and use of HBCD is currently being phased out globally after its inclusion on the Stockholm Convention's list of POPs in 2016 [40].

Despite discontinued use and production of many BFR compounds in some countries, global contamination continues today. For instance, the ban on octa- and penta-BDE technical mixtures has led to a dramatic increase in the production of

deca-BDE mixtures in Asia [41, 42]. The continued use of deca-BDE mixtures and the large reservoirs of BDE-209 found in soils and sediments worldwide is now a major cause for concern [43, 44]. BDE-209 is highly susceptible to degradation, via photolytic, microsomal and/or metabolic debromination pathways which can yield the lower brominated congeners such nona-, octa- and hepta-BDEs, shown to be more mobile and toxic [45-47]. In addition, many older products manufactured prior to the introduction of bans/restrictions, still contain BFRs and are in use today. These products therefore act as a continuous source of environmental contamination during their lifetime, after disposal and at electronic waste (e-waste) recycling sites [48-54]. Furthermore, and often overlooked, within the EU use of deca-BDE and HBCD is currently still permitted in textiles and in expanded/extruded polystyrene in buildings, respectively [40, 55].

Currently there are no restrictions on the production of TBBPA or its derivatives. It is the most widely used BFR representing approximately 60% of the total BFR market [56], amounting to over 120,000 tonnes in 2001 and over 170,000 tonnes in 2004 [57, 58]. The primary application (90%) of TBBPA is as a reactive flame retardant covalently bound to the epoxy and polycarbonate resins of electronic circuit boards. It is also used to a lesser extent (10%) as an additive flame retardant in acrylonitrile-butadiene-styrene (ABS) resin and high-impact polystyrene, used in automotive parts, pipes, fittings and refrigerators [27]. Despite the primary use of TBBPA as a reactive flame retardant, it has been identified in occupational, household and environmental dust samples [59-61], in sewage sludge [62], river sediments [63] and the water phase of lakes and rivers [64, 65]. TBBPA has also been detected in

various biotic samples such as human breast milk and plasma, eggs of predatory birds, marine mammals, fish and invertebrates [66-70].

Numerous *in vivo* studies have found that several individual PBDE congeners, PBDE technical mixtures, HBCD and TBBPA can alter circulating levels of T3 and/or T4 [71-81]. Given the detrimental effects resulting from subtle changes in TH status, particularly during crucial developmental windows [82], even relatively low environmental levels of BFRs pose a potential risk to the health of humans and wildlife. It is therefore imperative to understand the mechanisms of BFR-induced thyroid disruption. It is suspected that BFRs may act to disrupt TH levels by binding with TRs. However, there have been considerable discrepancies reported in the literature, with both agonistic and antagonistic effects of BFRs on TH-signalling detected in various cell-based assays [83-88]. To date the development of fish specific high-throughput reporter gene assays for testing the transcriptional activation of TRs by BFRs *in vitro* is limited, with studies largely focusing on TRs derived from mammals and several frog species. Results from these studies have been inconclusive, with TR- responsiveness to BFRs different depending on the species tested and the sensitivity of the cell line [83-89]. One recent study, however, assessed the effect of TBBPA on the transcriptional activity of TRs derived from the Japanese medaka (*Oryzias latipes*) and found that TBBPA inhibited the T3-induced activation of TRα [90].

It is clear that further work on the mechanisms of thyroid disruption by BFRs is required in order to assess their potential health and environmental risks, which cannot be predicted from investigating TR activation in one or two vertebrate

species. Differences in the responsiveness to estrogenic compounds has even been demonstrated between different fish species [91]. Here we developed *in vitro* thyroid-responsive reporter gene assays for two zebrafish (*Danio rerio*) TRs zfTRα and zfTRβ, to analyse agonistic activities of environmentally relevant BFRs, including PBDEs, TBBPA and HBCD.

4.3 Materials and Methods

4.3.1 Materials and reagents

3,3',5-Triiodo-L-thyronine (T3; CAS 6893-02-3) (purity ≥ 95%), 3,3′,5,5′-Tetrabromobisphenol A (TBBPA; CAS 79-94-7) (purity 97%) and 1,2,5,6,9,10-hexabromocyclododecane (HBCD; CAS 3194-55-6) (purity 95%) were obtained from Sigma-Aldrich Inc (UK). 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47; purity 99.8%), 2,2′,4,4′,6-penta-bromodiphenyl ether (BDE-100; purity 99.9%), 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183; purity 99.9%) and deca-bromodiphenyl ether (BDE-209; purity 98.5%) were provided by Ulrika Winnberg, Jorke Kamstra and Kees Swart on behalf of Dr. Juliette Legler from VU University Amsterdam, The Netherlands. All chemicals were dissolved in dimethylsulfoxide (DMSO).

4.3.2 Construction and cloning of zebrafish thyroid receptors

Adult wild-type zebrafish (*wik* strain) were obtained from breeding stocks at the University of Exeter. Total RNA was isolated from whole homogenised zebrafish samples at 120 hours post fertilisation using Tri-Reagent following the manufacturer's instructions. RNA (1 µg) was treated with RQ1 RNase-Free DNase (Promega, UK) and subsequently reverse transcribed to complementary DNA

(cDNA) using random hexamers (Eurofins MWG Operon, Germany) and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. This cDNA was used as the template in an initial PCR reaction using Phusion High Fidelity DNA Polymerase (New England Biolabs, NEB, UK) to obtain a *thraa* (zfTRα; Genbank accession number NM_131396.1) sequence of 1560 base pairs (bp). The following PCR protocol was employed: 98˚C for 30 seconds, followed by 35 cycles of denaturation at 98˚C for 10 seconds, annealing at 65˚C for 30 seconds and elongation at 72˚C for 45 seconds using the primers zfTRα_F1: CGC GTT GTT TTG GAA GCA GT and zfTRα_R1: GAT GCT TTC GGG GGA GTC TG. This amplified cDNA sequence was used in an additional nested PCR reaction to obtain the 1284 bp full coding sequence of zfTRα using the following nested PCR protocol: 98˚C for 30 seconds, followed by 35 cycles of denaturation at 98˚C for 10 seconds, annealing at 69˚C for 30 seconds and elongation at 72˚C for 45 seconds with the primers zfTRα_F2: ATG GAA AAC ACA GAG CAG GAG C and zfTRα_R2: TCA CCT TAA GCA GGA ACC GTC T. The resulting PCR product was purified using GeneJET PCR purification columns (ThermoScientific, UK) according to the manufacturer's instructions. Sequencing of the product (Source Bioscience, UK) and characterisation using BLAST (NCBI) and Clustal Omega (EMBL-EBI) confirmed it was zfTRα (Fig. S1 and Fig. S2). A-tailing was subsequently carried out with the purified zfTRα PCR product using Taq Polymerase (NEB) according to the manufacturer's instructions and the product purified again as described above. The purified PCR zfTRα product was initially ligated into pGEM-T Easy vector (Promega) following the manufacturer's recommendations. The *thrb* gene (zfTRβ; Genbank accession number NM_131340.1) was synthesised by Eurofins Genomics and provided in a PEX-K4 vector. The zfTRα and zfTRβ sequences were subsequently

sub-cloned into the mammalian expression vector pcDNA3.1 (+) and pcDNA3.1 (-), respectively, (Invitrogen) by incubation of 1 µl vector DNA and 2 µl insert DNA with the appropriate restriction enzymes (Not*I* for zfTRα, EcoR*I* and Xho*I* for zfTRβ; NEB) and T4 DNA ligase (Promega) at room temperature for 1 hour. zfTRβ in pcDNA3.1 (+) vector was sequenced (Source Bioscience, UK), characterised using BLAST (NCBI) and Clustal Omega (EMBL-EBI) to confirm it was zfTRβ (Fig. S3 and Fig. S4).

4.3.3 Construction of TRE-PAL/DR4 reporter plasmids

The TH-regulated reporter vectors containing a TRE were prepared using oligo DNA primers TRE-DR4_Fwd: CTC GCT AGC CTC GAG AGG TCA CTT GAG GTC AGA TGA GCT TCA GGT CAT GTA AGG TCA CCC TGC CCT A and TRE-DR4-Rev: TCT AAG CTT AGA TCT TGA CCT CAA GTG ACC TAG GAC TGA AGT GAC CTC AGT TGA CCT TAG GGC AGG GTG ACC TTA CA. These were amplified (10 µM each) with Primestar Gxl (Takara) under the following PCR conditions: 98˚C for 10 seconds, 60˚C for 15 seconds, 68˚C for 15 seconds for 30 cycles. DNA was purified and sub-cloned into pCR4 Blunt TOPO vector (Invitrogen). The DNA fragment was cut using restriction enzymes Nhe*I* and Hind*III* and subsequently sub-cloned into pGL4.24 (Promega). The TRE-PAL reporter vector was constructed according to Oka *et al* (2013) [90]. Briefly, the vector contained four palindromes (AGGTCATGACCT) and was constructed by sub-cloning of oligonucleotides having 4× TRE into the Nhe*I*–Hind*III* site of pGL4.24 (Promega).

4.3.4 Culture of HEK cells

Unless stated differently, all reagents used for cell culture were obtained from Invitrogen (UK). Human embryonic kidney 293 (HEK 293) cells were obtained from LGC Standards (UK). HEK cells were cultured in phenol red free Dulbeccco's Modified Eagles medium (DMEM) containing 1000 mg l^{-1} D-Glucose, with 10% heat inactivated foetal bovine serum (FBS), 1% L-glutamine (Sigma) and 1% penicillin–streptomycin (Sigma). Cells were sub-cultured when reaching approximately 80% confluence. For sub-culturing, cells were rinsed with calcium and magnesium-free Dulbecco's phosphate-buffered saline (DPBS) and dissociated with 0.05% trypsin/EDTA for 3 minutes. The trypsin was subsequently neutralised with fresh cell culture medium and cells collected by centrifugation for 3 min at 1000 rpm at room temperature. Cells were re-suspended in culture medium, counted and seeded into 75 cm^3 cell culture flasks (at a ratio of 1:6-1:10) or into 24 well plates for subsequent reporter gene assays (see below).

4.3.5 Transfection for reporter gene assays

HEK cells were seeded in 24-well plates at 5×10^4 cells per well in DMEM with 10% charcoal/dextran-treated FBS (Hyclone) and penicillin–streptomycin. After 24 hours, cells were transfected with 400 ng of reporter vector (pGL4.24-PAL or pGL4.24-DR4), 100 ng of pRL-TK [contains the strong herpes simplex virus thymidine kinase promoter driving sea pansy (*Renilla reniformis)* luciferase gene as an internal control to normalise variations in transfection efficiency] (Promega), and 200 ng of either pcDNA3.1-TRα or pcDNA3.1-TRβ using Fugene HD transfection reagent (Promega).

4.3.6 Chemical screening

Initially transcriptional assays were used to establish dose-response curves for the receptor agonist, T3, in the concentration ranges between 10^{-12} and 10^{-7} M for both zfTRα and zfTRβ. At the same time, we compared the functions of two separate reporter constructs, pGL4.24-PAL and pGL4.24-DR4. The pGL4.24-DR4 construct showed greater induction by both zfTRα and zfTRβ and was used in subsequent exposures as the reporter construct. The sensitivity of the TRs to TBBPA, HBCD, BDE-47, BDE- 100, BDE-183 and BDE-209 was examined in the concentration ranges of 10^{-12} M to 10^{-6} M for both zfTRα and zfTRβ. All compounds were applied to the medium at non-toxic concentrations for cells after 4 hours of incubation post transfection. The concentration of DMSO in the culture medium was 0.1% and control wells were dosed with 0.1% DMSO only.

4.3.7 Dual luciferase assays

After an incubation of 40 hours post dosing (i.e. 44 hours after transfection), the luciferase activity of the cells was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, cells were rinsed with 1 ml PBS and subsequently lysed with 100 µl 1x passive lysis buffer. 10 µl of lysed cells were added to 50 µl substrate buffer and firefly (*Photinus pyralis*) luciferase (reporter) activity measured. Subsequently, this luminescence was quenched by addition of 50 µl Stop & Glo® reagent and sea pansy luciferase activity was measured. Measurements were carried out using an Infinite M200 plate reader (Tecan, Austria). Promoter activity was normalised by calculating firefly / sea pansy luciferase activity and expressed as fold activation relative to the control group.

4.3.8 Data analyses

Data are presented throughout as mean fold change ± standard error of the mean (SEM). Transfections were performed in triplicate and repeated three times on cells with different passage numbers. Dose–response data and half maximal effective concentration (EC-50) values were analysed using GraphPad Prism (Graph Pad Software Inc.). All other statistical analyses were carried out using R Studio. A general linear model (GLM) with Gaussian errors structures was used to compare the T3-induced luciferase activity in the zfTRα and zfTRβ assays and to compare the functions of two reporter constructs, pGL4.24-DR4 and pGL4.24-PAL. Data was log-transformed where necessary to ensure linearity and models were checked for homoscedasticity and normality of residuals. Differences were considered significant at $p<0.05$.

4.4 Results

4.4.1 Comparison of reporter plasmids

In order to establish an effective reporter gene assay, we initially examined the induction of transcriptional activity of zfTRα and zfTRβ by the natural ligand T3. Simultaneously, the responsiveness of two constructed reporter vectors, pGL4.24-PAL and pGL4.24-DR4, was examined. Independently of the reporter vector, T3 was effective in inducing luciferase activity of both TRs and zfTRβ was more sensitive than zfTRα ($p<0.05$). The test systems transfected with the pGL4.24-DR4 and pGL4.24-PAL reporter vectors showed significant activation of zfTRα in cells exposed to concentrations of T3 greater than 10^{-10} M and 10^{-9} M, respectively ($p<0.05$; Fig. 1A). Using the pGL4.24-DR4 construct as reporter vector, TRα was

activated by T3 with a maximal effect (E_{max}) of 5.1- fold relative to the control at 10 nM and the EC-50 for T3 was 1.73 nM (Table 1). Using the pGL4.24-PAL construct, TRα was activated with an E_{max} of 2.8- fold relative to the control and the EC-50 was 3.33 nM (Table 1). The test systems transfected with the pGL4.24-DR4 and pGL4.24-PAL reporter vectors showed significant activation of zfTRβ in cells exposed to concentrations of T3 greater than 10^{-10} M, respectively ($p<0.05$; Fig. 1B). TRβ was activated by T3 with an E_{max} of 15.5- fold relative to the control and EC-50 of 1.56 nM using the pGL4.24-DR4 construct as reporter vector (Table 1). Using the pGL4.24-PAL construct, TRβ was activated by T3 with an E_{max} of 11.5- fold relative to the control and EC-50 of 1.87 nM (Table 1). As a result of its greater sensitivity, pGL4.24-DR4 was used as the response vector in subsequent BFR dose response reporter gene assays.

4.4.2 BFR dose responses

The thyroid hormonal activity of six BFR compounds (TBBPA, HBCD, BDE-47, BDE-100, BDE-183 and BDE-209) was subsequently examined using the zebrafish TR-dependent reporter gene assay, however, none of the BFRs tested induced the transactivation of zfTRα (Fig. 2) or zfTRβ (Fig. 3) at any of the concentrations tested.

4.5 Discussion

A reporter gene transcriptional assay was established for both zebrafish TRα and TRβ receptors. We found that zfTRα was activated by T3 with an E_{max} of 5.1- fold relative to the control and EC-50 of 1.73 nM using the pGL4.24-DR4 construct, and an E_{max} of 2.8- fold relative to the control and EC-50 of 3.33 nM using the pGL4.24-

PAL construct. No previous data are available on the transactivation of zebrafish TRs by T3 to help confirm the stability and robustness of the established system. However, a similar reporter gene study has been carried out to assess the effect of T3 on medaka TR transcriptional activity in HEK cells. In that study only a pGL4.24-PAL response construct was used [90] and the authors of that work found that medaka TRα activity exhibited a 10- fold greater sensitivity to T3 exposure (EC-50 of 0.37 nM) when compared to the zfTRα activity observed using the pGL4.24-PAL construct in the present study. Nevertheless, the E_{max} values from both studies are in a similar range (9- fold increase relative to the control in medaka). Considering that different fish species show differences in their responsiveness to estrogenic chemicals [91], it may also be the case that they show differences in their responsiveness to thyroid chemicals though this has yet to be examined in any detail. A number of amino acid substitutions in the LBD of the estrogen receptor alpha (ERα) of different vertebrate species are suggested to be a contributing factor to their varying sensitivities to the different estrogens [91, 92] and may also explain the observed differences in the responsiveness of TRα to T3 between medaka and zebrafish (Fig. S5 and S6 show amino acid alignments of medaka, human and zebrafish TRα-LBDs and TRβ-LBDs).

Regarding the zfTRβ activity in the present study, we found that T3 induced an E_{max} of 15.5- fold relative to the control and EC-50 of 1.56 nM using the pGL4.24-DR4. The E_{max} (11.5- fold relative to the control) and EC-50 (1.87 nM) were similar using the pGL4.24-PAL construct. The sensitivity of zfTRβ to T3 exposure is comparable to results seen for the medaka (EC-50 of 3.32 nM) and *Xenopus laevis* (EC-50 of 1.5 - 2.94nM) [88, 90]. Human TRβ activity appears less sensitive to T3 exposure (EC-50

values between 10^{-8} and 10^{-5}) in reporter gene assays carried out in Chinese hamster ovary cells (CHO-K1) and green monkey kidney fibroblast cells (CV-1) [83, 93] but a similar sensitivity was observed in assays using HEK-cells (EC-50 of 1.37 nM) [90]. It appears that some of the differences seen could be a result of the different cell types utilised in various reporter gene assay studies. Across different cell lines there may be differences in the expression of endogenous proteins that act as co-repressors and co-activators, which play a critical role in modulating the transcriptional activity of TRs. In addition, different cell types may have different metabolic capabilities which may influence the responses observed in transactivation assays

Here we found that the T3-induced transcriptional activity of both zebrafish TRs was greater when using the pGL4.24-DR4 construct as reporter vector compared with the pGL4.24-PAL construct. In mammal studies it has become increasingly clear that TRs exhibit preferences for different configurations of TREs (DR4, PAL or IP) depending on their conformational state (heterodimer, homodimer or monomer). Therefore the type of response element used in reporter gene assays can influence the observed TR transcriptional activity [94, 95]. As far as we are aware no information is available on the preferences of fish TRs for different TREs. In the context of mammalian transcriptional regulation however, it has been shown that heterodimers of RXRs and TRs bind to DNA with a strong preference for DR4 response elements [26, 96]. Given that no endogenous expression of RXR occurs in HEK cells [97] and the transfected reporter vectors lacked an RXR construct, we can conclude that binding as a heterodimer to the TREs would not have been possible in our study. Since the TRE half-sites are arranged in pairs in both PAL and DR4

constructs, we consequently assume binding occurred as a homodimer. Given that TRβ preferentially forms homodimers while TRα forms homodimers poorly [98], this may explain the observed differences in the T3-induced luciferase activity between TRα and TRβ in the present study. Both zebrafish TRs activated gene transcription by binding preferentially to the DR4 response element. In mammals, both TRs bind as monomers to TREs with no discernible preference, while TRβ homodimers bind preferentially to IP response elements, weakly to DR4 and not at all to PAL [96, 99, 100]. Meanwhile, TRα homodimers bind TREs, at best, only weakly. Given that TRβ readily forms homodimers, this may explain the preferential binding to DR4 observed in the present study. The preferential binding of TRα to the DR4 element, observed here in a fish species, remains to be explained and perhaps species-specific TRE preferences exist, however this has yet to be examined.

None of the six BFRs tested here had an effect on zebrafish TR-mediated transcriptional activity in a luciferase reporter gene assay performed in a HEK-cell line. Our results are in line with a comprehensive study on the effect of PBDEs and TBBPA on human TR activity, which found no TH agonistic effect in an *in vitro* assay using CHO-K1 cells [86]. Similarly, HBCD and TBBPA had no agonistic effect on human TRα1 activity in HeLaTR cells [87]. In the only previous study examining fish TR activity, no agonistic effect of TBBPA was seen on medaka TR activity in an assay performed in HEK cells [90]. In contrast to these studies, BDE-28 was found to induce *X. laevis* TRβ activity in a reporter gene assay performed in a CV-1 cell line, though no effect on TRα was observed [88].

There does, however, appear to be sufficient evidence that several BFRs have an antagonistic effect on TR-mediated transcription based on various *in vitro* studies. For instance, the hydroxylated PBDE congener 4-HO-BDE-90 was shown to have a weak antagonistic effect on the activity of both human TRs in CHO-K1 cells [86], while BDE-206 showed antagonistic activation of both *X. laevis* TRs [88]. In addition, antagonistic effects of TBBPA on both human TRs were observed in CHO-K1 cells [83], as well as on medaka TRβ in HEK cells [90]. A study carried out using the rat pituitary tumour GH3 cell line found that at low levels (up to 1 μM) TBBPA acted as a weak agonist on rat TRs but at higher levels (above 5 μM) acted as an antagonist [84]. The observed BFR suppression of TR-mediated transcription *in vitro* may be a result of several mechanisms including the inhibition of TR-TRE binding, the recruitment of co-repressor proteins to TRs and/or the dissociation of co-activators from TRs. Interestingly, a recent study demonstrated that several PBDEs (BDE-209, BDE-100, BDE-153, BDE-154) suppressed TR-mediated gene expression and in the case of BDE-209 attributed the disruption to the partial dissociation of TRs from the TRE [85]. Examining the antagonistic effects of BFRs on zebrafish TRs, though beyond the scope of this study, is a key area of interest for future work.

There is a high degree of variations in the results derived from various *in vitro* assays regarding the effect of BFR compounds on TR-mediated transcription. This may be a result of the large range of BFR compounds tested, the different species and receptor types (TRα or TRβ) examined, as well as the different cell systems and reporter constructs utilised. It should also be noted that BFR compounds are highly hydrophobic and easily disassociate from aqueous solutions, and in *in vitro* reporter gene assays carried out in plastic 24-well plates the exposure concentrations may be

significantly lower than nominal concentration. This makes interpreting the available data challenging. Nevertheless, based on the observations in the current study and prior work with mammal TRs, we tentatively conclude that BFRs have limited agonistic effects on TR-mediated gene transcription when studied *in vitro.* In contrast, a number of studies have found that various BFRs are capable of altering the transcription of TH-responsive genes in both mammals and fish species *in vivo.* For instance, exposure to BDE-47, has been shown to induce a suite of TH-responsive genes in the brain and liver of Wister rat pups, despite the fact that in that same study BDE-47 did not interfere with TRβ transcriptional activity *in vitro* [101]. In contrast to rodents, limited information is available on the specific genes controlled by THs in fish [102]. Given that the genes for the TRs themselves contain TREs and are auto-induced by T3, the TR transcripts are good markers for assessing TH-induced gene transcription. For instance, zebrafish larvae exposed to BDE-47 had reduced TRβ transcript levels and enhanced TRα transcript levels [103]. Meanwhile in the same study, TBBPA exposure resulted in elevated TRα transcript levels, but no changes in TRβ transcript levels were observed [103]. Similarly, exposure of adult fathead minnows (*Pimephales promelas*) to BDE-47 was shown to reduce the transcript abundance of TRβ as well as the TH-responsive transcription factor basic transcription element-binding protein (BTEB) in the brain, while in the brain of females transcript levels of TRα were elevated [104]. Again, exposure to BDE-209 elevated the transcript levels of both TRs in fathead minnows and zebrafish [105, 106]. However it is highly plausible that altered TR-mediated transcription, is in fact a consequence of increased T3 levels in target tissues (resulting from increased deiodinase activity) and not due to direct interaction with BFRs.

Taking the findings of both *in vitro* and *in vivo* studies together, it appears that increased transcript levels of TH-responsive genes may not be the result of a direct interference with TR activity but perhaps are a consequence of altered TH levels, resulting from increased metabolism and excretion of THs and/or changes in the production of TH by the thyroid glands/follicles. On the other hand, in terms of decreased transcript levels of TH-responsive genes, it appears that BFRs may alter transcription by directly disrupting TR activity and/or through multiple other mechanisms involved in altering circulating TH levels.

Overall the data presented in this study suggests that the transactivation reporter gene assay developed for zebrafish TRs is a useful and sensitive assay for examining the activation of TRs, as shown by responses to the natural ligand T3 and may be applied to further screen environmentally relevant compounds, suspected of causing thyroid disruption, for zebrafish TR activation. *In vitro* screens reduce the use of animal models and allow for more a targeted approach in identifying and assessing the mechanistic pathways of substances suspected of adversely effecting human and wildlife health. Despite the expanding knowledge on thyroid disruption by BFRs, further investigations are still greatly needed in order to fully understand their mechanisms of action, particularly in fish species.

Figures and Tables

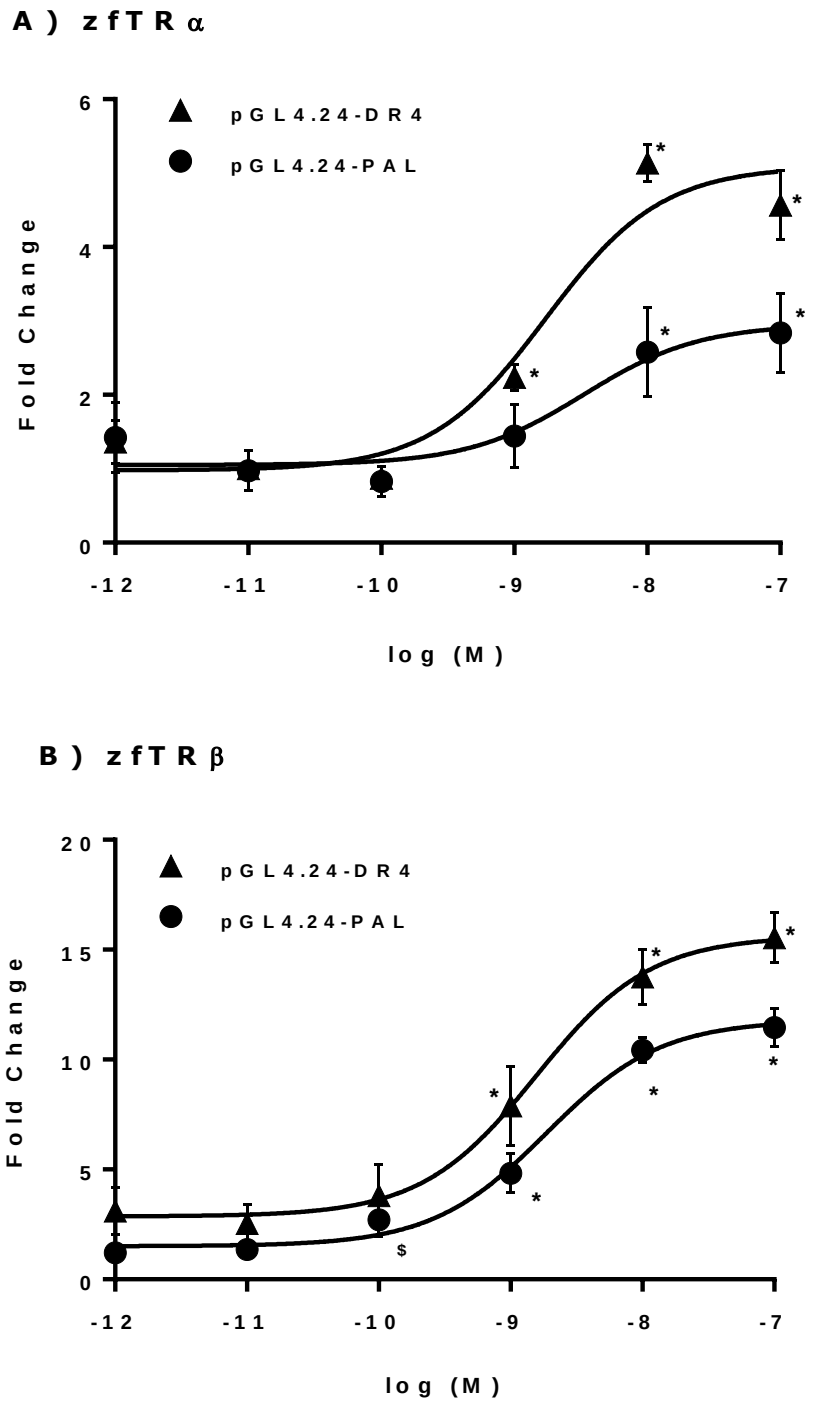

Figure 1: The transcriptional activity of zebrafish TRs exposed to 3,3′,5-triiodo-L-thyronine (T3). Dose response profile for (A) zfTRα and (B) zfTRβ using the reporter vectors pGL4.24-DR4 and pGL4.24-PAL. The results are represented as means ±

SEM and each group was analysed in triplicate. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S1. Both zfTRα and zfTRβ transcriptional activities were significantly induced by T3 ($p<0.05$). An asterisk above a data point denotes that the treatment was significantly different from the DMSO control ($p<0.05$). A dollar sign above a data point denotes that the treatment was approaching significant ($p=0.0514$)

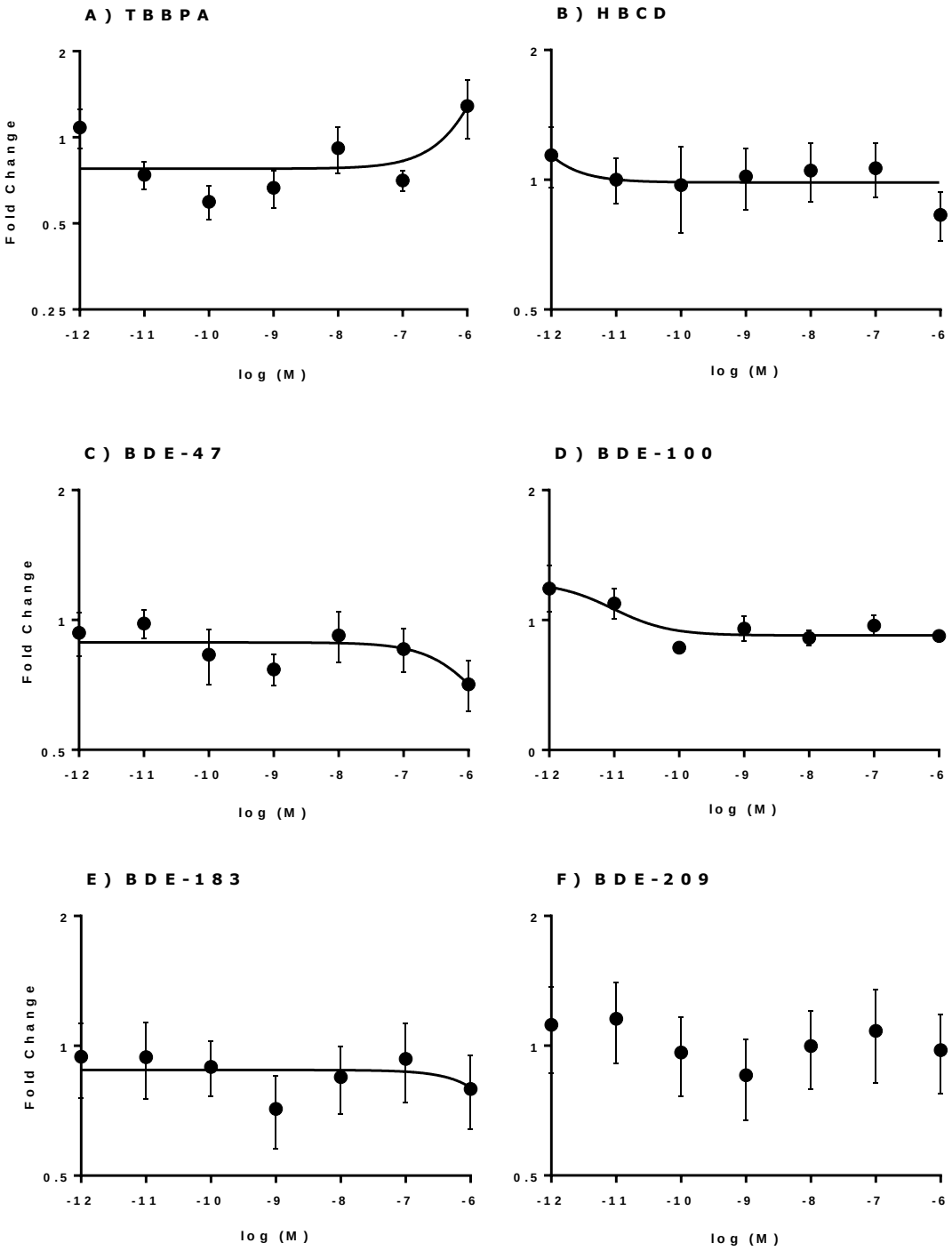

Figure 2: Transcriptional activities of zebrafish TRα exposed to brominated flame retardants using the reporter vector pGL4.24-DR4. Concentration-response profiles for (A) tetrabromobisphenol A (TBBPA), (B) hexabromocyclododecane (HBCD), (C) 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), (D) 2,2′,4,4′,6-penta-bromodiphenyl

ether (BDE-100), (E) 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183) and (F) deca-bromodiphenyl ether (BDE-209). The results are represented as means ± SEM and each group was analysed in triplicate. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S1. There was no significant effect of BFR treatments on TRα transcriptional activity.

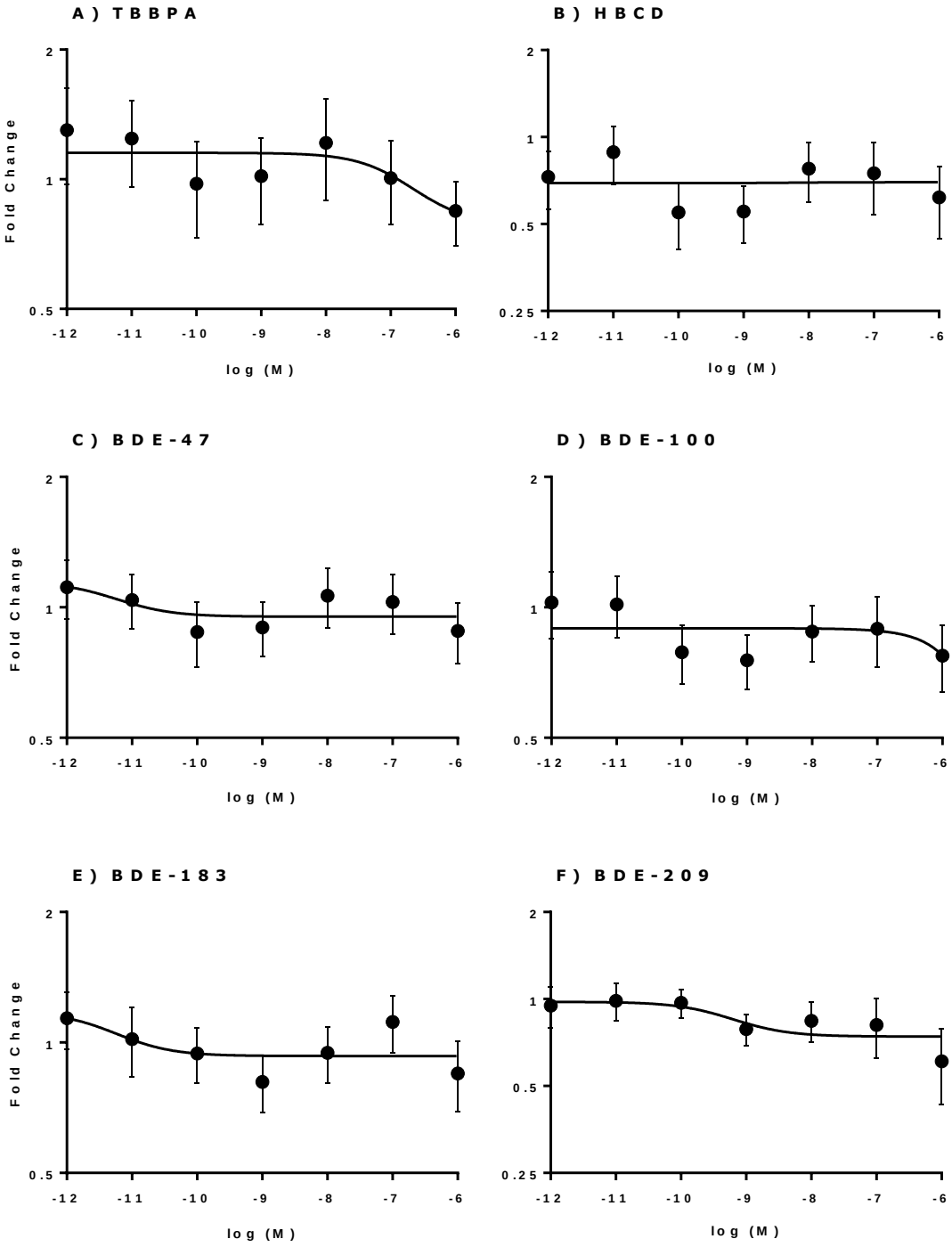

Figure 3: Transcriptional activities of zebrafish TRβ exposed to brominated flame retardants using the reporter vector pGL4.24-DR4. Concentration-response profiles for (A) tetrabromobisphenol A (TBBPA), (B) hexabromocyclododecane (HBCD), (C) 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), (D) 2,2′,4,4′,6-penta-bromodiphenyl

ether (BDE-100), (E) 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183) and (F) deca-bromodiphenyl ether (BDE-209). The results are represented as means ± SEM and each group was analysed in triplicate. Statistics were carried out using general linear models (GLM; R) with model details reported in Table S1. There was no significant effect of BFR treatments on TRβ transcriptional activity.

Table 1: Transcriptional activity of 3,3′,5-triiodo-L-thyronine (T3) mediated by zebrafish thyroid receptors TRα and TRβ.

	Vector	EC-50[a] (nM)	95% CI (M)[b]	E_{max}[c]
zfTRα	pGL4.24-DR4	1.73	1.917×10^{-10} to 1.559×10^{-8}	5.1
	pGL4.24-PAL	3.33	2.432×10^{-10} to 4.558×10^{-8}	2.8
zfTRβ	pGL4.24-DR4	1.56	1.063×10^{-9} to 2.278×10^{-9}	15.5
	pGL4.24-PAL	1.87	8.473×10^{-10} to 4.109×10^{-9}	11.5

a half maximal effective concentration (EC-50)

b 95% CI: 95% confidence intervals of EC-50

c E_{max}: Maximal effect (as fold change relative to control)

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Chapter 4

Supplementary Material

Development of a transactivation reporter gene assay for zebrafish (*Danio rerio*) thyroid receptors TRα and TRβ and an investigation into their interactions with brominated flame retardants.

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Supplementary Figures

```
zfTRα(pcDNA3.1)     ATGGAAAACACAGAGCAGGAGCACAACCTGCCAGAAGGAGATGAGACGCAATGGCCAAAT
NM_131396.1         ATGGAAAACACAGAGCAGGAGCACAACCTGCCAGAAGGAGATGAGACGCAATGGCCAAAT

zfTRα(pcDNA3.1)     GGAGTGAAGAGGAAAAGAAAGAATAGTCAATGCTCTATGAACAGCACATCCGACAAGAGC
NM_131396.1         GGAGTGAAGAGGAAAAGAAAGAATAGTCAATGCTCTATGAACAGCACATCCGACAAGAGC

zfTRα(pcDNA3.1)     ATCTCTGTTCCTGGGTACGTTCCCAGTTATCTGGAGAAAGATGAGCCGTGTGTGGTGTGC
NM_131396.1         ATCTCTGTTCCTGGGTACGTTCCCAGTTATCTGGAGAAAGATGAGCCGTGTGTGGTGTGC

zfTRα(pcDNA3.1)     GGAGACAAGGCCACTGGCTATCATTACCGCTGCATCACATGTGAGGGCTGCAAGGGTTTC
NM_131396.1         GGAGACAAGGCCACTGGCTATCATTACCGCTGCATCACATGTGAGGGCTGCAAGGGTTTC

zfTRα(pcDNA3.1)     TTCAGGAGGACAATACAGAAAAACCTTCATCCGTCCTATTCCTGTAAATATGACAGCTGC
NM_131396.1         TTCAGGAGGACAATACAGAAAAACCTTCATCCGTCCTATTCCTGTAAATATGACAGCTGC

zfTRα(pcDNA3.1)     TGCATCATTGACAAAATCACCCGCAACCAGTGCCAGCTGTGCCGCTTCAGGAAGTGCATC
NM_131396.1         TGCATCATTGACAAAATCACCCGCAACCAGTGCCAGCTGTGCCGCTTCAGGAAGTGCATC

zfTRα(pcDNA3.1)     TCAGTGGGCATGGCCATGGACTTGGTGCTGGATGATTCGAAGCGCGTGGCCAAGAGGCGT
NM_131396.1         TCAGTGGGCATGGCCATGGACTTGGTGCTGGATGATTCGAAGCGCGTGGCCAAGAGGCGT

zfTRα(pcDNA3.1)     CTGATCGAAGAAAATCGGGAGAAGAGAAAGAAAGAGGAGATTGTGAAGACGTTACACAAT
NM_131396.1         CTGATCGAAGAAAATCGGGAGAAGAGAAAGAAAGAGGAGATTGTGAAGACGTTACACAAT

zfTRα(pcDNA3.1)     CGACCTGAGCCCACCGTGTCAGAGTGGGAGCTCATTCGTATGGTGACGGAGGCTCATCGG
NM_131396.1         CGACCTGAGCCCACCGTGTCAGAGTGGGAACTCATTCGTATGGTGACGGAGGCTCATCGG

zfTRα(pcDNA3.1)     CACACCAATGCCCAGGGCCCTCACTGGAAACAGAAACGCAAGTTCCTACCAGAAGACATT
NM_131396.1         CACACCAATGCCCAGGGCCCTCACTGGAAACAGAAACGCAAGTTCCTACCAGAAGACATT

zfTRα(pcDNA3.1)     GGACAGTCTCCAGCTCCGACATCAGACAATGATAAAGTGGACCTGGAGGCCTTCAGTGAG
NM_131396.1         GGACAGTCTCCAGCCCCGACATCAGACAATGATAAAGTGGACCTGGAGGCCTTCAGTGAG

zfTRα(pcDNA3.1)     TTTACCAAGATCATTACGCCTGCCATCACACGAGTTGTGGACTTTGCCAAAAAACTGCCC
NM_131396.1         TTTACCAAGATCATTACGCCTGCCATCACACGAGTTGTGGACTTTGCCAAAAAACTGCCC

zfTRα(pcDNA3.1)     ATGTTCTCTGAGCTGCCCTGTGAAGTCCAGATCATCTTGCTGAAAGGCTGCTGTATGGAG
NM_131396.1         ATGTTCTCTGAGCTGCCCTGTGAAGACCAGATCATCTTGCTGAAAGGCTGCTGTATGGAG

zfTRα(pcDNA3.1)     ATCATGTCATTGCGAGCAGCGGTGCGGTACGACCCCGAGAGCGAGACTCTGACCCTGAGC
NM_131396.1         ATCATGTCATTGCGAGCAGCGGTGCGGTACGACCCCGAGAGCGAGACTCTGACCCTGAGC

zfTRα(pcDNA3.1)     GGAGAGATGGCCGTCAGTCGAGAGCAGCTGAAAAACGGAGGGCTCGGAGTGGTTTCTGAT
NM_131396.1         GGAGAGATGGCCGTCAGTCGAGAGCAGCTGAAAAACGGAGGGCTCGGAGTGGTTTCTGAT

zfTRα(pcDNA3.1)     GCCATCTTTGATTTGGGGAAGAGCCTGTCACAGTTTAACCTGGATGACTCTGAGGTGGCA
NM_131396.1         GCCATCTTTGATTTGGGGAAGAGCCTGTCACAGTTTAACCTGGATGACTCTGAGGTGGCA

zfTRα(pcDNA3.1)     CTGCTACAAGCTGTGCTGCTCATGAGCTCAGATCGCTCTGGACTGACATGTGTGGAGAAG
NM_131396.1         CTGCTACAAGCTGTGCTGCTCATGAGCTCAGATCGCTCTGGACTGACATGTGTGGAGAAG

zfTRα(pcDNA3.1)     ATCGAGAAGTGTCAGGAGATGTACCTGTTGGCATTCGAGCACTACATCAACCACCGCAAG
NM_131396.1         ATCGAGAAGTGTCAGGAGATGTACCTGTTGGCATTCGAGCACTACATCAACCACCGCAAG

zfTRα(pcDNA3.1)     CACAACATCTCCCACTTCTGGCCCAAGCTGCTGATGAAGGTGACGAACCTGCGCATGATC
NM_131396.1         CACAACATCTCCCACTTCTGGCCCAAGCTGCTGATGAAGGTGACGAACCTGCGCATGATC

zfTRα(pcDNA3.1)     GGCGCCTGCCACGCCAGCCGCTTCCTGCACATGAAGGTGGAGTGTCCAACAGAACTGTTC
NM_131396.1         GGCGCCTGCCACGCCAGCCGCTTCCTGCACATGAAGGTGGAGTGTCCAACAGAACTGTTC

zfTRα(pcDNA3.1)     CCGCCGCTCTTCCTGGAGGTCTTCGAGGATCAGGAGGGAAGCACTGGAGTGGCAGCACAG
NM_131396.1         CCGCCGCTCTTCCTGGAGGTCTTCGAGGATCAGGAGGGAAGCACTGGAGTGGCAGCACAG

zfTRα(pcDNA3.1)     GAAGACGGTTCCTGCTTAAGGTGA
NM_131396.1         GAAGACGGTTCCTGCTTAAGGTGA
```

Figure S1: Nucleotide sequence alignment of PCR amplified zfTRα sequence in pcDNA3.1 vector with known sequence (NM_131396.1), with conserved residues highlighted in grey and non-conserved residues in white.

Figure S2: Amino acid alignment of zfTRα protein sequence and known protein sequence (NP_571471.1), with conserved residues highlighted in grey and non-conserved residues in white.

```
zfTRβ(pcDNA3.1)   ATGTCAGAGCAAGCAGACAAATGCAACTCCCGCTGGAAAGATGAGGCCATGCAGAATGGG
NM_131340.1       ATGTCAGAGCAAGCAGACAAATGCAACTCCCGCTGGAAAGATGAGGCCATGCAGAATGGG

zfTRβ(pcDNA3.1)   TACATACCAAGTTACCTGGACAAGGATGAGCTGTGTGTTGTTTGTGGAGACAAAGCAACT
NM_131340.1       TACATACCAAGTTACCTGGACAAGGATGAGCTGTGTGTTGTTTGTGGAGACAAAGCAACT

zfTRβ(pcDNA3.1)   GGTTATCACTATCGCTGCATTACATGTGAGGGCTGCAAAGGTTTCTTTCGACGCACAATT
NM_131340.1       GGTTATCACTATCGCTGCATTACATGTGAGGGCTGCAAAGGTTTCTTTCGACGCACAATT

zfTRβ(pcDNA3.1)   CAGAAGAACTTAAACCCAACATACGCATGCAAGTATGAAGGAAAGTGTGTCATTGACAAA
NM_131340.1       CAGAAGAACTTAAACCCAACATACGCATGCAAGTATGAAGGAAAGTGTGTCATTGACAAA

zfTRβ(pcDNA3.1)   GTTACCCGAAACCAGTGCCAAGAATGTCGCTTCAAGAAATGCATCGCTGTTGGCATGGCT
NM_131340.1       GTTACCCGAAACCAGTGCCAAGAATGTCGCTTCAAGAAATGCATCGCTGTTGGCATGGCT

zfTRβ(pcDNA3.1)   ACAGACTTGGTATTGGATGACAGTAAACGTTTAGCCAAGCGGAAGCTGATTGAGGAGAAC
NM_131340.1       ACAGACTTGGTATTGGATGACAGTAAACGTTTAGCCAAGCGGAAGCTGATTGAGGAGAAC

zfTRβ(pcDNA3.1)   CGTGAACGCCGACGGCGTGAGGAGCTGCAGAAGACTGTATGGGATCGACCAGAGCCCACA
NM_131340.1       CGTGAACGCCGACGGCGTGAGGAGCTGCAGAAGACTGTATGGGATCGACCAGAGCCCACA

zfTRβ(pcDNA3.1)   CAGGAAGAGTGGGAGATGATACGGGTTGTCACAGAGGCCCATATGGCCACAAATGCCCAA
NM_131340.1       CAGGAAGAGTGGGAGATGATACGGGTTGTCACAGAGGCCCATATGGCCACAAATGCCCAA

zfTRβ(pcDNA3.1)   GGCAACCACTGGAAACAAAAGCGCAAATTCCTGCCAGAAGACATTGGATCGGCACCTATT
NM_131340.1       GGCAACCACTGGAAACAAAAGCGCAAATTCCTGCCAGAAGACATTGGATCGGCACCTATT

zfTRβ(pcDNA3.1)   GTCAATGCACCAGAGGGGAACAAAGTGGACATTGAAGCCTTCAGTCAGTTTACAAAAATT
NM_131340.1       GTCAATGCACCAGAGGGGAACAAAGTGGACATTGAAGCCTTCAGTCAGTTTACAAAAATT

zfTRβ(pcDNA3.1)   ATCACCCCCGCCATCACTCGTGTCGTGGATTTTGCCAAAAAGCTACCCATGTTCTGTGAG
NM_131340.1       ATCACCCCCGCCATCACTCGTGTCGTGGATTTTGCCAAAAAGCTACCCATGTTCTGTGAG

zfTRβ(pcDNA3.1)   CTGCCTTGTGAGGACCAGATCATTTTGCTGAAGGGTTGTTGCATGGAGATCATGTCTCTT
NM_131340.1       CTGCCTTGTGAGGACCAGATCATTTTGCTGAAGGGTTGTTGCATGGAGATCATGTCTCTT

zfTRβ(pcDNA3.1)   CGAGCAGCAGTGCGTTATGACCCCGAGAGCGATACTCTGACGCTAAACGGGGAGATGGCT
NM_131340.1       CGAGCAGCAGTGCGTTATGACCCCGAGAGCGATACTCTGACGCTAAACGGGGAGATGGCT

zfTRβ(pcDNA3.1)   GTCACACGGGGCCAGCTCAAGAATGGGGGTTTGGGTGTTGTCTCAGATGCCATCTTTGAT
NM_131340.1       GTCACACGGGGCCAGCTCAAGAATGGGGGTTTGGGTGTTGTCTCAGATGCCATCTTTGAT

zfTRβ(pcDNA3.1)   CTGGGTGTCTCGCTGTCCTCTTTTAACTTGGACGATTCAGAGGTGGCGTTGTTACAAGCC
NM_131340.1       CTGGGTGTCTCGCTGTCCTCTTTTAACTTGGACGATTCAGAGGTGGCGTTGTTACAAGCC

zfTRβ(pcDNA3.1)   GTGATTCTGCTTTCCTCTGATCGTCCAGGTTTAACAAGCGTTGAGCGGATAGAGCGTTGT
NM_131340.1       GTGATTCTGCTTTCCTCTGATCGTCCAGGTTTAACAAGCGTTGAGCGGATAGAGCGTTGT

zfTRβ(pcDNA3.1)   CAGGAGGAGTTTCTTTTGGCCTTTGAACATTACATCAACTACCGTAAGCACAAGGTGGCT
NM_131340.1       CAGGAGGAGTTTCTTTTGGCCTTTGAACATTACATCAACTACCGTAAGCACAAGGTGGCT

zfTRβ(pcDNA3.1)   CACTTTTGGCCCAAACTGCTGATGAAGGTGACAGACCTGCGCATGATTGGTGCCTGCCAT
NM_131340.1       CACTTTTGGCCCAAACTGCTGATGAAGGTGACAGACCTGCGCATGATTGGTGCCTGCCAT

zfTRβ(pcDNA3.1)   GCCAGCCGCTTCCTGCACATGAAGGTGGAATGTCCCACAGAGCTCTTTCCGCCCCTCTTC
NM_131340.1       GCCAGCCGCTTCCTGCACATGAAGGTGGAATGTCCCACAGAGCTCTTTCCGCCCCTCTTC

zfTRβ(pcDNA3.1)   CTGGAAGTGTTTGAAGACTGA
NM_131340.1       CTGGAAGTGTTTGAAGACTGA
```

Figure S3: Nucleotide sequence alignment of zfTRβ sequence in pcDNA3.1 vector and known sequence (NM_131340.1), with conserved residues highlighted in grey.

```
ZfTRβ_protein        MSEQADKCNSRWKDEAMQNGYIPSYLDKDELCVVCGDKATGYHYRCITCEGCKGFFRRTI
NP_571415.1          MSEQADKCNSRWKDEAMQNGYIPSYLDKDELCVVCGDKATGYHYRCITCEGCKGFFRRTI

ZfTRβ_protein        QKNLNPTYACKYEGKCVIDKVTRNQCQECRFKKCIAVGMATDLVLDDSKRLAKRKLIEEN
NP_571415.1          QKNLNPTYACKYEGKCVIDKVTRNQCQECRFKKCIAVGMATDLVLDDSKRLAKRKLIEEN

ZfTRβ_protein        RERRRREELQKTVWDRPEPTQEEWEMIRVVTEAHMATNAQGNHWKQKRKFLPEDIGSAPI
NP_571415.1          RERRRREELQKTVWDRPEPTQEEWEMIRVVTEAHMATNAQGNHWKQKRKFLPEDIGSAPI

ZfTRβ_protein        VNAPEGNKVDIEAFSQFTKIITPAITRVVDFAKKLPMFCELPCEDQIILLKGCCMEIMSL
NP_571415.1          VNAPEGNKVDIEAFSQFTKIITPAITRVVDFAKKLPMFCELPCEDQIILLKGCCMEIMSL

ZfTRβ_protein        RAAVRYDPESDTLTLNGEMAVTRGQLKNGGLGVVSDAIFDLGVSLSSFNLDDSEVALLQA
NP_571415.1          RAAVRYDPESDTLTLNGEMAVTRGQLKNGGLGVVSDAIFDLGVSLSSFNLDDSEVALLQA

ZfTRβ_protein        VILLSSDRPGLTSVERIERCQEEFLLAFEHYINYRKHKVAHFWPKLLMKVTDLRMIGACH
NP_571415.1          VILLSSDRPGLTSVERIERCQEEFLLAFEHYINYRKHKVAHFWPKLLMKVTDLRMIGACH

ZfTRβ_protein        ASRFLHMKVECPTELFPPLFLEVFED
NP_571415.1          ASRFLHMKVECPTELFPPLFLEVFED
```

Figure S4: Amino acid alignment of zfTRβ protein sequence and known protein sequence (NP_571415.1), with conserved residues highlighted in grey.

A

TRα	Zebrafish	Medaka	Human
Zebrafish	100.00	93.59	90.00
Medaka		100.00	90.06
Human			100.00

B

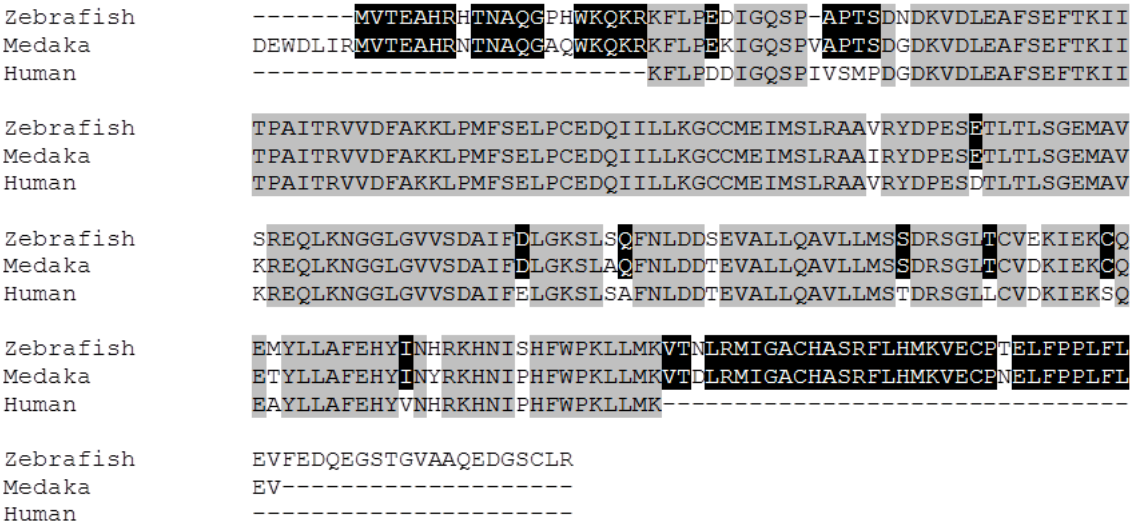

Figure S5. (A) Percent similarity of the ligand binding domain (LBD) structure of zebrafish TRα (*D. rerio*), medaka TRα (*O. latipes*) and human TRα (*H. sapiens*). (B) Amino acid alignment of medaka and human TRα-LBDs with the zebrafish TRα-LBD. Residues highlighted in grey are fully conserved between all of the species. Residues highlighted in black are conserved between the two fish species. The GenBank accession numbers of the TRα sequences in this alignment are; zebrafish NP_571471.1, medaka BAD11772.1 and human NP_955366.1.

A

TRβ	Zebrafish	Medaka	Human
Zebrafish	100.00	91.36	90.53
Medaka		100.00	86.42
Human			100.00

B

```
Zebrafish      QEEWEMIRVVTEAHMATNAQGNHWKQKRKFL---------PEDIGSAPIVNAPEGNKVDI
Medaka         QEEWDLIRLVTEAHTTTNAQGSHWKQKRKFLSQAGGKETKPEDFGQSSVVSSPDGNKVDI
Human          DEEWELIKTVTEAHVATNAQGSHWKQKRKFL---------PEDIGQAPIVNAPEGGKVDL

Zebrafish      EAFSQFTKIITPAITRVVDFAKKLPMFCELPCEDQIILLKGCCMEIMSLRAAVRYDPESD
Medaka         EAFSQFTKIITPAITRVVDFAKKLPMFCELPCEDQIILLKGCCMEIMSLRAAVRYDPESE
Human          EAFSHFTKIITPAITRVVDFAKKLPMFCELPCEDQIILLKGCCMEIMSLRAAVRYDPESE

Zebrafish      TLTLNGEMAVTRGQLKNGGLGVVSDAIFDLGVSLSSFNLDDSEVALLQAVILLSSDRPGL
Medaka         TLTLNGEMAVTRDQLKNGGLGVVSDAIFDLGVSLSSFNLDDSEVALLQAVILLSSDRPGL
Human          TLTLNGEMAVTRGQLKNGGLGVVSDAIFDLGMSLSSFNLDDTEVALLQAVLLMSSDRPGL

Zebrafish      TSVERIERCQEEFLLAFEHYINYRKHKVAHFWPKLLMKVTDLRMIGACHASRFLHMKVEC
Medaka         SSTDRIERCQEEFLLAFEHYINHRKHKVAHFWPKLLMKVTDLRMIGACHASRFLHLKVEC
Human          ACVERIEKYQDSFLLAFEHYINYRKHHVTHFWPKLLMKVTDLRMIGACHASRFLHMKVEC

Zebrafish      PTELFPPLFLEV
Medaka         PTELFPPLFLEV
Human          PTELFPPLFLEV
```

Figure S6. A) Percent similarity of the ligand binding domain (LBD) structure of zebrafish TRβ (*D. rerio*), medaka TRβ (*O. latipes*) and human TRβ (*H. sapiens*). B) Amino acid alignment of medaka and human TRβ-LBDs with the zebrafish TRβ-LBD. Residues highlighted in grey are fully conserved between all of the species. Residues highlighted in black are conserved between the two fish species. The GenBank accession numbers of the TRβ sequences in this alignment are: zebrafish NP_571415.1, medaka BAD11773.1 and human AAI06930.1.

Supplementary Tables

Table S1: General linear models of the relationship between T3/ BFRs and zebrafish thyroid receptor (TRα and TRβ) transcriptional activity. Minimum adequate models (F-value) for the activity of the two TRs are shown for the following chemicals: 3,3′,5-triiodo-L-thyronine (T3), tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), 2,2′,4,4′,6-penta-bromodiphenyl ether (BDE-100), 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183) and deca-bromodiphenyl ether (BDE-209). Transcriptional activity was measured in assays utilizing either palindrome (PAL) or direct repeat (DR4) thyroid response elements (TREs).

		TRα		TRβ	
Treatment	**TRE**	**df**	**Minimum Adequate Model**	**df**	**Minimum Adequate Model**
T3	PAL	64	3.24**	64	59.47***
	DR4	64	40.06***	64	24.91***
TBBPA	DR4	64	NS	64	NS
HBCD	DR4	64	NS	64	NS
BDE-47	DR4	64	NS	64	NS
BDE-100	DR4	64	NS	64	NS
BDE-183	DR4	64	NS	64	NS
BDE-209	DR4	64	NS	64	NS

CHAPTER 5

General Discussion

Fish are especially vulnerable to the exposure of anthropogenic pollutants. There are approximately 20,000 different species of fish - they are the most diverse group of vertebrates - occupying all aquatic niches. Understanding the actions of environmental pollutants on fish is important in evaluating the health of the aquatic environment and can indicate potential impacts on human health. While fish have long been the focus of field and laboratory studies into some endocrine disrupting chemicals (EDCs), most notably environmental oestrogens and anti- androgens which constitute a threat to reproductive health, thyroid disrupting chemicals (TDCs) have principally been assessed in amphibians and mammals. Given that thyroid hormones (THs) play a key role in a wide range of developmental processes and physiological functions in fish, it is important to consider how the actions of THs can potentially be disrupted by TDCs. In fish, the molecular mechanisms underlying TH disruption by brominated flame retardants (BFRs) and the effects on TH-sensitive tissues during early life stages are not fully understood. This has been limited by the lack of fundamental knowledge on the TH system of fish and the difficulties associated with examining transcriptional changes in discrete embryonic-larval tissues.

The first objective of this PhD was to establish the ontogeny, expression patterns and regulation of genes in the hypothalamic-thyroid-pituitary (HPT) axis of zebrafish during early life stages to enhance our understanding of the role of THs in fish and the potential targets of TDCs. The second objective was to examine the molecular mechanisms and target tissues of thyroid disruption by BFRs in zebrafish using both *in vivo* and *in vitro* assays.

In this discussion I provide a critical analysis of the studies conducted in this thesis to meet these objectives and review the key findings. I also include a discussion on the challenges and limitations of this work and discuss the relevance of these data in the wider context of toxicology research and possible directions for future research.

5.1 Key Findings

Chapter 2

In this thesis work, I have shown that thyroid receptors (alpha and beta) mRNAs show different and dynamic patterns of expression across early zebrafish developmental stages. These results suggest a role for THs in the development/functions of multiple tissues including the otic vesicles, brain, intestine and craniofacial tissues. The genes encoding deiodinase enzymes (*dio1* and *dio2*) were co-expressed in multiple TH target tissues, including the brain, intestine, pituitary and craniofacial tissues, consistent with their role in peripheral TH regulation. In addition, *dio3* expression was observed in the brain, kidneys and liver and therefore was implicated in controlling circulating and peripheral TH levels.

Examining the regulation of genes in the HPT axis by T3 during zebrafish early life stages, I established that exogenous exposure to T3 altered the expression of nine out of the ten genes selected (in whole body samples). The transthyretin encoding *ttr* gene was the only gene which was non-responsive to T3. We also found that transcriptional changes were tissue- and developmental stage-specific with TR-mediated signalling in the pineal gland, brain, pectoral fins, liver, intestine and craniofacial tissue sensitive to T3 exposure. This information increases our

understanding of the potential roles of various different components of the HPT axis, and it also highlights target tissues and/or functions which are potentially vulnerable to TH disruption by environmental pollutants.

Chapter 3

In Chapter 3, it was demonstrated that tetrabromobisphenol A (TBBPA) and 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), two important brominated flame retardants, disrupt TH homeostasis in zebrafish embryo-larvae after short sub-acute exposures. Until recently, the effect of xenobiotics on fish thyroid functions have typically been assessed using measures of TH biosynthesis, circulating TH levels and/or thyroid size. Unlike in mammals, the thyroid system of fish is not driven primarily by the central HPT axis. In fish the primary role of the HPT axis is to ensure T4 homeostasis, whereas T3 production and homeostasis is regulated in peripheral tissues by deiodinase enzymes. Therefore, the aforementioned indices relate mainly to the efficacy of TH release from the thyroid follicles to the blood. Here we have shown that TBBPA and BDE-47 disrupted the expression of genes associated with TH conjugation and clearance, thyroid follicle development, TH transport and TH signalling.

We also showed that TBBPA and BDE-47 altered gene transcription in a tissue-specific manner in zebrafish embryo-larvae. Previous research has shown that BFR compounds can disrupt transcriptional regulation of genes in the HPT axis of fish embryo-larvae [1-3], but as far as we are aware no other studies to date have assessed the effect of TBBPA and BDE-47 on TH genes at the tissue level. By examining changes in the expression pattern of TRs, we suggest that TR-mediated

regulation of target genes in the brain, liver, pronephric ducts and craniofacial tissues of zebrafish embryo-larvae may be particularly vulnerable to TBBPA and BDE-47 exposure. Potential compensatory responses in TH production/ metabolism by deiodinase enzymes were also evident in the brain, intestinal bulb, liver and pituitary of exposed embryo-larvae.

A key finding of this chapter was that responses to TBBPA, in terms of transcript profiles and expression patterns of several thyroid related genes, depended on the developmental stage examined. The effect of TBBPA appeared more pronounced in larvae at 96 hpf, compared with at 48 hpf and 120 hpf. Previous studies have demonstrated thyroid related genes to be transiently altered by BDE-209 in adult fish [4]. Here it was shown that TBBPA effected thyroid gene transcription which varied across even very early developmental life stages. This finding highlights the importance of life stage selection in experiments designed to examine the effects of environmental pollutants on the highly complex thyroid system.

The effect of TBBPA on the transcription of TH-responsive genes is not straightforward. Previous *in vivo* (in tadpoles) and *in vitro* studies have found that TBBPA acts as an agonist on several TH-responsive genes in the absence of THs, but acts an antagonist in co-exposed groups [5-7]. However, Chapter 3 demonstrated that TBBPA enhanced the T3-induced up-regulation of several genes in the HPT axis (*thrb, dio2* and *dio3)* of zebrafish larvae following a 96 hour exposure from fertilisation. Depending on the concentrations examined, TBBPA also had both stimulatory and suppressive effects on T3-induced up-regulation of *thraa* transcription.

Chapter 4

It has been proposed that environmental pollutants can disrupt TH signalling in wildlife by disrupting the activity of thyroid receptors (TRs), ligand-binding transcription factors, which mediate the genomic actions of THs. Here, I developed a functional and sensitive *in vitro* reporter gene assay for screening the ability of environmental compounds to disrupt zebrafish TR-mediated transcription. To date, only one similar assay has been developed using fish TRs, [in medaka (*Oryzias latipes*)] and the effect of TBBPA was examined [8]. We demonstrated that six brominated flame retardants including TBBPA, BDE-47, hexabromocyclododecane (HBCD), 2,2′,4,4′,6-penta-bromodiphenyl ether (BDE-100), 2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether (BDE-183) and deca-bromodiphenyl ether (BDE-209) had no agonistic effects on zebrafish TRα- and TRβ-mediated transcriptional activity in HEK cells. These results suggest that these BFR compounds do not directly interfere with TR-activity *in vitro*.

5.2 Challenges and Considerations

5.2.1 Examining tissue-specific effects of BFR exposures

The mechanistic actions of environmental pollutants are not usually restricted to one specific process but rather act via multiple sites and with multiple mechanisms involved. For the most part, studies assessing BFR-induced transcriptional changes in the HPT axis of fish early life stages have largely relied on qRT-PCR on pooled whole animal samples, while tissue-specific effects have been limited to adult studies where isolating organs/tissues of interest is relatively straightforward. This has significantly hindered our understanding of the mechanistic pathways and potential

targets of BFR compounds. One of the most important advantages of using whole mount *in situ* hybridisation is that it allows us to identify transcriptional changes in specific tissues during very early life stages. Here, in this thesis work I detected changes in the expression of thyroid related genes in the brain, pronephric ducts, pineal gland, liver, craniofacial tissues, otic vesicles, pectoral fins, intestinal bulb and yolk syncytial layer. Interestingly, I was able to show that in the case of some target genes, transcriptional changes were observed in WISH assays but with no effect observed in qRT-PCR assays. Often it is assumed that WISH is a less sensitive technique to qRT-PCR and indeed this may well be so when comparing responses for genes within the same tissues or for genes with high basal levels of expression. Changes in the expression of genes which are restricted to very small and specific sites within the embryo-larvae are, however, less likely to be detected by qRT-PCR when diluted in non-responsive organ/tissues of the whole animal, as they were in the present body of work. This thesis work highlights the value of a combination approach, using both qRT-PCR and WISH, when examining gene transcriptional changes in fish following chemical exposures.

It is important to note that WISH is a time consuming assay, particularly when large numbers of genes are examined in multiple exposure conditions and across several developmental stages (as was the case throughout this body of work). In addition, one of the major limitations of using WISH for assessing transcriptional changes by environmental chemicals is the difficulties associated with reliably quantifying changes in staining intensity within various tissues. While computer software packages such as Adobe Photoshop and Image J can reasonably quantify staining intensities in immunohistochemistry and fluorescent samples, it is less straight

forward with digoxigenin labelled RNA probes [9]. This is particularly true for tissues located deep in the body cavity (e.g. the pituitary) and/or orientated in an awkward position (e.g. the pronephric ducts are narrow and run the length of the trunk). I attempted on numerous occasions to create florescent anti-sense RNA probes for the thyroid related genes used in the WISH assays, however problems with non-specific binding meant this was not a viable alternative to the digoxigenin probes. While radioactive detection methods of WISH samples are perhaps best suited for answering questions relating to differential gene expression (yielding semi-quantitative measurements of mRNA levels), they themselves present challenges and shortcomings such as laboratory safety, waste disposal challenges and need for expensive analytical software.

In recent years, transgenic fish have increasingly been applied in the field of ecotoxicology allowing us to visualise and quantify tissue responsiveness to chemical exposures in real time and in intact organisms [10]. While numerous transgenic fish have been developed to examine the effect of contaminants on various pathways, as far as we are aware only one has been developed with the aim of studying TH disruption [11]. Ji *et al.,* (2012) generated a transgenic zebrafish with enhanced green fluorescent protein (eGFP) driven by the TSHβ (thyrotropin stimulating hormone β) promoter. Given the complex nature of peripheral TH regulation in fish, this model is limited in its ability to assess disruption at the central HPT axis rather than at TH target genes in specific tissues. Future work would be well placed to focus on the development of transgenic fish for detecting thyroid disrupting chemicals using the thyroid response elements (TRE), which binds the thyroid receptor-ligand complex, and controls thyroid-responsive genes. While it is

known that thyroid receptors in mammals exhibit preferences for different configurations of TREs (DR4, PAL or IP) depending on their conformational state (heterodimer, homodimer or monomer), the preferences of fish TRs for the various different TREs is not well understood. Prior to the development of a transgenic fish line, a basic understanding of these preferences/interactions would be extremely valuable in informing the design of suitable TRE constructs.

5.2.2 Adverse effects of BFR-induced transcriptional changes

Here transcriptional changes in the HPT axis were examined following BFR exposures but changes in protein levels and/or activity were not assessed. Although mRNA and protein expression are tightly linked through translation, transcriptional changes don't always seamlessly correlate with changes in protein activity which are often influenced by complex feedback and compensatory effects at multiple levels of biological organisation. In terms of increasing our understanding of the adverse health effects and mechanistic pathways of BFR-induced thyroid disruption in fish, future research needs to consider both the fundamental processes underlying protein regulation in the HPT axis and how xenobiotics alter protein regulation at the pre- and post- translational level. Although pre- and post- translational reactions for many proteins in the HPT axis are fairly well characterised in mammals, this is not the case for many of these proteins in fish species. In terms of environmental relevance, isolated data on transcriptional changes has limitations, but in combination with other endpoints (such as protein, physiological and morphological changes) this information can be used to develop adverse outcome pathways (AOPs) of chemical exposure and assess potential adverse health effects of environmental stressors.

5.2.3 Exposure conditions

One of the major limitations in carrying out this work was the availability of BFR compounds (with the exception of TBBPA), which are often sold as a stock solution in toluene. Given the toxic properties of this solvent, several PBDE congeners were kindly synthesised by collaborators and provided in stocks of DMSO. Having said this, the stock solutions were at relatively low concentrations and a limited volume was provided, creating logistical problems for exposures carried out on fish embryo-larvae. Compared to *in vitro* assays which are carried out in 24 well plates (1ml of solution per well), *in vivo* assays required relatively large volumes of exposure solution (1ml per embryo) and 50% of this solution was replaced every 24 hours. This limited the concentration I could assess in FET tests, the number of replicates I was able to carry out in sub-acute exposures and the number of BFR compounds I was able to assess.

For the *in vivo* exposures carried out in Chapter 3, I selected three nominal concentrations of TBBPA (25, 100 and 250 µg/ L) and BDE-47 (0.0564, 0.564 and 5.64 mg/ L) to test. Based on FET tests and on existing evidence in the literature, these concentrations were non- toxic and did not induce significant morphological defects in zebrafish embryo-larvae after 96 hour exposures. It was established that TBBPA (≥25 µg/ L) and BDE-47 (≥0.564 mg/L) were capable of altering the expression of several important genes in the HPT axis of zebrafish embryo-larvae after relatively short exposure periods (48-120 hours). Although usually below the limit of detection in the water phase, TBBPA has repeatedly been detected in fish species from around the world, commonly at concentrations between 0.01-50 µg/ kg

lipid weight (lw) [12-17]. The highest concentration of TBBPA recorded in fish to date was 245 µg/ kg lw observed in whiting (*Merlangius merlangus*) muscle from the North Sea [18]. BDE-47 has been detected in various fish from around the world, with particularly high levels observed in North America [19]. For example, BDE-47 concentrations often exceeded 1 mg/ kg lw in freshwater fish sampled from various rivers across Virginia, USA [20]. Therefore, while the selected BDE-47 concentrations were environmentally relevant, the selected TBBPA concentrations were at the higher end of the scale in terms of known environmental levels. I justified the use of high exposure concentrations in this work in order to examine potential mechanistic pathways of TBBPA-induced thyroid disruption. Effects at very low concentrations may be less pronounced and, therefore difficult to identify, particularly using WISH assays. Although 25 µg TBBPA/ L is unlikely to occur regularly in surface waters at the present time, TBBPA currently has the highest production volume of BFRs in the world. In addition, the levels of TBBPA have been shown to vary seasonally, with relatively high concentrations (up to 5 µg/ L) detected in water bodies during the summer months. Our results highlight the potential vulnerability of the thyroid system of wild fish populations to spikes in environmental TBBPA concentrations and raises the question of what effects may result from chronic low levels exposures.

The (sub-acute) BFR exposures in Chapter 3 of this thesis were carried out via the water. Given that the main route of exposure to these compounds for fish is via the diet, it could be argued that a diet based exposure regime would have been more environmentally relevant. However, I was particularly interested in the effects of BFRs on early embryonic-larval life stages, prior to the onset of feeding (at around 4-

5 days post fertilisation). Exposures could have started following the onset of feeding, however, *in situ* hybridisation becomes extremely difficult to carry out beyond the 120 hour stage (RNA probe unable to penetrate to deeper tissues) and therefore it would not have been possible to assess tissue-specific transcriptional changes using this approach.

5.2.4 Assessing the effects of TDCs using the zebrafish model

While the general architecture of the thyroid system appears to be evolutionary conserved across vertebrates, there are important difference between mammals and fish in terms of the functions and regulations of various proteins of the HPT axis. Therefore, understanding the mechanisms of thyroid disruption by BFRs in fish is important, and one shouldn't assume that an effect observed (or lack of an effect) in mammals/amphibian models is necessarily the case in fish. While there are numerous practical advantages to using small fish model species, such as zebrafish, for examining the mechanisms underlying the toxicity of TDC in a laboratory setting, it is important to note that their biology is somewhat different to many wild fish species and this may have important implications in interpreting/extrapolating toxicology results to wild fish populations.

5.2.5 *In-vitro* assays

In toxicology, *in vitro* screens offer several advantages over studies with whole animals. Dosing cell cultures is easier, more reproducible and produces less toxic waste. Results are obtained more rapidly than with intact animals and with less cost. In addition, experiments with cell cultures satisfy the need to reduce the use of animals in toxicology testing. Finally, they allow for a more targeted approach for

assessing the mechanistic pathways of substances suspected of adversely effecting human and wildlife health, without the complexities such as bioaccumulation and depuration that arise from *in vivo* studies. Reporter gene assays based on TR-mediated transcription are particularly useful in that they indicate whether environmental pollutants can directly interfere with the transcription of TH-target genes. It is important to stress however that *in vitro* bioassays have limitations and care should be taken in extrapolating data based on *in vitro* test systems to explain *in vivo* conditions. As discussed in Chapter 4, co-repressor and co-activator proteins play a critical role in modulating the transcriptional activity of TRs and differences in the endogenous expression of these proteins in various cells lines may have a significant effect on the observed outcomes of *in vitro* assays. In addition, different cell types may have different metabolic capabilities which may also influence the observed responses. Here I performed reporter gene assays using zebrafish TRs in a human embryonic kidney cell line. It would have been better to carry out this assay in a cell line derived from zebrafish tissues (or tissues from an alternative fish species) in order to better reflect the inter-cellular properties within fish. However, mammalian cells require higher temperatures (34-37°C) for optimal cell growth compared to fish cells (18-22°C), resulting in faster growth rates in mammalian cells. For this reason and the larger volume of information available on mammalian cell lines (e.g. nutritional requirements, differentiation capacity, direct immortalisation, cell lineage position and transfection) meant their application in our current research was preferable to fish cell lines. While *in vitro* bioassays are never going to entirely replace *in vivo* studies on whole fish, when carried out in conjunction with other assays they can greatly assist our understanding of the mechanistic actions of environmental contaminants in fish.

5.2.6 Non-thyroid hormone targets of BFRs

While this thesis has specifically focused on the interaction of BFRs with the thyroid system in fish, it is worth noting that BFRs also have the potential to disrupt endocrine systems at other target sites and can adversely affect various other biological systems. For example, *in vitro* and *in vivo* studies have demonstrated that various BFR compounds interact with the estrogen, androgen and progesterone signalling pathways [21, 22], can cause developmental neurotoxicity [23] and can induce morphological effects in the liver and kidney (reviewed in [24]). Therefore it is important to remember that the BFR-induced changes to TH signalling observed here are not likely to have occurred in isolation, and that BFRs may have also interfered with interacting signalling pathways.

5.3 Future Research

5.3.1 Biological significance of BFR-induced thyroid disruption

Altered TH signalling during early development has been shown to cause severe motor deficiencies, irreversible cognitive impairments and found to impair reproductive, muscle and skeletal development [25-27]. In recent years there has been a growing concern about the potential relationship between the increasing prevalence of neurodevelopmental disorders in humans, and the almost exponential increase in exposure to environmental pollutants. Previous studies in fish and rodents have shown that early developmental exposure to BFRs can result in a wide range of long-lasting neurobehavioral alterations, such as decreased habituation, increased locomotor activity/hyperactivity, cognitive impairment, and altered motor development [28-32]. In addition, strong correlations between foetal PBDE exposure

and later neurodevelopment have been observed in human epidemiological studies [33, 34]. Compared to the number of studies examining the effects of xenobiotics on neurodevelopmental parameter, relatively few have focused on the effect of these compounds on thyroid dependant tissues such as the skeleton or cardiac tissues. Nevertheless, several BFRs have been found to impact skeletal development in fish, rodents and bird studies with bone density, length, mineral content, total area, cortical area and cortical thickness affected [35-39]. Corroborative evidence of BFR-mediated bone alteration has also been reported in wildlife species exposed to PBDEs. Specifically, negative correlative relationships between PBDE exposure and bone mineral density were observed in polar bears (*Ursus maritimus*) [40] and ring-billed gulls (*Larus delawarensis*) [41]. While it is extremely difficult to prove any causal link between developmental exposure to xenobiotics and adverse developmental effects, it has been hypothesised that thyroid disruption may be a possible mechanistic link [42].

While the reporter gene assay developed here (Chapter 4) indicate that several BFR compounds do not directly affect zebrafish TR-mediated transcription, the *in vivo* exposures (Chapter 3) showed that TBBPA and BDE-47 exposures altered TR transcription, with expression in the brain and skeletal tissues particularly sensitive to disruption. In order to examine the significance of these transcriptional changes in terms of potential neuro- and skeletal development impairments, future studies need to explore several research avenues. Firstly, an important knowledge gap that needs addressing is whether TR transcriptional changes correlate with changes in receptor protein levels and receptor activity. Secondly, while THs appear to regulate fate specification of early cortical neurons [43], migration of cortical [44] and cerebellar

neurons [45], synaptogenesis [46] and apoptosis [47], the genes directly regulated by TH via TRs during these processes and the characterisation of their TR binding regions are not well described in the literature. Recently, RNA-based functional genomics and chromatin immunoprecipitation (ChIP) sequencing approaches have been developed in mammalian cell lines [48] and isolated mammalian tissues [49] in order to shed light on these processes. As far as I am aware in fish the information on TH target genes at this scale is non-existent. Therefore, an important area for future research is establishing the key downstream target genes of TRs in specific tissues such as brains and cartilage/bone and whether the expression of these genes is affected by BFR exposures.

5.3.2 Non-genomic actions of THs

Until recently, the developmental effects of THs have largely been viewed as mediated by genomic mechanisms (via TR proteins in the nucleus). However, a number of non-genomic mechanisms of TH action have been demonstrated over the last decade (reviewed in [50]). These actions are by definition independent of nuclear uptake of T3 and do not require a direct interaction of T3 with the transcriptionally active nuclear receptors. Instead the mechanisms of non-genomic actions of THs depend upon cellular signal transduction systems, novel cell surface receptors such as integrin alpha V beta 3 (αVβ3) and/or cytoplasmic TRs. The functions of non-genomic of TH actions have mainly been considered to relate to homeostasis, such as actions on plasma membrane ion transporters or maintenance of the cytoskeleton. They are however also involved in the regulation of cancer-related angiogenesis and cancer cell survival pathways [51]. As far as I have seen only one study to date has examined the effect of an environmental compound

(BPA) on the non-genomic actions of THs [52]. Given that BFRs have been shown to alter circulating levels of THs in various vertebrate species, future work should examine whether disrupted TH levels result in downstream non-genomic effects.

5.3.3 BFR mixtures

Here we examined the effect of individual BFR compounds on the thyroid system, however wildlife and humans are often exposed to a mixture of endocrine disrupting compound with the potential for additive, antagonistic or synergistic effects. Compared with estrogens and anti-androgens, TDCs are the least well studied endocrine disruptor and therefore it is not surprising that few studies have examined the effect of TDC mixtures. The limited animal studies which have examined the impact of BFR mixtures have largely focused on reproductive endpoints and not thyroid disruption endpoints. What is clear from these studies is that mixtures of endocrine disrupting chemicals may produce effects that are not observed when the components are administered individually (reviewed by Kortenkamp, 2007) and therefore understanding the effects and potential dangers of exposure to complex, environmentally relevant BFR mixtures is critical and should be a focus for future research.

5.3.4 Test guidelines for TDCs

While there is growing evidence that the HPT axis is a target of endocrine-disrupting compounds (EDCs), this is not reflected adequately in current screening and assessment procedures for endocrine activity. In addition, the majority of screens to date have focused on the general parameters of thyroid function. For example in mammals, TSH secretion is commonly used to assess thyroid disruption. Meanwhile

the Amphibian Metamorphosis Assay (AMA) using *Xenopus laevis*, an accepted OECD guideline for the examining thyroid disruption in amphibians [53], is based on T3-dependent metamorphosis in tadpoles and examines tail resorption, forelimb emergence and histological alterations in the thyroid gland and TSH expression. Currently, there is no assay or validated protocol for the assessment of thyroid disrupting compounds in fish nor is there any functional *in vitro* screening test. The thyroid systems of fish, amphibians and mammals are similar in many respects, and one might consider adapting the aforementioned assays to examine thyroid disruption in fish. Indeed, the effects of xenobiotic on fish thyroid function have typically been assessed from changes in TH biosynthesis or TH secretion or plasma TH levels. It is important to stress that the thyroid system in fish is not driven primarily by the central HPT axis, with T3 production and homeostasis regulated in peripheral tissues (as previously explained). Therefore, it can be assumed that the aforementioned indices relate mainly to the efficacy of T4 release from the thyroid follicles to the blood and don't necessarily detect disruption of TH metabolism or receptor and post-receptor TH effects in peripheral tissues [54]. In recent years, the development of peripheral deiodinase activity assays in teleosts and flounder metamorphic assays have begun to bridge the gap. The disadvantage of these assays lies in the fact that they don't consider other components of the fish thyroid cascade, such as central T4 production or receptor mediated effects of T3 on target cells. Given the complex and unique nature of the thyroid system (among endocrine systems), it is important to recognise that in terms of measuring altered thyroidal status, no single biomarker can examine all aspects of thyroid function in fish. Here we carried out *in vivo* assays in order to examine the potential mechanisms of BFR-induced thyroid disruption, but did not assess whether exposure resulted in an

adverse biological effect. Therefore, these assays in the present form may not be ideal for test guideline development. Notwithstanding that, some elements of our assay should be considered in future test guideline development such the examination of multiple levels of the thyroid cascade at both a whole body and tissue specific level and the transient nature of thyroid disruption. Currently there are no satisfactory assays for evaluating post-receptor effects of T3, however biological responses that are unique to thyroid function such as parr-smolt transformation, flounder metamorphosis, and early developmental events (e.g. gut development and pigmentation) with additional research could become effective TH screens/tests. It is important to note that in order to develop a suite of screens and tests that are capable of identifying toxicants that may disrupt the thyroid system of fish, assays must be developed based on what is known about fish thyroid endocrinology. At this stage, we have relatively little knowledge of the thyroid system in fish, particularly in teleost species. As BFRs will continue to appear both in industrial applications and, even if the production has ceased, in our environment, there is a continued need for effects studies on BFRs.

5.3.5 Novel BFRs: Have we replaced one TDC with another?

Due to the bans and restrictions on PBDEs and HBCDs an increasing number of alternative brominate flame retardant have been introduced to the market in order that consumer product fire safety standards are met. These compounds include decabromodiphenyl ethane (DBDPE), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), bis(2-ethylhexyl)-3,4,5,6-tetrabromo-phthalate (TBPH) and tetrabromobisphenol A-bis (2,3-dibromopropylether) (TBBPA-DBPE) [55]. Currently there is a lack of reliable data

on the levels of production of these novel BFRs and their uses, however like their predecessors, they have been detected in various matrices in the environment [55]. Knowledge on the toxic effects and associated molecular mechanisms of these chemicals *in vivo* is currently very limited. In order to address these knowledge gaps, future research would be well placed in applying whole genome analyses such as microarrays and live cell arrays to establish potential models of toxicity and the potential for endocrine disruption.

5.4 Concluding Remarks

During the course of this PhD, I have established various assays, both *in vivo* and *in vitro*, in order to examine fundamental elements of the thyroid system of zebrafish and the molecular mechanisms of BFR-induced thyroid disruption. Here I established and validated the first thyroid- specific reporter gene transcription assay for zebrafish thyroid receptors. While none of the BFRs examined here had an agonistic effect on TR-mediated transcriptional activity, these *in vitro* assays are useful tools to further screen environmentally relevant compounds suspected of causing thyroid disruption.

Only recently has WISH been utilised to examine the effect of environmental compounds on tissue specific gene expression patterns. Here I have established the first comprehensive WISH analysis of thyroid- related genes in zebrafish embryo-larvae following exposure to BFRs. These results, which include effects induced at environmentally relevant concentrations, demonstrate that WISH is a sensitive technique for detecting changes in gene expression within distinct developing tissues. Having said that, the inherent difficulties in quantifying gene expression changes in WISH assays could limit their use in future toxicology research. The

thyroid system is a complex and highly sophisticated pathway involved in multiple developmental pathways in vertebrates. Therefore, it is important that environmental scientists continue to focus on tissue-specific changes to thyroid functions during early life stages, whether this is via fluorescent WISH probes, immunohistochemistry and/or the development of thyroid responsive transgenic fish in order to determine the potential targets and adverse effects following exposure to thyroid disrupting chemicals.

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