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Detailed review paper (DRP) on the thyroid hormone system in fish and identification of potential thyroid hormone system related endpoints for inclusion in existing OECD fish Test Guidelines

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Detailed review paper (DRP) on the thyroid hormone system in fish and identification of potential thyroid hormone system related endpoints for inclusion in existing OECD fish test guidelines

Foreword

This document contains the Detailed Review Paper (DRP) on the thyroid hormone system in fish and identification of potential thyroid hormone system related endpoints for inclusion in existing OECD fish test guidelines.
This DRP is the result of project 2.64 of the Test Guidelines Programme (TGP), led by Denmark, Belgium and Germany, which was included in the TGP workplan in 2019.
the Validation Management Group on Ecotoxicity testing (VMG-Eco) discussed this project in the 2020 VMG-Eco meeting, with subsequent discussion during the AG-EDTA meeting in May 2021 and the 2021 VMG-Eco meeting. Initial commenting by the AG-EDTA and VMG-Eco took place in April-May 2022 and an amended document was further discussed at the 17th VMG-Eco meeting in November 2022, where the VMG-Eco concluded that the draft DRP could be forwarded to the WNT-review stage.
The WNT review and commenting round took place from November – December 2022, and the document was subsequently approved by the WNT at its 35th meeting in April 2023. The Chemicals and Biotechnology Committee agreed to its declassification on 20 June 2023.

This document is published under the responsibility of the Chemicals and Biotechnology Committee.

Abbreviations

AC	Adenylate cyclase
ACTH	Adrenocorticotropin
AMA	Amphibian metamorphosis assay
AOP(s)	Adverse outcome pathway(s)
ATP	Adenosine triphosphate
BDE	Brominated diphenyl ether
BDE-47	2,2′,4,4′-Tetrabromodiphenyl ether
BDE-99	2,2′,4,4′,5-Pentabromodiphenyl ether
BDE-209	2,2′,3,3′,4,4′,5,5′,6,6′-Decabromodiphenyl ether
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
CaMK	Calmodulin-dependent protein kinase
cAMP	Cyclic 3’,5’-adenosine monophosphate
Cas	CRISPR associated sequence
CRH	Corticotropin releasing hormone
CRISPR	Clustered regularly interspaced short palindromic repeats
DAG	Diacylglycerol
DE-71	A mixture of polybrominated diphenyl ethers
DEHP	Di-(2-ethylhexyl) phthalate
DIO(s)	Deiodinase(s)
DIO1	Type 1 deiodinase
DIO2	Type 2 deiodinase
DIO3	Type 3 deiodinase
DIT	Diiodotyrosine
DOPO	9,10-Dihydro-9-oxa-10-phosphaphenanthrene-10-oxdie
Dpf	Days post fertilisation
Dph	Days post hatch
DRP	Detailed review paper
EAS	Estrogen, androgen, steroidogenesis
EC50	Median effective concentration
EDTA	Advisory group on endocrine disrupters testing and assessment
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EU	European Union
F-53B	6:2 Chlorinated polyfluorinated ether sulfonate
FELS	Fish early life stage
FET	Fish embryo acute toxicity
FSH	Follicle stimulating hormone
GCL	Ganglion cell layer
Hpf	Hours post fertilisation
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
IGF-I	Insulin-like growth factor I
INL	Inner nuclear layer
IPL	Inner plexiform layer

IPS	Inositol 1,4,5-triphosphate
KE(s)	Key event(s)
LAGDA	Larval amphibian growth and development assay
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LH	Luteinising hormone
LOEC	Lowest observed effect concentration
MCT(s)	Monocarboxylate transporter(s)
MEHP	Mono-(2-ethylhexyl) phthalate
MEOGRT	Medaka extended one generation reproduction test
MIE(s)	Molecular initiating event(s)
MIT	Monoiodotyrosine
MO	Morpholino
MOA	Mode(s) of action
NIS	Sodium iodide symporter
NOEC	No observed effect concentration
OECD	Organisation for Economic Cooperation and Development
OKR	Optokinetic response
OMR	Optomotor response
ONL	Outer nuclear layer
o,p'-DDT	1,1-Trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl) ethane
OPL	Outer plexiform layer
PBDE	Polybrominated diphenyl ether
PBDE-47	2,2′,4,4′-Tetrabromodiphenyl ether
PFBA	Perfluorobutyric acid
PFDoA	Perfluorododecanoic acid
PFECA	Perfluoropolyether carboxylic acid
PFNA	Perfluorononanoate
PFO3OA	Perfluoro (3,5,7-trioxaoctanoic) acid
PFO4DA	Perfluoro (3,5,7,9-tetraoxadecanoic) acid
PFO5DoDA	Perfluoro (3,5,7,9,11-pentaoxadodecanoic) acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PLC	Phospholipase C
p,p'-DDE	1,1-Dichloro-2,2-bis (p-chlorophenyl)-ethylene
PTU	Propylthiouracil
qRT-PCR	Quantitative real-time polymerase chain reaction
RIA	Radioimmunoassay
RPE	Retinal pigment epithelium
rT3	Reverse T3
RXR(s)	Retinoid X receptor(s)
T2	3,3'-Diiodothyronine
T3	3,3',5-Triiodo-L-thyronine
T4	Thyroxine or 3,3',5,5'-tetraiodo-L-thyronine
TBBPA	Tetrabromobisphenol A
TBG	Thyroxine-binding globulin
TBP	2,4,6-Tribromophenol
TDCPP	Tris(1,3-dichloro-2-propyl)phosphate
Tg	Thyroglobulin

TG(s)	Test guideline(s)
TH(s)	Thyroid hormone(s)
THS	Thyroid hormone system
TPO	Thyroperoxidase or thyroid peroxidase
TR(s)	Thyroid hormone receptor(s)
TRE(s)	Thyroid hormone response element(s)
TSH	Thyroid stimulating hormone or thyrotropin
TSHβ	Thyroid stimulating hormone subunit β
TSHR	Thyroid stimulating hormone receptor
TTR	Transthyretin
UGT	UDP-glucuronosyltransferase
VMG-Eco	Validation management group for ecotoxicology
Wpf	Weeks post fertilisation
XETA	Xenopus eleutheroembryonic thyroid assay
ZEOGRT	Zebrafish extended one generation reproduction test
ZFN	Zinc-finger nucleases

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1. OECD fish test guidelines for endocrine disrupting chemicals

International standardised test guidelines (TGs) with sensitive endpoints form the basis for hazard identification and characterisation and risk assessment used in the regulation of chemicals. In 1997, at the request of member countries and the international industry, the Organisation for Economic Cooperation and Development (OECD) initiated the Special Activity on Endocrine Disrupters Testing and Assessment (EDTA) with the aim of providing a suite of standardised and internationally accepted test guidelines (TGs) and assessment strategies for regulatory application (OECD, 2001). Under the supervision of the EDTA Advisory Group (EDTA AG), Validation Management Groups for mammalian (VMG-mammalian), ecotoxicity (VMG-eco) and non-animal (VMG-non-animal) testing were established in 1999, 2001 and 2002, respectively. Overseen by the Working Group of National Coordinators of the Test Guidelines Programme (WNT), these groups have continuously managed the development of TGs for the evaluation of endocrine disrupters. OECD TGs are covered by the Mutual Acceptance of Data (MAD) system in which the OECD member countries and other full adherents have agreed that the results of testing performed in accordance with OECD TGs and principles of Good Laboratory Practice (or their equivalents) in one country must be accepted by other countries.

The EDTA AG has developed the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupters (Table 1). The Conceptual Framework ranks, into five levels of increasing biological organisation, the OECD TGs and standardised test methods available, under development or proposed that can be used to evaluate chemicals for endocrine disruption. The Conceptual Framework helps to evaluate the overall strength of the evidence that a chemical may be acting as an endocrine disrupter and to determine additional testing demands.

The Conceptual Framework includes several OECD fish TGs at different levels. However, these TGs do not include THS related endpoints.

Table 1. The OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals (updated from OECD, 2018)

Mammalian and non-mammalian toxicology		
Level 1 Existing data and existing or new non-test information	– Physical and chemical properties, e.g. molecular weight reactivity, volatility, biodegradability – All available (eco)toxicological data from standardised or non-standardised tests – Read-across, chemical categories, quantitative structure activity relationships and other *in silico* predictions, and absorption, distribution, metabolism and excretion model predictions	
Level 2 *In vitro* assays providing data about selected endocrine mechanism(s)/ pathway(s) (mammalian and non-mammalian methods)	– Estrogen (OECD TG 493) or androgen receptor binding affinity (US EPA TG OPPTS 890.1150) – Estrogen receptor transactivation (OECD TG 455, ISO 19040-3), yeast estrogen screen (ISO 19040-1 & 2) – Androgen receptor transactivation (OECD TG 458) – Steroidogenesis *in vitro* (OECD TG 456) – Aromatase assay (US EPA TG OPPTS 890.1200) – Thyroid disruption assays (e.g. thyroperoxidase inhibition, transthyretin binding) – Retinoid receptor transactivation assays – Other hormone receptors assays as appropriate – High-throughput screens	
	Mammalian toxicology[3]	**Non-mammalian toxicology[3]**
Level 3 *In vivo* assays providing data about selected endocrine mechanism(s)/ pathway(s)[1]	– Uterotrophic Assay (OECD TG 440) – Hershberger assay (OECD TG 441)	– Amphibian metamorphosis assay (AMA) (OECD TG 231) – Fish short-term reproduction assay (FSTRA) (OECD TG 229)[2] – 21-day fish assay (OECD TG 230) – Androgenised female stickleback screen (AFSS) (OECD GD 148) – EASZY Assay. Detection of Substances Acting through Estrogen Receptors using Transgenic cyp19a1b GFP Zebrafish Embryos (OECD TG 250) – *Xenopus* embryonic thyroid signalling assay (XETA) (OECD TG 248) – Juvenile medaka anti-androgen screening assay (JMASA) (draft OECD GD) – Short-term juvenile hormone activity screening assay using *Daphnia magna* (draft OECD TG) – Rapid androgen disruption adverse outcome reporter (RADAR) assay (OECD TG 251)
Level 4 *In vivo* assays providing data on adverse effects on endocrine-relevant endpoints[2]	– Repeated dose 28-day study (OECD TG 407) – Repeated dose 90-day study (OECD TG 408) – Pubertal development and thyroid function assay in peripubertal male rats (PP male assay) (US EPA TG OPPTS 890.1500) – Pubertal development and thyroid function assay in peripubertal female rats (PP female assay) (US EPA TG OPPTS 890.1450) – Prenatal developmental toxicity study (OECD TG 414) – Combined chronic toxicity and carcinogenicity studies (OECD TG 451-453) – Reproduction/developmental toxicity screening test (OECD TG 421) – Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 422) – Developmental neurotoxicity study (OECD TG 426) – Repeated dose dermal toxicity: 21/28-day study (OECD TG 410) – Subchronic dermal toxicity: 90-day study (OECD TG 411) – 28-day (subacute) inhalation toxicity study (OECD TG 412) – Subchronic inhalation toxicity: 90-day study (OECD TG 413) – Repeated dose 90-day oral toxicity study in non-rodents (OECD TG 409)	– Fish sexual development test (FSDT) (OECD TG 234) – Larval amphibian growth and development assay (LAGDA) (OECD TG 241) – Avian reproduction assay (OECD TG 206) – Fish early life stage (FELS) toxicity test (OECD TG 210) – New guidance document on harpacticoid copepod development and reproduction test with *Amphiascus* (OECD GD 201)[2] – *Potamopyrgus antipodarum* reproduction test (OECD TG 242)[4] – *Lymnaea stagnalis* reproduction test (OECD TG 243)[4] – Chironomid toxicity test (OECD TG 218-219)[4] – *Daphnia magna* reproduction test (with male induction) (OECD TG 211)[4] – Earthworm reproduction test (OECD TG 222)[4] – Enchytraeid reproduction test (OECD TG 220)[4] – Sediment water *Lumbriculus* toxicity test using spiked sediment (OECD TG 225)[4] – Predatory mite reproduction test in soil (OECD TG 226)[4] – Collembolan reproduction test in soil (TG OECD 232)[4]
Level 5 *In vivo* assays providing more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism[2]	– Extended one-generation reproductive toxicity study (EOGRTS) (OECD TG 443)[5] – Two-generation reproduction toxicity study (OECD TG 416, most recent update)	– Fish life cycle toxicity test (FLCTT) (US EPA TG OPPTS 850.1500) – Medaka extended one-generation reproduction test (MEOGRT) (OECD TG 240) – Avian two-generation toxicity test in the Japanese quail (ATGT) (US EPA TG OCSPP 890.2100/740-C-15-003) – Sediment water chironomid life cycle toxicity test (OECD TG 233)[4] – *Daphnia* multigeneration test for assessment of EDCs (draft OECD TG)[4] – Zebrafish extended one-generation reproduction test (ZEOGRT) (draft OECD TG)

Given the crucial role that the thyroid hormones (THs) play in normal development of vertebrate species, the identification of chemicals disrupting the thyroid hormone system (THS) is warranted.

Lack of knowledge of chemicals affecting thyroid specific endpoints and thereby lack of identification of THS disrupting chemicals could potentially cause negative impacts on the environment and human health.

A detailed understanding of mechanisms causing THS disruption is key for reliable prediction of results across species and classes (Holbech et al., 2020; Knapen et al., 2020). Wherever possible, the same chemical should only be tested in different species where a low degree of conservation of TH signalling in the respective species has been found.

Currently, rodents, dogs and rabbits are the main test organisms for assessing hazard and risks of chemicals to human health with respect to thyroidal modalities, while amphibians represent the main

test organism used for that purpose in the context of environmental hazard and risk assessment. However, existing amphibian-based TGs may not cover all potential THS disrupting molecular initiating events (MIEs, see section 4.1) and life stages that might be affected. The TG 231 (Amphibian Metamorphosis Assay, AMA; OECD, 2009) is a Level 3 test covering 3 weeks of the metamorphic phase of the life cycle from developmental stage 51, according to Nieuwkoop and Faber (NF) (≤17 days post fertilisation (dpf)). The scientific basis of TG 231 can be found in the detailed review paper on amphibian metamorphosis assay for the detection of thyroid active substances (OECD, 2004), in which endpoints are described to reflect thyroid dysfunction and their sensitivity to thyroid stimulation and inhibition. The TG 248 (Xenopus Eleutheroembryonic Thyroid Assay, XETA; OECD, 2019) is a Level 3 screening test in which tadpoles are exposed to the test chemical at NF stage 45 for a duration of 72 h. The current version of TG 248 covers TR (ant)agonism, TH clearance, TH metabolism and potentially modulation of TH transport via interaction with TH plasma binding proteins and inhibition of TH transmembrane transporters but it does not cover NIS inhibition and TPO inhibition. The TG 241 (Larval Amphibian Growth and Development Assay, LAGDA; OECD, 2015b) is a Level 4 test covering 16 weeks of the metamorphic phase and juvenile development and it provides data on adverse effects on endocrine-relevant endpoints. The LAGDA includes the period of the AMA and is, therefore, expected to respond to chemicals affecting the endpoints in the AMA. Although benzophenone-2, which is a putative TPO inhibitor, was tested in a single laboratory during the validation (Haselman et al., 2016), the LAGDA has not been validated using a suite of model thyroid active chemicals with known MIEs (NIS-, TPO- and deiodinase (DIO-)inhibition, and interaction with TH binding). It is, therefore, not yet validated if the LAGDA reveals adverse population-relevant effects of all MIEs of THS disruption.

Even though fish represent the most used non-mammalian vertebrate test organisms for other endocrine modalities (i.e. estrogen, androgen, steroidogenesis (EAS)), until now, testing approaches for THS disruption do not include fish.

The rationale behind inclusion of THS relevant endpoint in fish TGs is to reduce parallel testing in fish and amphibians; and it is envisioned to include endpoints appropriate to reveal all main THS disrupting MIEs in the fish TG(s) covering more lifestages.

Unlike investigation of effects on the THS in wildlife, which is mostly based on amphibian models, investigation of effects on the sex hormone system in wildlife is mostly based on fish models. However, some chemicals may be suspected to interfere with either the THS or the sex hormone system. In these cases, it is currently necessary to test the chemical in both fish and amphibians to clarify the concerns for endocrine disruption, since none of the existing fish TGs include endpoints specific for THS disruption (and the amphibian models are not well validated for investigation of effects on the sex hormone system). If fish TGs were refined to also - and with the same sensitivity - detect THS disrupters it would probably in many cases make testing of endocrine disrupting chemicals more efficient and potentially lead to resource savings.

Addition of THS related endpoints to fish studies is intended to reduce parallel testing in amphibians and fish and may reduce the number of animals used for testing of chemicals. The goal is to identify and select unambiguous THS related endpoints which can be evaluated preferably without increasing the complexity of experimental protocols of fish TGs or the number of fish used. However, power analyses should be performed to ensure adequate power for those endpoints, and it cannot be ruled out that there could be a need to increase the number of fish used within existing tests (as well as other resources and time) to be able to conduct the additional THS related assessments. Furthermore, for some chemicals, it could potentially be difficult to establish concentration spacing in a single study that would reliably capture the metric of interest (e.g. NOEC and LOEC) for each endpoint of interest; though this may not be a problem if the aim is to capture the most sensitive endpoints rather than to understand effects more fully on individual endpoints. Finally, it should be noted that certain chemicals raising specific concerns for amphibian populations should still be tested in amphibians for protection of this vertebrate class.

Reduction of vertebrate testing is coherent with the 3R principles advocating replacement, reduction and refinement of animal testing. However, the complexity of the THS has been considered to preclude the sole use of *in vitro* systems for reliable detection of THS disrupting chemicals (Zoeller and Tan, 2007). All though there is increased activity in the development of *in vitro* methods (Noyes et al., 2019), due to the complexity of the THS, the use of *in vitro* data for purposes beyond screening is challenging, and replacement of *in vivo* testing in this field represents major challenges. Hence, apart from ongoing efforts to develop, expand, and refine *in vitro* assays to measure chemical interactions that lead to a perturbation of the TH pathway, the possibilities for reduction and refinement of *in vivo* testing procedures offer great advantages.

The lack of thyroid endpoints in fish tests was raised as a point of concern by the OECD VMG-Eco in 2016 and Denmark suggested to initiate the evaluation of thyroid endpoints for inclusion in existing OECD fish TGs.

The lack of thyroid endpoints was likewise recognised as a serious gap in existing fish TGs at two EU workshops, “Setting Priorities for Further Development and Validation of Test Methods and Testing Approaches” and “Supporting the Organization of a Workshop on Thyroid Disruption” in 2017.

In 2019, “Inclusion of thyroid endpoints in OECD fish Test Guidelines” was adopted as Project 2.64 in the OECD Work Plan for the Test Guidelines Programme with Denmark as lead country. In 2021 Belgium and Germany entered project 2.64 as co-lead countries. The purpose of this project is to include endpoints for THS disrupting chemicals in existing OECD fish TGs - with OECD TG 234 (Fish Sexual Development Test) as the main test model. It is recognised that several OECD TGs are relevant and especially OECD TG 210 also covering the developmental stages and OECD TG 236 as an embryonic alternative should be explored for possible inclusion of THS related endpoints.

1.1. Fish model species for identification of THS disruption

While anuran amphibians (particularly *Xenopus laevis*) have historically been the preferred non-mammalian models in THS research, in the latest two decades fish (particularly zebrafish, *Danio rerio*) have been widely used as models.

The anuran amphibians and the zebrafish share common advantages: the ability to easily produce large numbers of free-living embryos, their accessibility during key stages of development, and the absence of continuous maternal hormonal influence on embryo and larvae development (Couderq et al., 2020).

During a five year period from 2014-2019, the zebrafish was the predominant non-mammalian model employed in scientific papers assessing THS disruption *in vivo* or discussing regulatory issues on the topic (Couderq et al., 2020). Out of 108 non-mammalian (eco)toxicology studies, 78 used fish, 29 used amphibians and 6 used birds. Of the 78 fish studies, 61 used zebrafish, 2 used medaka and 9 used various other species.

Zebrafish represent multiple advantages as experimental animals, including a well characterised development (Kimmel et al., 1995). Further experimental advantages are cost-efficiency and ease of culture and breeding, large number of offspring, short generation time, visibility of organ development due to transparency of embryos and larvae, and access to the fully sequenced zebrafish genome (Howe et al., 2013; McArdle et al., 2020). One female zebrafish can produce over 200 eggs per mating (Chakraborty et al., 2009); and the zebrafish reach maturity at about 2-3 months of age (Lawrence et al., 2012; Nasiadka and Clark, 2012). Amenability to various forms of genetic manipulation like **1**) morpholino (MO) based gene knockdown technique, **2**) targeted gene knockout technology using zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) or clustered regularly interspaced short palindromic repeats/CRISPR associated sequence (CRISPR/Cas) and **3**) generation

of transgenic zebrafish lines with fluorescent reporter genes further increases the value of zebrafish as model species (Marelli and Persani, 2017; Liu et al., 2019a).

Zebrafish are widely used as a model species in many different research fields including basic developmental biology, neurobiology, immunology, human diseases, biomedical research and toxicity testing of chemicals (Key and Devine, 2003; Hill et al., 2005; Chakraborty et al., 2009; Sipes et al., 2011; Marelli and Persani, 2017; Korzh et al., 2018; García-Moreno et al., 2019; Teame et al., 2019). The high level of conservation of endocrine systems that exists between zebrafish and higher vertebrates (McGonnell and Fowkes, 2006) has likewise promoted the use of zebrafish in endocrine disruption research. The THS is highly conserved between vertebrates (Heijlen et al., 2013). Most components of the zebrafish THS have been characterised and their structure and function have been shown to resemble those of higher vertebrates. This has made zebrafish a frequently used model species in vertebrate developmental biology and THS disruption research, with relevance to both human health and (eco)toxicology studies (Heijlen et al. 2013; Couderq et al., 2020).

The zebrafish is unique with respect to the level of available knowledge and technology (Scholz et al., 2008; Porazzi et al., 2009; Heijlen et al., 2013; Marelli and Persani, 2017). Although the other small fish species traditionally employed in OECD TGs, i.e. Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) may be equally suited test species for development of THS related endpoints, the availability of detailed information on the functioning of the zebrafish THS, together with the above-mentioned advantages, make zebrafish a promising valuable laboratory model species for development of THS related endpoints. Thus, the current DRP focuses primarily on the zebrafish as the model species. However, other fish species may offer other advantages compared to the zebrafish (sex determination, size, flatfish metamorphosis from symmetric pelagic larvae to asymmetric benthic juveniles etc.) and relevant information from other species has been included. The effects of THS disrupting chemicals on zebrafish should be compared to other species in the future.

Furthermore, although the zebrafish is a good model for identifying the effects of chemicals on the THS, the effects in mammals could be different and should also be examined.

1.2. OECD fish TGs suitable for inclusion of THS related endpoints

Multiple OECD TGs using fish for assessment of the endocrine disrupting potential of chemicals could be suitable for inclusion of TH related endpoints, i.e. TG 210 (Fish Early Life Stage Toxicity test (OECD, 2013a)), TG 229 (Fish Short Term Reproduction Assay (OECD, 2012)) and TG 234 (Fish Sexual Development Test (OECD, 2011)). Extension of TG 236 (Fish Embryo Acute Toxicity (FET) test (OECD, 2013b)) with THS related endpoints could further potentially provide a valuable test for THS disruption. TG 240 (Medaka Extended One Generation Reproduction Test (OECD, 2015a)) using Japanese medaka and the draft Zebrafish Extended One Generation Reproduction Test also covers sensitive life stages responsive to THS disruption. Finally, although it is not officially under validation, the proposed combination of TG 229 and TG 234 into the integrated Fish Endocrine Disruptor Test (iFEDT, EU tender project No. 07.0203/2018/794670/ETU/ENV.B.2) could be a valuable test for THS disruption in the future.

An enhancement of the current TGs by adding specific THS related endpoints could potentially increase the diagnostic value of the tests.

1.2.1. TG 234 (Fish Sexual Development Test)

TG 234 (OECD, 2011) was adopted by OECD in 2011 as the first fish test guideline with specific endpoints investigating adverse effects of endocrine disrupting chemicals on the sex hormone system. The test has been fully validated for zebrafish, three-spined stickleback (*Gasterosteus aculeatus*) and

Japanese medaka, and partially validated for fathead minnow. In the test, fish are exposed from newly fertilised eggs until the completion of sexual differentiation (60 days post hatch (dph) for zebrafish, stickleback and medaka; up to 120 dph for fathead minnow) to at least three concentrations of the test chemical. A biological sample (blood plasma, liver or head/tail homogenate) is collected for vitellogenin analysis from each fish and the remaining part is fixed for histological evaluation of the gonads to determine the phenotypic sex; optionally, histopathology (e.g. staging of gonads, severity of intersex) can be performed. In addition, body length and weight should be measured and survival, hatching success, abnormal behaviour and morphological abnormalities should be recorded.

An effect on sex ratio demonstrates that the test chemical causes an adverse apical effect relevant at the population level; and the measurement of vitellogenin can show the endocrine activity of the chemical (estrogenic, anti-estrogenic, steroidogenesis related or possibly androgenic activity). The combined analysis of vitellogenin and sex ratio provides information on the endocrine mode of action (MOA).

The test method is applicable for use as a Level 4 assay as described in the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (Table 1).

TG 234 is used in combination with other lines of evidence for identification and assessment of endocrine disrupting chemicals affecting the hypothalamic-pituitary-gonadal (HPG) axis in fish especially with respect to estrogen, androgen and steroidogenesis (EAS) modalities.

The inclusion of THS related endpoints in the TG 234 and other relevant fish TGs (section 1.2.2-1.2.7) would improve the possibilities for simultaneous identification of endocrine disrupting chemicals affecting the hypothalamic-pituitary-thyroid (HPT) axis by covering also thyroidal modalities.

1.2.2. TG 210 (Fish Early Life Stage Toxicity Test)

TG 210 (OECD, 2013a) investigates the lethal and sub-lethal effects of chemicals on fish early life stages (FELS). Validated species are zebrafish, medaka, fathead minnow, sheepshead minnow (*Cyprinodon variegatus*), rainbow trout (*Onchorhynchus mykiss*) and silverside (*Menidia* spp). Fertilised eggs are placed in test chambers and exposed to the test chemical for a species specific time period that is necessary for the control fish to reach a juvenile life stage (~30 dph for zebrafish). The main endpoints include mortality, time to hatching, hatching success, growth, morphological abnormalities and abnormal behaviour.

Although not being designed to give evidence of endocrine effects, TG 210 gives information on parameters that may be susceptible to endocrine disruption (such as hatchability and development) and is included at Level 4 in the OECD Conceptual Framework (Table 1).

TG 210 is an extensively used bioassay for standard (sub)chronic fish toxicity testing. In Europe, it is a standard data requirement for plant protection products (Regulation EC1107/2009) supporting aquatic ecotoxicological risk assessments and chemical management.

Since the TG 210 covers the thyroid-relevant life stage of larval-to-juvenile metamorphosis, it offers the possibility for evaluating THS related developmental events occurring during this transition. Developmental effects observed in the TG 210 test may support the case that a chemical is a possible THS disrupter but cannot on their own be used to reach such a conclusion (OECD, 2018). Furthermore, since the test does not cover the full juvenile stage and does not include EAS-mediated parameters either, other fish TGs may be more obvious candidates for inclusion of THS related endpoints.

1.2.3. TG 229 (Fish Short Term Reproduction Assay)

The TG 229 (OECD, 2012) is an *in vivo* screening assay in which sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life cycle (21 days).

At termination of the 21-day exposure period, two biomarker endpoints that may be associated specifically with endocrine signalling are measured in males and females as indicators of endocrine activity of the test chemical: vitellogenin and secondary sexual characteristics. Vitellogenin is measured in zebrafish, Japanese medaka and fathead minnow, and secondary sex characteristics are additionally measured in Japanese medaka and fathead minnow. Furthermore, quantitative fecundity is monitored daily throughout the test. Moreover, gonad histopathology may be evaluated to add to the weight of evidence of other endpoints.

The TG 229 serves as an *in vivo* screening assay for endocrine disruption activity and reproductive effects. However, reproductive effects, like most apical effects except sex ratio changes below systemic toxicity levels, may be caused by non-endocrine mechanisms.

TG 229 is a Level 3 test according to the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (Table 1).

The TG 229 is widely used to identify compounds that have potential to interfere with the hypothalamic-pituitary-gonadal axis, and/or endocrine active chemicals and others toxic to the process of reproduction itself.

1.2.4. TG 236 (Fish Embryo Acute Toxicity (FET) test)

TG 236 (OECD, 2013b) is a validated test to assess acute toxicity of compounds in zebrafish embryos. Newly fertilised zebrafish eggs are exposed to different doses of the test chemical for a period of 96 h. Every 24 h, up to four apical observations are recorded as indicators of lethal toxicity of the test chemical: **1**) coagulation of fertilised eggs, **2**) lack of somite formation, **3**) lack of detachment of the tail-bud from the yolk-sac and **4**) lack of heartbeat. At the end of the exposure period, acute fish early-life developmental toxicity of the compound is determined based on the dose-response relationships of the apical observations, and the LC50 is calculated.

TG 236 is used in combination with other information to quickly and reproducibly assess acute fish toxicity of chemicals for regulatory purposes.

TG 236 is currently limited to observations of lethal endpoints. However, research has shown that a suite of exposure related sublethal endpoints can be included in an extended test (Braunbeck et al., 2015; von Hellfeld et al., 2020). Extension of the TG with THS related endpoints could potentially provide a valuable test for THS disruption. The use of TG 236 would be in accordance with the EU 3R principles, because zebrafish embryos are not regarded as protected stages until the onset of independent feeding (EU, 2010) i.e. up to 120 h post fertilisation (hpf) (Strähle et al., 2012).

1.2.5. TG 240 (Medaka Extended One Generation Reproduction Test (MEOGRT))

TG 240 (OECD, 2015a) is started by exposing sexually mature Japanese medaka males and females (at least 12 weeks post fertilisation (wpf)) in breeding pairs for 3 weeks. As near as possible to the first day of the fourth week, eggs are collected to start the F1 generation. During rearing of the F1 generation (a total of 15 weeks), hatchability and survival are assessed. In addition, fish are sampled at 9-10 wpf for developmental endpoints and spawning is assessed for three weeks from 12 through 14 wpf. An F2 generation is started after the third week of the reproduction assessment and reared until completion of hatching.

TG 240 measures several biological endpoints. Primary emphasis is given to potential adverse effects on population-relevant parameters including survival, gross development, growth and reproduction. Secondarily, to provide mechanistic information, where there is a posteriori evidence for a chemical having potential endocrine disrupter activity then other useful information is obtained by measuring

vitellogenin, phenotypic secondary sex characteristics as related to genetic sex, and evaluating histopathology (OECD, 2015a).

The test can be used to evaluate the potential chronic effects of chemicals, including potential endocrine disrupting chemicals, on fish. The data can be used at Level 5 of the OECD Conceptual Framework (Table 1).

The TG 240 can be used at any stage in the hazard assessment process. However, the most likely use scenario is when there are already some data available to suggest possible endocrine disrupting properties. In these cases, the TG 240 can be used to investigate whether such potential properties result in adverse apical effects on development, growth or reproduction over an entire life cycle (OECD, 2018).

1.2.6. Draft TG on Zebrafish Extended One Generation Reproduction Test (ZEOGRT)

Since TG 240 was designed and validated for the medaka as a single test species there has been an initiative from Germany to further develop an analogous test protocol with zebrafish as a test species. The development of a Zebrafish Extended One Generation Reproduction Test (ZEOGRT) was adopted as project 2.59 on the OECD Work Plan for the Test Guidelines Programme in 2016 and is currently in validation by the OECD.

Like the MEOGRT, the ZEOGRT is a comprehensive test using fish exposed continuously from the adult stage of the first generation (F0) to the newly hatched stage of the third generation (F2) (OECD, 2018). The ZEOGRT differs from the MEOGRT in that zebrafish can be group spawners, whereas medaka are pair spawners. In the ZEOGRT, spawning groups of 5 female and 5 male sexually mature F0 zebrafish (approximately 15 wpf) are allowed to reproduce for 3 weeks, their F1 offspring are brought to sexual maturity, the F1 adults are allowed to breed, and finally their offspring (F2) are followed to hatching (up to 14 dpf) (OECD, 2018).

The main emphasis of the ZEOGRT concerns population-relevant apical endpoints (e.g. survival, development, growth, sex ratio and reproduction). However, to obtain mechanistic information, additional endpoints include measurements of vitellogenin and histopathology (OECD, 2018). Unlike the MEOGRT, the test may be able to detect relatively small changes in the sex ratio of the F1 generation since it includes a large number of F1 fish (36 per replicate) (OECD, 2018). On the other hand, it does not have a genetic sex endpoint, which may diminish the power to measure changes in sex ratio (OECD, 2018).

If successfully validated, the ZEOGRT could contribute useful evidence regarding the probable causality of apical effects, which is a key issue in the definition of endocrine disrupting chemicals (OECD, 2018). With the inclusion of thyroid specific endpoints, the ZEOGRT could potentially provide a valuable alternative to the MEOGRT as a Level 5 test for THS disruption. The choice between the two TGs should be made based on available data on the chemicals being tested, and on the endpoint and species sensitivities (ECHA/EFSA/JRC, 2018; OECD, 2018).

1.2.7. Proposed integrated Fish Endocrine Disruptor Test (iFEDT)

EU tender project No. 07.0203/2018/794670/ETU/ENV.B.2 suggests merging OECD TGs 229 and 234 and adding THS related endpoints for fish. Merging these two tests into the “integrated Fish Endocrine Disruptor Test” (iFEDT) allows the development of a mid-tier OECD test (partial life cycle) that covers reproduction, early development and sexual differentiation and that will provide information about potential endocrine disrupting effects for regulatory purposes. Additionally, since the iFEDT will cover multiple life stages and endocrine disrupting mechanisms, this approach will contribute to the 3R principle by reducing fish testing in general, as several aspects can be investigated in a single test.

1.2.8. Considerations on the various OECD fish TGs

The OECD fish TGs cover various life stages offering different advantages and disadvantages in relation to informing about mechanistic and/or adverse effects of endocrine active chemicals.

TG 236 includes the embryonic-to-larval transition (see section 3.2), which may be a developmental window particularly susceptible to disruption of the THS. TG 236 could for 3R reasons be a valuable screen for identification of THS activity of chemicals.

TGs including later life stages, i.e. TG 210, TG 234, TG 240 and the ZEOGRT, could be used for more definitive adverse effect determination.

TG 210 is systematically conducted for regulatory risk assessment, which could make it a valuable candidate TG for inclusion of THS relevant endpoints. TG 210 covers the relevant life stage of larval-to-juvenile metamorphosis and thus offers the possibility for evaluating important developmental events occurring during this transition. The larval-to-juvenile transition in zebrafish includes inflation of the anterior swim bladder chamber, formation of the adult pigmentation pattern, development of adult fins and fin rays and scale formation (McMenamin and Parichy, 2013). However, TG 210 does not cover the full juvenile stage and will, therefore, not be able to reveal potential THS disrupting MOA affecting specifically the later part of this potentially sensitive window. In addition, TG 210, although being the standard chronic test in many cases, does not include EAS-mediated parameters either.

Since TG 234 is already known to be informative for endocrine disruption and since it is a more integrative test covering more life stages and potentially offering additional applicable endpoints, TG 234 could be considered to hold stronger promise for assessment of THS disrupting chemicals than TG 210. However, effects on THS related endpoints induced in the fish early life stages may not persist to the sexually differentiated juvenile stage if the chronic exposure induces compensatory mechanisms in the THS (Crane et al., 2006).

Finally, it should be noted that neither TG 236, TG 210 or TG 234 cover the reproductive phase. Thus, endpoints related to this life cycle phase could be examined in TG 229, the ZEOGRT (when validated) and the MEOGRT when the Japanese medaka has been evaluated for THS relevant endpoints. However, as many reproductive effects may be caused by non-endocrine mechanisms, the added benefits of conducting these tests should be carefully evaluated.

1.2.9. Applicability of transgenic fish lines

The zebrafish is the fish species most employed as transgenic model, those that carry exogenously derived DNA intentionally inserted into their genome (Lee et al., 2015). Transgenic zebrafish have been commonly employed in studies of developmental biology, in pharmaceutical research and recently as biosensors in ecotoxicology. Transgenic zebrafish lines offering specific tools for identifying THS disrupting chemicals have also been generated. For example, zebrafish with fluorescent reporter gene proteins under the control of thyroid stimulating hormone subunit β (TSHβ) (Ji et al., 2012) and thyroglobulin (Tg) (Opitz et al., 2012; Fetter et al., 2015; Jarque et al., 2018) gene regulatory elements are available. In the transgenic zebrafish embryos, THS disruption is readily detectable by assessment of fluorescence using microscopy or plate readers. However, transgenic zebrafish lines are not available for all modes of THS disruptive action. It may be useful with time, to generate reporter systems on multiple genes susceptible to THS related disruption and combine these systems in the same transgenic fish line, thereby permitting the detection of THS disrupting compounds acting through various MIEs simultaneously. If transgenic fish are to be used in OECD fish TGs, the fish should be bred from a homozygous parental generation to ensure that all offspring are transgenic.

2. The fish thyroid hormone system

THs influence most physiological processes in fish. Like in other vertebrates, the normal functioning of the THS is essential for the control of early development/differentiation, growth, metabolism and reproduction. However, the detailed mechanisms through which THs control these activities are not known. The actions of THs may be direct, as well as permissive by facilitation or augmentation of other signal transduction pathways including the growth hormone/insulin-like growth factor I (GH/IGF-I) axis (Orozco and Valverde, 2005; Brent, 2012; Molla et al., 2019; Deal and Volkoff, 2020).

The THS is highly conserved throughout all vertebrate classes (McArdle et al., 2020). Similarly to mammals, birds, reptiles and amphibians, the fish THS comprises three principal components: **1**) a central HPT axis regulating the biosynthesis, storage and secretion of mainly the TH, L-thyroxine (T4), **2**) peripheral deiodinases regulating the tissue levels of by far the most biologically active TH, 3,3',5-triiodo-L-thyronine (T3) and **3**) thyroid hormone receptors (TRs) mediating effects of THs on physiological processes (Eales and Brown, 1993; Eales et al. 1999, Blanton and Specker, 2007).

THS functioning is dependent on **1**) thyroid stimulating hormone (TSH) receptor (TSHR) activation, **2**) iodine uptake, **3**) TH synthesis and secretion, **4**) TH distribution, **5**) tissue-specific T4 deiodination and synthesis of T3, **6**) binding of THs to TH nuclear receptors (TRs), **7**) TH metabolism and **8**) TH feedback mechanisms as illustrated in Fig. 1.

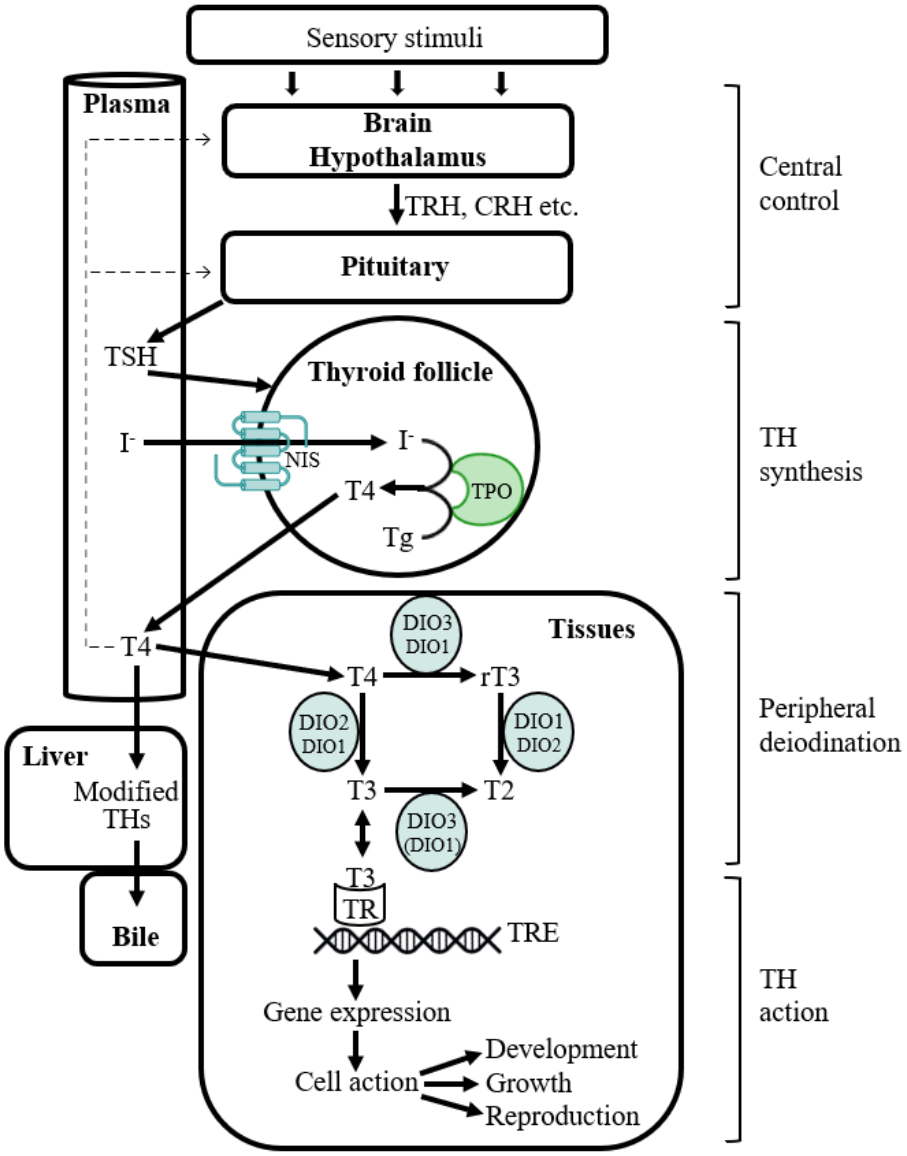

Fig. 1. Simplified outline of the HPT axis, peripheral deiodination and hormonal action in fish. The release of TSH from the pituitary is regulated by various TSH releasing factors from especially the hypothalamus. TSH regulates the synthesis of THs, primarily T4, in the thyroid follicles. T4 is converted to T3 by most, if not all, tissues. T3 binds to TR (in various receptor complexes) and induces gene transcription. Circulating THs feed back on the hypothalamus and the pituitary to maintain TH homeostasis. (Based on Eales et al., 1999 and Blanton and Specker, 2007).

2.1. Hypothalamic and pituitary control of TH levels

Endocrine systems are generally influenced by the hypothalamic-pituitary axis. Within this axis, the hypothalamus conveys specific signals to the pituitary gland, which in turn releases hormones directed towards target tissues. Pituitary hormones are secreted by different hormone-producing cell types: **1**) thyrotropes producing thyroid stimulating hormone (thyrotropin, TSH); **2**) corticotropes producing adrenocorticotropin (ACTH); **3**) somatotropes producing growth hormone (GH); **4**) melanotropes producing melanocyte stimulating hormone; **5**) gonadotropes producing follicle stimulating hormone (FSH) and luteinising hormone (LH) and **6**) lactotropes producing prolactin (Pogoda and

Hammerschmidt, 2007). The pituitary hormones affect various target tissues including the thyroid tissue, interrenal tissue, liver and gonads. These tissues release additional hormones which control key physiological processes like growth, metabolism, reproduction and stress (Blanco et al. 2020).

The hypothalamic-pituitary axis plays an important role in the control of TH synthesis in vertebrates by responding to various environmental or internal stimuli (reviewed in Norris and Carr, 2013). TSH releasing factors act on the thyrotropic cells in the pituitary stimulating the synthesis and secretion of TSH which in turn stimulates the thyroid follicles to synthesise and secrete THs (Norris and Carr, 2013).

In mammals, thyrotropin-releasing hormone (TRH) from the hypothalamus is considered the key regulator of pituitary TSH release. However, this is not always the case for non-mammalian vertebrates (Gorbman and Hyder, 1973; Larsen et al., 1998; De Groef et al., 2006; Galas et al., 2009). In teleosts, the present data indicates that the TSH releasing factor is species-dependent and may include TRH, corticotropin releasing hormone (CRH) or other hypothalamic or non-hypothalamic endocrine factors (Larsen et al., 1998; Chowdhury et al., 2004; De Groef et al., 2006; Galas et al., 2009; Watanabe et al., 2016; Campinho, 2019; Deal and Volkoff, 2020). Furthermore, some studies have indicated, that TSH secretion in fish is regulated mainly through inhibitory hypothalamic control (Peter and McKeown, 1975; Larsen et al., 1998; Chowdhury et al., 2004; MacKenzie et al., 2009).

TSH is a glycoprotein composed of an α- and a β-subunit (MacKenzie et al., 2009). The α-subunit of TSH is nearly identical to that of FSH and LH. The β-subunit is structurally distinct and thus defines the functional specificity of the hormone (Pierce and Parsons, 1981; Maugars et al., 2014).

TSH released by the pituitary is the key physiological regulator of thyroid function, stimulating TH biosynthesis (Norris and Carr, 2013).

2.2. TH synthesis in the thyroid

The thyroid activity is under the combined regulation of TSH from the pituitary gland and availability of iodine originating from external sources.

The functional unit of the thyroid tissue in all vertebrates is the thyroid follicle, which entails a monolayer of polarised follicular epithelial cells (thyrocytes) with the apical surface facing the follicular lumen and the basolateral membrane facing the bloodstream.

2.2.1. TSH receptor (TSHR) activation and signalling pathways

Regulation of thyroid function by TSH is mediated via binding to G protein coupled TSHRs located at the basolateral membrane of the thyroid follicular epithelial cells. TSHR activation results in intracellular signalling via different G protein subtypes activating several second messenger systems. In humans, mainly the Gs and Gq subtypes are activated by TSHR activation (Allgeier et al., 1994; Kleinau et al., 2017; Tuncel, 2017). Gs activates the adenylate cyclase/cyclic 3',5'-adenosine monophosphate (AC/cAMP) pathway which leads to activation of the transcription of genes involved in TH synthesis: *slc5a5* (*nis*), *tg* (thyroglobulin) and *tpo* (Van Heuverswyn et al., 1985; Chazenbalk et al., 1987; Riedel et al., 2001). Gq activates the phospholipase C/Ca^{2+} (PLC/Ca^{2+}) pathway which regulates iodide apical efflux, H_2O_2 production and thyroglobulin (Tg) iodination (Corvilain et al., 1994; Van Sande et al., 2006; Pereira et al., 2020).

The intracellular pathways activated by TSH have been shown to differ among mammals (Song et al., 2010). E.g. in dogs, TSH activates only the cAMP cascade but not the PLC cascade. These differences may imply different toxicological consequences in different species.

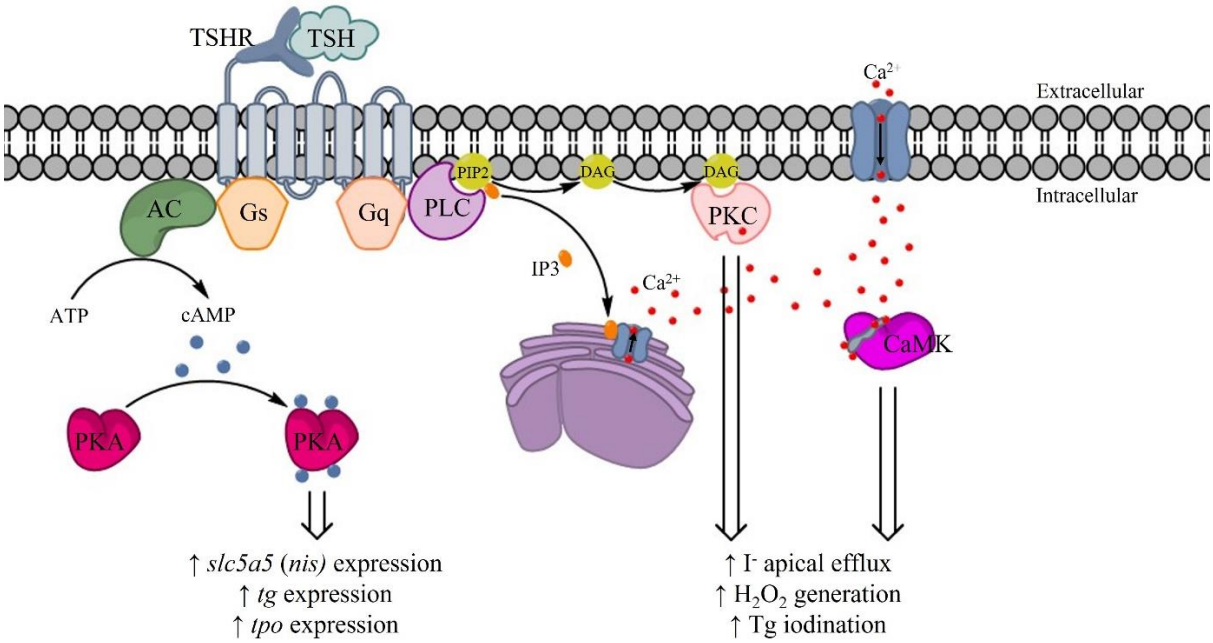

Fig. 2. Signalling pathways activated by TSH in human thyroid follicular epithelial cells. TSH binds to the TSHR, activating Gs and Gq which activate two different regulatory pathways: AC/cAMP and PLC/Ca^{2+} pathways, respectively.

AC/cAMP pathway: Gs activates AC, which converts adenosine triphosphate (ATP) to cAMP, which activates protein kinase A (PKA), which activates transcription of genes involved in TH production: *slc5a5* (*nis*), *tg* and *tpo*.

PLC pathway: Gq activation stimulates PLC that hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 binds to its endoplasmic reticulum (ER) receptors releasing Ca^{2+}. Increased intracellular Ca^{2+} is followed by an increase of Ca^{2+} from the extracellular medium and calmodulin-dependent protein kinases (CaMK) activation. DAG activates the protein kinase C (PKC). CaMK and PKC activation regulate the iodide apical efflux, H_2O_2 generation and Tg iodination. (Based on Pereira et al., 2020).

Compared to mammals, less information is available concerning the TSHR in fish. However, studies have shown that TSHR primary structure, functional properties and aspects of signalling are conserved between fish and mammals (Kumar et al, 2000; Oba et al., 2001; Farid and Szkudlinski, 2004; MacKenzie et al., 2009; Ponce et al., 2010; Opitz et al., 2011; Raldúa et al., 2012; Gao et al., 2016).

2.2.2. Iodine uptake

Biosynthesis of THs in the thyroid follicles requires the presence of adequate amounts of iodine (I) originating from external sources. Terrestrial vertebrates obtain almost all their iodine from the diet via efficient absorption of iodide (I^-) to the bloodstream from the gastrointestinal tract (where all iodine is converted to iodide). In mammals, dietary iodide absorption is mediated by the NIS in the small intestine (Nicola et al., 2009). However, the primary source of iodine in fish is debated and probably differs between species. Dietary iodine enrichment studies suggest that fish, like terrestrial vertebrates, accumulate iodine from the diet via the gastrointestinal tract (Ribeiro et al., 2011). Additionally, marine fish drink seawater, which is a rich source of iodine potentially capable of ensuring adequate gastrointestinal iodide supplies. *In situ* hybridisation experiments have demonstrated *slc5a5* (*nis*) expression in the gastrointestinal tract of zebrafish, further supporting this as a key site of iodine

transport into the general circulation (Holloway et al., 2021). Furthermore, studies suggest that fish may take up iodine from the ambient water across the gills (Hunn and Fromm, 1966). However, specific iodine transport mechanisms in the gills have not been established and little evidence has been found for *slc5a5* (*nis*) expression in gills (Li et al., 2011c).

Absorbed iodide is transported in the plasma to the thyroid tissue. Plasma iodide concentrations vary between fish species but are generally considerably higher in fish than in mammals (Eales and Brown, 1993).

2.2.3. TH biosynthesis and secretion

The synthesis of THs takes place in the thyroid follicles, which are made up of a monolayer of epithelial cells surrounding an extracellular lumen filled with proteinaceous colloid (Fig. 3).

TH synthesis requires two precursors: iodide and Tg (Pereira et al., 2020) and further involves TPO and H_2O_2 (Citterio et al., 2019).

A first step in TH biosynthesis is the active uptake of circulating iodide in the thyroid tissue by NIS. The iodide is further transported into the colloid in the thyroid follicular lumen.

The glycoprotein Tg is the substrate upon which THs are synthesised. It is expressed exclusively in the thyroid follicular epithelial cells and is the predominant protein produced in the thyroid follicles (Marino et al., 2000; Citterio et al., 2019). The overall structure of Tg is conserved in all vertebrates (Citterio et al., 2019). Within the thyroid follicular epithelial cells, newly synthesised Tg is packaged as dimers in secretory vesicles and excreted into the colloid by exocytosis.

In the presence of H_2O_2 provided by the dual-function oxidase (DUOX) system, TPO oxidises iodide, and subsequently iodinates the phenolic ring of specific hormonogenic tyrosine residues in Tg (Citterio et al., 2019). This results in the formation of diiodotyrosine (DIT) and monoiodotyrosine (MIT), which are subsequently coupled by TPO resulting in the generation of mainly T4 (derived from two DITs) and to a lesser extent T3 (derived from one DIT and one MIT) on Tg (Citterio et al., 2019; Marinò et al., 2000). After endocytosis of Tg into the thyroid follicular epithelial cells, T4 and T3 are cleaved from the Tg backbone. The THs are secreted into the bloodstream via transporters such as monocarboxylate transporters (MCTs) at the basolateral plasma membrane (Citterio et al., 2019). The I^- contained within uncoupled MIT and DIT is recycled by iodotyrosine dehalogenase 1 (Citterio et al., 2019).

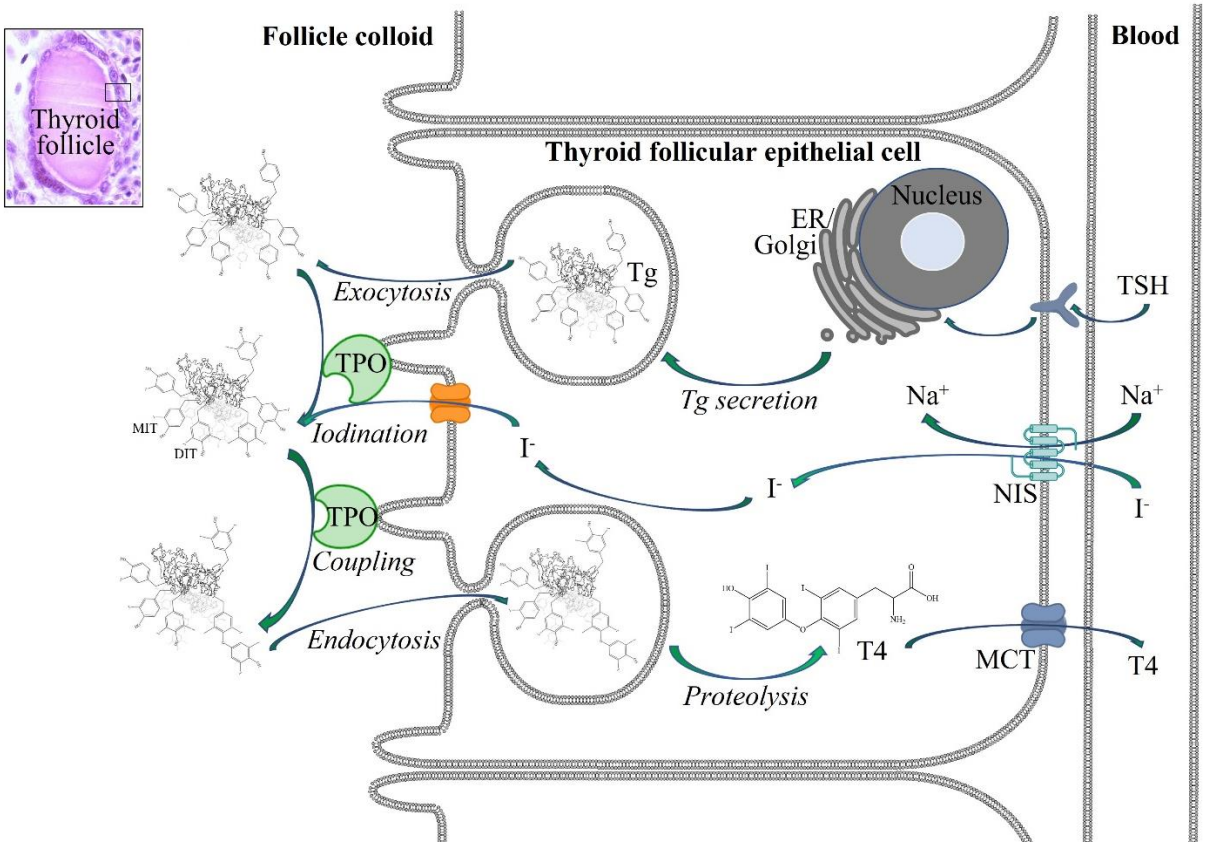

Fig. 3. T4 synthesis. (Based on Barrett, 2003; Raldúa et al., 2012; Mondal et al., 2016).

2.3. TH distribution

After release to the bloodstream, THs, which are lipophilic, readily binds to TH distributor proteins. The function of these proteins is to keep THs in the circulatory system to ensure consistent distribution of TH to the target tissues by counteracting the tendency of THs to partition non-specifically into cell membranes. Less than 1% of the THs have been estimated to appear in the free (non-protein bound) form in the blood of both fish and mammals (Eales and Shostak, 1985; Rabah et al., 2019). The rest of the THs are reversibly bound to TH distributor proteins.

Three major TH distributor proteins have been identified in the blood of vertebrates: albumin, transthyretin (TTR), and thyroxine-binding globulin (TBG). However, not all three proteins are found in all vertebrate classes.

All adult vertebrates studied have albumin as a TH distributor protein in their blood (Richardson et al., 1994; reviewed in Richardson, 2002). For some groups of adult vertebrates (in general: fish, amphibians, reptiles, monotremes and some polyprotodont marsupials), albumin is the only TH distributor protein. Other groups of adult vertebrates (in general: birds, diprotodont marsupials and some eutherians) possess both albumin and TTR in the blood. Some eutherians, including humans, have all three TH distributor proteins in the blood.

During specific stages of development, some groups of vertebrates have an additional TH distributor protein present in their blood which is not present in the blood of adults. In some fish, amphibians, reptiles and polyprotodont marsupials, where albumin is the only TH distributor protein in adults, TTR has been shown to be additionally present during specific stages of development (Yamauchi et al., 1998;

1999; Prapunpoj et al., 2000; Richardson et al., 2005). In a diprotodont marsupial, TBG has been detected during development in addition to the albumin and TTR present in adults (Richardson et al., 2005).

The additional TH distributor proteins increase the TH distribution capacity, and their appearance seems to coincide with the transient surges of elevated TH levels in the blood that all vertebrates experience at specific stages during development (Hulbert, 2000; Richardson et al*.,* 2005). In fish and amphibians, the rise in TH levels is often related to a metamorphic event (Hulbert, 2000; Yamauchi and Ishihara, 2009), and the appearance of the additional TH distributor proteins may be associated with the capacity to meet increased TH requirements to support TH-dependent tissue transformations during metamorphosis.

The relative contribution of each of the TH distributor proteins to the total TH distribution capacity differs between species. In humans, albumin is the most abundant TH distributor protein but binds THs with the lowest affinity, TTR appears at intermediate quantities and binds with intermediate affinity, and TBG is present in the lowest concentration but has the highest affinity for THs (reviewed in Rabah et al., 2019).

Both albumin and TBG have a higher affinity for T4 than for T3 in all studied species. Likewise, TTR binds T4 with a higher affinity than T3 in mammals. Conversely, TTR has a higher affinity for T3 than for T4 in birds, reptiles, amphibians and fish (reviewed in Power et al., 2000 and Rabah et al., 2019). Some studies suggest that in fish, T4 is distributed by albumin and T3 is distributed by TTR, based on the affinity of the proteins for T4 and T3 (Yamauchi et al., 1999; Power et al., 2000).

The TH distributor proteins provide a buffering like system for maintaining the blood levels of physiologically relevant free THs that can enter cells (Hulbert, 2000; Rabah et al, 2019). Studies in different mammalian species have shown that the half-life of both T3 and T4 depends on the abundance of the high or low affinity transport proteins (Lewandowski et al., 2004). Perturbations of the thyroid axis are expected to be more likely to happen, the shorter the half-life of the TH is.

2.4. Peripheral TH uptake and deiodination

In the peripheral organs, the local concentrations of the THs are regulated relatively independent of the TH levels in the circulation by means of transmembrane transporters that facilitate the uptake and efflux of THs, and the intracellular DIO enzymes.

Free TH from the circulation enters cells facilitated by specific transmembrane TH transporter proteins, including the monocarboxylate transporter 8 (MCT8), the monocarboxylate transporter 10 (MCT10) and the organic anion transporting polypeptide 1C1 (OATP1C1) (Arjona et al., 2011; Heijlen et al., 2013; Vatine et al., 2013; Zada et al., 2013; Campinho et al., 2014; de Vrieze et al., 2014; Silva et al., 2017; Zada et al., 2017; Groeneweg et al., 2019; Vancamp et al., 2019; Walter et al., 2019a; 2019b; 2019c; Admati et al., 2020). In medaka, studies have shown that the TH status influences the expression of *mct8*, *mct10* and several OATP genes including *oatp1c1* (Muzzio et al., 2014).

Intracellular TH levels are regulated by specific deiodination processes which either activate or inactivate THs (Fig. 4). Three types of selenoprotein iodothyronine DIOs are expressed in zebrafish (Dong et al., 2013).

T4 is regarded as a comparatively inactive prohormone for T3 in genomic actions of THs. T3 is usually more potent than T4 in binding to the TR and regulating gene transcription. T3 is synthesised from circulating T4 in most peripheral tissues via the action of DIOs (Orozco and Valverde-R, 2005). DIO2 is considered to be the major TH activating DIO enzyme, catalysing the conversion of T4 to T3 via outer-ring deiodination. DIO3 on the other hand performs inner-ring deiodination thereby converting T4 to TR

inactive reverse T3 (rT3) or T3 to inactive 3,3'-diiodothyronine (T2). DIO1 can perform both reactions, although it prefers rT3 over T4 and T3 as a substrate (Sanders et al., 1997). While DIO1 and DIO3 are plasma membrane proteins, DIO2 is an endoplasmic reticulum (ER) resident protein (Bianco and Larsen, 2005).

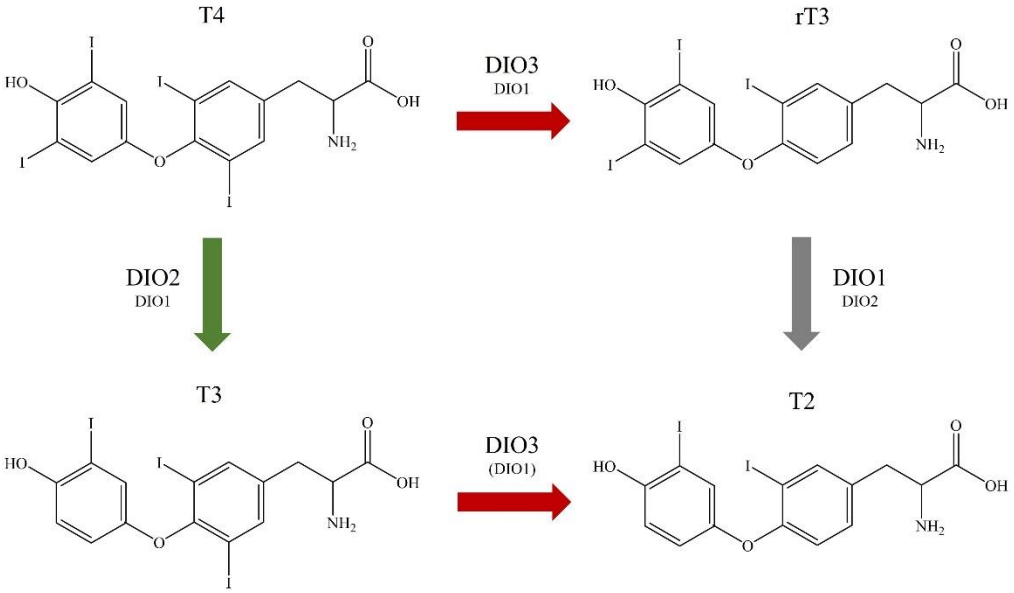

Fig. 4. Overview of the major pathways of TH deiodination. The font size indicates the substrate preferences of the three different deiodinase isoforms, DIO1, DIO2 and DIO3. Green arrow indicates activation, while red arrows indicate inactivation. (Based on Darras and Van Herck, 2012).

The expressions and activities of the different deiodinases may vary among species and among tissues and are major determinants of the plasma TH levels and of the availability of active hormones for TR binding (Orozco and Valverde, 2005; Gereben et al., 2015).

DIOs are responsive to TH concentrations. DIO2 and DIO3 expression and activity respond to thyroidal status in an opposite way. In general, high levels of THs promote decreases in *dio2* expression and DIO2 enzyme activity, while the contrary is the case for DIO3. Low levels of THs induce the opposite effect (Jarque and Piña, 2014). The mechanism through which thyroidal status regulates DIO activity is not known, although experiments suggest that the regulation occurs at a pre-translational level (Orozco and Valverde, 2005).

The response of DIO1 to TH levels is variable, especially in fish (Darras and Van Herck, 2012). DIO1 is very inefficient in converting T4 into T3 when compared to DIO2 (Darras and Van Herck, 2012). Studies suggest that DIO1 has a subordinate role compared to DIO2 in zebrafish embryonic development under normal (euthyroid) conditions, since knockdown of *dio1* alone does not have apparent effects on development prior to hatching (Walpita et al., 2010). By contrast, both DIO2 as well as DIO3 gene knockdown/knockout affect zebrafish development (Heijlen et al., 2014; Bagci et al, 2015; Houbrechts et al, 2016a; 2016b). While DIO2 is essential for the local generation of sufficient levels of T3 available for tissue differentiation, DIO3 has the proposed functions of protecting tissues from excessive T3 levels and favouring cell proliferation (Galton, 2005; Bouzaffour et al., 2010; Dentice and Salvatore, 2011; Heijlen et al., 2014).

2.5. TH binding to TRs

The major pathway of initiation of biological actions of T3 is via binding to nuclear TRs in target cells. To exert genomic effects, cytoplasmic T3 enters the nucleus and binds to nuclear TRs with high affinity and specificity.

Most vertebrates only have two nuclear TR genes, encoding TRα (*thra* (or *thrα* or *trα*)) and TRβ (*thrb* (or *thrβ* or *trβ*)), respectively. However, as a result of ancestral teleost specific genome duplication, the zebrafish genome contains two TRα genes (*thraa* and *thrab*), together with a single TRβ gene (*thrb*) (García-Cegarra et al., 2013). The *thraa* gene encodes at least two receptor isoforms (TRα1S (or TRαA1) and TRα1L (or TR αA1-2)) and *thrab* encodes a single receptor (TRαB) with possibly limited transactivation activity (Takayama et al., 2008; Darras et al., 2011; Marelli et al., 2016; Marelli and Persani, 2018). The *thrb* gene encodes three isoforms (TRβ1S, TRβ1L and TRβ2) (Marelli et al., 2016; Marelli and Persani, 2018).

TRs function as hormone-activated transcription factors that upon activation by TH control the expression of a broad range of genes. The specific genomic actions of TRs are influenced by the local availability of T3, by the relative presence of the TR isoforms and nuclear receptor corepressors and coactivators, and by the sequence and location of the TH response element (TRE) (Brent, 2012).

TRs form various receptor complexes (monomers, homodimers and heterodimers (e.g. with retinoid X receptors (RXRs)). However, their specific roles may vary depending upon the ligand availability and gene context (Singh et al., 2018).

A high level of sequence homology between mammalian and fish TRs indicates that they probably bind THs, undergo dimerisation and regulate gene transcription in a similar mode (Porazzi et al., 2009).

In the classical model of TR action, unliganded TR binds to RXR to form a heterodimer which binds to a TRE and then to a corepressor, repressing gene expression. T3 binding to the ligand-binding domain results in release of the corepressor and promotion of coactivator binding which initiates gene transcription (Brent, 2012; Yen, 2015; Mendoza and Hollenberg, 2017).

Furthermore, TH can negatively regulate some target genes, although the molecular mechanism is uncertain (Menzoza and Hollenberg, 2017).

In addition to TH actions involving direct regulation of transcription by TR, non-genomic actions initiated by THs at receptors in the plasma membrane, at cytoplasmic hormone binding proteins, or in mitochondria have been identified (Davis et al., 2018).

2.6. TH metabolism

The main classical pathways of TH metabolism are deiodination, sulfation and glucuronidation (van der Spek et al., 2017).

DIO3 is regarded as the major TH inactivating deiodinase catalysing inner-ring deiodination of T3 and T4, resulting in T2 and rT3 (Fig. 4).

DIO3 is the main deiodinase expressed during embryonic life (Parsons et al., 2020), and its activity is usually much higher than that detected in adult tissues (Orozco et al., 2012). DIO3 is assumed to protect tissues from an overload of THs during ontogeny and favour cell proliferation (Galton, 2005; Bouzaffour et al., 2010; Dentice and Salvatore, 2011; Heijlen et al., 2014).

In adult animals, the brain is usually the tissue with the highest DIO3 activity (Sutija and Joss, 2006), whereas hepatic expression of DIO3 is limited to embryonic life in most vertebrates (Orozco et al., 2012).

A second main pathway in TH metabolism is sulfation of the phenolic hydroxyl group; a reaction catalysed by phenol sulfotransferase (SULT). Sulfate conjugation of THs primarily occurs in the liver (van der Spek et al., 2017). Sulfation facilitates deiodination of T4 to inactive rT3 by DIO1, while sulfation of T4 blocks the outer ring deiodination by DIO1 and thereby the conversion to T3 (Moreno et al., 1994; Wu et al., 2005).

Although DIO1 can deiodinate both the inner- and the outer-ring of T4 and T3, it prefers rT3, sulfated T3 and sulfated T4 over T3 and T4 as substrates (Moreno et al., 1994). Consequently, the main role of DIO1 is to degrade biologically inactivated TH (van der Spek et al., 2017).

Another main pathway in TH metabolism is glucuronidation in which a glucuronic group is conjugated to the phenolic hydroxyl group in THs. This coupling reaction is catalysed by uridinediphosphate-glucuronosyltransferase (UGT) with UDP-glucoronic acid as a cofactor. Glucoronidation takes place in the liver and kidneys, and increases the water solubility of THs, which facilitates the excretion through the bile and urine (van der Spek et al., 2017).

Relatively minor pathways in TH metabolism include ether-link cleavage and oxidative deamination or decarboxylation of the alanine side chain of THs (van der Spek et al., 2017).

2.7. TH homeostasis

The thyroid cascade has a substantial capacity to compensate for disturbances that would otherwise disrupt TH homeostasis, which in the present context implies the maintenance of TH levels suitable for a given physiological state.

In mammals, both T3 and T4 levels are regulated via negative feedback by both T3 and T4 on the HPT axis. Circulating TH feeds back on the hypothalamus and the pituitary to inhibit the release of TRH and TSH, respectively (Chiamolera and Wondisford, 2009).

The relative significance of the HPT axis and the peripheral deiodination activity in maintaining TH homeostasis in fish is debated. It has been proposed that the HPT axis mainly controls T4 homeostasis, while T3 homeostasis in both plasma and tissues is mainly controlled by regulation of peripheral T4 deiodination (Eales et al., 1999). Other studies examining the regulation of TSH expression in fish have supported a role for both T3 and T4 in central negative feedback (MacKenzie et al., 2009). Both T3 and T4 appear to modify the transcription of the β unit of TSH in the pituitary gland, which regulates the release of TSH and thereby the maintenance of stable TH concentrations in the circulation (Pradet-Balade et al., 1997; 1999; Yoshiura et al., 1999).

3. The role of the thyroid hormone system in zebrafish development

Zebrafish development proceeds from fertilisation of the egg through embryo, larva and juvenile stages to adulthood (Parichy et al., 2009). The THS has been shown (e.g. via MO knockdown) to play an essential role in different stages of zebrafish development, although the exact molecular role of THs during development has not been fully characterised (Bohnsack and Kahana, 2013). Furthermore, other signalling pathways, e.g. retinoic acid and IGF-1, may also interact with the THS in the regulation of the development (Bohnsack and Kahana, 2013; Molla et al., 2019).

3.1. The THS during embryonic development

The period of embryonic development includes a pre-hatching stage and an eleutheroembryo stage, i.e., the stage between hatching and onset of exogenous feeding (Verbueken et al., 2018).

Until embryonic TH synthesis is active, the earliest stages of teleost fish are dependent on maternally provided THs stored in the yolk sac (Porazzi et al, 2009). T4 and T3 concentrations of around 11 and 6 ng/g, respectively, have been measured in the yolk of chum salmon (*Oncorhynchus keta*) at the time of hatching (Tagawa and Hirano, 1989). However, in zebrafish, both *tpo, ttr, dio1*, *dio2*, *dio3a*, *dio3b*, *thra* and *thrb* are expressed very early in pre-hatch embryos prior to thyroid development, suggesting that not all TH present prior to the appearance of thyroid follicles is derived from maternal sources (Walpita et al., 2007; Vergauwen et al., 2018).

The zebrafish thyroid tissue originates from the pharyngeal endoderm (Zada et al., 2017). The onset of thyroid development in fish resembles that of higher vertebrates, with the key transcription factors for thyroid differentiation, hhex (hematopoietically expressed homeobox), nkx2.1a/nkx2.4b (NK2 class homeobox) and pax2a (paired box 2.1), appearing at around 24 hpf (Rohr and Concha, 2000; Elsalini et al., 2003; Porazzi et al., 2009). In addition, from about 28 hpf, pax8 (paired box 8) is expressed in the thyroid precursor cells of zebrafish (Wendl et al., 2002). Thyroid development begins at 32 hpf, when evagination from the pharyngeal epithelium is completed to form the thyroid primordium (Rehberger et al., 2018). The first thyroid follicle differentiates at around 55 hpf (Alt et al., 2006) and is positive for T4 by 60 hpf (Wendl et al., 2007). During subsequent development, an increasing number of follicles appear further posterior along the ventral aorta (Wendl et al., 2002; Elsalini et al., 2003; Alt et al., 2006).

Zebrafish usually hatch around 48-72 hpf at a temperature of 28.5°C (Kimmel et al., 1995) and findings suggest an inhibitory role of THs in hatching (Reddy and Lam, 1991). The observed decline in T4 contents of zebrafish eggs from 48-72 hpf (Chang et al., 2012) possibly facilitates hatching.

Sustained endogenous T4 production in the thyroid follicles by 72 hpf is able to compensate for the diminishing pool of maternally derived T4 (Porazzi et al., 2009). In the zebrafish pituitary thyrotropes, *tshβ* and *dio2* are coexpressed by 48 hpf and TH-driven negative feedback regulation of *tshβ* transcription occurs in the thyroid axis by 96 hpf (Tonyushkina et al., 2014).

3.2. Role of THs in embryonic-to-larval transition

At around 3-7 dpf (Liu and Chan, 2002; Verbueken et al., 2018; Vergauwen et al., 2018), depending on rearing conditions (Wilson, 2012), zebrafish undergo an embryonic-to-larval transition. Major developmental transformations are required to prepare for active locomotion and exogenous feeding. These include cartilagination of the pectoral fins and finfolds for active swimming, inflation of the posterior swim bladder for buoyancy control, resorption of the yolk sac, and structural and functional maturation of the mouth and gastrointestinal tract to cope with live prey (Liu and Chan, 2002).

The embryonic-to-larval transition depends on TH signalling (Liu and Chan, 2002; Chang et al, 2012; Walter et al., 2019c). A rapid increase in zebrafish whole-body T4 content is observed from 3-5 dpf, which is presumably related to the embryonic-to-larval transition (Chang et al., 2012). Expression levels of *slc5a5* (*nis*), *tpo*, *tg*, *ttr*, *dio1*, *dio2*, *dio3a*, *thra* and *thrb* in zebrafish increase throughout the transition period, further reflecting the importance of the THS in this developmental event (Vergauwen et al., 2018).

3.3. Role of THs in larval-to-juvenile transition (metamorphosis)

The larval-to-juvenile transition occurs around 14-33 dpf in zebrafish (Brown, 1997; Liu and Chan, 2002; Parichy and Turner, 2003; Vergauwen et al., 2018). It is a gradual process with no fixed borders since it depends on rearing conditions and on the morphological endpoint analysed (Alfonso et al., 2020).

Major changes occurring during the larval-to-juvenile transition include the development of the adult pigmentation pattern, the formation of scales, the development of adult fin morphology, the ossification of the axial skeleton and the inflation of the anterior swim bladder chamber (Brown, 1997; Liu and Chan, 2002).

THs are viewed to be essential in the larval-to-juvenile transition of fish as reviewed by Campinho (2019). A rapid increase in whole-body T4 levels from 14 to 21 dpf has been observed in zebrafish (Chang et al., 2012), which indicates that T4 is correlated with the transition. Furthermore, increases in whole-body *dio1* and *dio2* expression levels and concomitant decrease in *dio3* expression levels have been observed in zebrafish during the larval-to-juvenile transition phase, suggesting an increased demand for activation of T4 to T3 (Vergauwen et al., 2018). Although the increase in TH levels during metamorphosis correlates with tissue-specific genetic responses, the detailed molecular and cellular events mediating each metamorphic event are not clear (Campino, 2019).

Juvenile morphology closely resembles adult morphology; however, the fish are not considered adults until they become sexually mature at around 2-3 months of age (Parichy et al., 2009; Lawrence et al., 2012; Nasiadka and Clark, 2012; Singleman and Holtzman, 2014).

4. Thyroid hormone system related endpoints

4.1. THS disruption

The normal functioning of the THS is essential for the control of early development, growth, metabolism and reproduction in all vertebrates.

Due to the complexity of the THS, chemicals with THS related MOA may exert impact on several points in the thyroid cascade. These include **1**) interferences with T4 synthesis (TSH, NIS, Tg, TPO), **2**) TH distribution, **3**) peripheral uptake (e.g. MCT8) and activation to T3 (DIO), **4**) interaction with TR, **5**) TH metabolism (liver conjugating enzymes) and **6**) feedback mechanisms. Thus, key MIEs (molecular initiating events) for THS disruption include **1**) NIS inhibition, **2**) TPO inhibition, **3**) DIO inhibition and **4**) interaction with TRs, TH distributor proteins and cell membrane transporters (e.g. MCT8).

In some mammals, induction of liver conjugating enzymes has been shown to lead to THS disruption, and phenobarbital, β-naphthoflavone and clofibric acid have been used as model compounds for this mechanism (Runge-Morris et al., 1998; Viollon-Abadie et al., 2000; Cunha and van Ravenzwaay, 2005; Noyes et al., 2019). However, it largely remains to be explored whether the THS of fish is affected when exposed to chemicals acting through this mechanism. Only two studies were found examining the effects of one of the three mammalian model compounds on the THS of fish. In one study, β-naphthoflavone significantly decreased plasma T4 levels in European eels (*Anguilla anguilla*), whereas T3 and TSH levels remained constant (Teles et al., 2005). In another study, T4 and T3 levels decreased, whereas TSH levels increased in Indian major carp (*Cirrhinus mrigala*) after exposure to clofibric acid (Saravanan et al., 2014).

Since endocrine disrupters are defined by both endocrine activity and a population-relevant adverse effect, with a (plausible) link between the two (WHO/IPCS, 2002; EFSA Scientific Committee, 2013; Munn and Goumenou, 2013), it is crucial to determine which endpoints can be considered population-relevant. Generally, survival, development, growth and reproduction are considered population-relevant endpoints (Weltje et al., 2013; Marty et al., 2017; OECD, 2018). However, while potentially of concern from the viewpoint of environmental hazard identification/characterisation, effects on survival or growth could be non-specific consequences of other toxic effects and, thus, may not be linked to an endocrine modality and would not on its own lead to the conclusion that the chemical is an endocrine disrupter in fish (OECD, 2018). Behavioural changes could in some cases be population-relevant, i.e. changes in sexual, feeding or fear response behaviour, but are not yet validated and with the currently available techniques difficult to quantify and relate to adversity.

Adverse outcome pathways (AOPs) connect MIEs to adverse outcomes in organisms via a series of intermediate cellular, organ and organism level key events (KEs) linked by KE relationships (OECD, 2017). MIEs and/or KEs may be shared by multiple AOPs, generating AOP networks, which add to the complexity of THS disruption.

The ideal endpoint for inclusion in fish TGs would be an easily assessed, reliable, robust, sensitive and specific KE covering all THS disrupting MIEs and linked to population-relevant adverse outcomes.

4.2. Identification of THS related effects from model compound studies

Several studies on potential THS related endpoints have been published during the recent years. The potential THS related endpoints have largely been identified through examination of typical effect patterns of model compounds disrupting the THS through different MIEs. In general, the various MIEs leading to THS disruption can be divided in those leading to effects on TH levels and those leading to effects on TH binding. Especially the effects of compounds influencing TH levels by inhibiting the enzymes involved in TH synthesis have been examined. The NIS inhibitor perchlorate and the TPO inhibitor (and possibly DIO inhibitor) propylthiouracil (PTU) are among the most used chemicals to determine the effects of inhibited thyroidal TH synthesis, while the DIO inhibitor iopanoic acid has been used to examine the effects of disrupted peripheral T3 regulation. These model compounds have been selected in this DRP as case studies representative of effects of disrupted TH levels on potential endpoints. Furthermore, tetrabromobisphenol A (TBBPA) has been selected as a case study representative of effects of disrupted TH binding on potential endpoints, based on the well-documented interaction of TBBPA with TRs and TTR (Meerts et al., 2000; Kitamura et al., 2002; Hamers et al., 2006).

4.2.1. Perchlorate: NIS inhibition

Perchlorate is a naturally occurring and manufactured oxidising agent applied in e.g. rocket fuels, explosives, fireworks and air bag inflators (Nizinski et al., 2021).

Perchlorate competitively inhibits iodide uptake by NIS in the thyroid follicles, thereby lowering the synthesis of THs.

Table 2 summarises the THS related effects of perchlorate observed in fish and is followed by a more detailed description of effects of perchlorate on potential THS related endpoints.

Table 2. Summarised THS related effects of perchlorate in fish

Endpoint	Species	Effect	NOEC	LOEC	Reference
Thyroid histopathology	Zebrafish (juveniles)	↑ Thyrocyte height ↓ Colloid ↑ Follicle number ↑ Thyrocyte hyperplasia	0.25 mg/L 0.125 mg/L 0.25 mg/L 0.25 mg/L	0.5 mg/L 0.25 mg/L 0.5 mg/L 0.5 mg/L	Schmidt et al., 2012
	Zebrafish (juveniles)	↑ Thyrocyte height ↓ Colloid	- -	10 mg/L 10 mg/L	Mukhi and Patiño, 2007
	Zebrafish (juveniles)	↑ Thyrocyte height ↓ Colloid	- -	100 mg/L 100 mg/L	Mukhi et al., 2007
	Zebrafish (juveniles)	↑ Thyrocyte height	0.5 mg/L	5 mg/L	Schmidt et al., 2017
	Zebrafish (juveniles)	↑ Thyrocyte height	-	100 mg/L	Sharma and Patiño, 2013
	Zebrafish (adults)	↑ Thyrocyte height ↓ Colloid ↑ Follicle area ↑ Thyrocyte hyperplasia	- - - -	10 mg/L 10 mg/L 10 mg/L 10 mg/L	Liu et al., 2006,
	Zebrafish (adults)	↑ Thyrocyte height ↓ Colloid	0.09 mg/L 0.011 mg/L	1.13 mg/L 0.09 mg/L	Mukhi et al., 2005
	Zebrafish (adults)	↑ Thyrocyte nuclear size ↓ Colloid ↑ Thyrocyte hyperplasia	- - -	18 mg/L 18 mg/L 18 mg/L	Patiño et al., 2003[a]
	Fathead minnow (larvae/juveniles)	↑ Thyrocyte height ↓ Colloid ↑ Thyrocyte hyperplasia	- - -	1 mg/L 1 mg/L 1 mg/L	Crane et al., 2005

Endpoint	Species	Effect	NOEC	LOEC	Reference
	Threespine stickleback (subadults and adults)	↑ Thyrocyte height (subadults only) ↓ Colloid ↓ Follicle area (adults only) ↑ Follicle number	- - - -	10 mg/L 10 mg/L 10 mg/L 10 mg/L	Gardell et al., 2017
	Threespine stickleback (larvae, juveniles, adults)	↑ Follicle number (larvae, juveniles, adults) ↓ Follicle size (adults) ↓ Thyrocyte number (adults) ↑ Thyrocyte height (juveniles) ↓ Thyrocyte height (adults)	- - - - -	100 mg/L 100 mg/L 100 mg/L 100 mg/L 100 mg/L	Petersen et al., 2015
	Threespine stickleback (adults)	↑ Follicle number ↓ Follicle size ↑ Thyrocyte height	- - 30 mg/L	30 mg/L 30 mg/L 100 mg/L	Furin et al., 2015[b]
	Eastern mosquitofish (adults)	↑ Thyrocyte height ↑ Colloid ↑ Thyrocyte hyperplasia	- - -	0.1 mg/L 0.1 mg/L 0.1 mg/L	Bradford et al., 2005
	Rainbow trout (immature, 5 months old)	↑ Thyrocyte height ↑ Number of thyroid follicles	- -	300 mg/L 300 mg/L	van Putten et al., 1983
		T			

Endpoint	Species	Effect	NOEC	LOEC	Reference
T4, T3 and TSH levels	Zebrafish (juveniles)	↓ Whole-body T4	0.5 mg/L	5 mg/L	Schmidt et al., 2012
	Zebrafish (adults)	↓ Whole-body T4 ↔ Whole-body T3	- 10 mg/L	10 mg/L -	Mukhi and Patiño, 2007
	Zebrafish (adults)	↔ Whole-body T4	11.48 mg/L	-	Mukhi et al., 2005
	Medaka (adults)	↓ Blood T4 ↔ Blood T3	- 100 mg/L	100 mg/L -	Lee et al., 2014
	Fathead minnow (larvae/juveniles)	↑ Whole-body T4 ↔ Whole-body T3 ↓ T3/T4 ratio	10 mg/L 100 mg/L 10 mg/L	100 mg/L - 100 mg/L	Crane et al., 2005
	Threespine stickleback (adults)	↔ Whole-body T4 ↔ Whole-body T3	30 mg/L 30 mg/L	- -	Furin et al., 2015[b]
	Threespine stickleback (adults)	↔ Whole-body T4 ↔ Whole-body T3	100 mg/L 100 mg/L	- -	Gardell et al., 2015
	Threespine stickleback (subadults, adults)	↔ Whole-body T4 ↔ Whole-body T3	100 mg/L 100mg/L	- -	Gardell et al., 2017
	Threespine stickleback (larvae, juveniles,	↔ Whole-body T4 ↔ Whole-body T3 (except at lowest conc.)	10 mg/L -	- 10 mg/L	Petersen et al., 2015

Endpoint	Species	Effect	NOEC	LOEC	Reference
	adults)				
	Chinese rare minnow (adults)	↔ Plasma T4 (males and females) ↓ Plasma T3 (males) ↔ Plasma T3 (females)	0.005 mg/L - 0.005 mg/L	- 0.05 mg/L -	Li et al., 2011b
	Eastern mosquitofish (adults)	↓ Whole-body T4	-	0.1 mg/L	Bradford et al., 2005
	Catfish (adults)	↑ Pituitary TSH	-	5 mg/fish	Singh et al., 1977
	Zebrafish (embryos)	↓ T4 in thyroid follicles	-	274 mg/L	Raldúa and Babin, 2009
	Zebrafish (embryos)	↓ T4 in thyroid follicles	-	0.4 mg/L	Thienpont et al., 2011
	Zebrafish (embryos)	↓ T4 in thyroid follicles	-	2000 mg/L	Li et al., 2012
	Zebrafish (embryos)	↓ T4 in thyroid follicles	-	250 mg/L	Alt et al., 2006
	Zebrafish (adults)	↓ Intensity of colloidal T4 ring in follicles	-	0.011 mg/L	Mukhi et al., 2005
Gene expression	Medaka (adults)	↓ *thra* ↓ *thrb* ↑ *dio2*	- - -	100 mg/L 100 mg/L 100 mg/L	Lee et al., 2014
	Chinese rare minnow (larvae, adults)	↓↑↔ *slc5a5* (depends on age and exp. time) ↓↔ *dio1* (depends on exposure time)	- - -	0.05 mg/L 0.05 mg/L 0.005 mg/L	Li et al., 2011b

Endpoint	Species	Effect	NOEC	LOEC	Reference
		↓↑↔ *dio2* (depends on age and exposure time) ↓↔ *dio3* (depends on exposure time)	-	0.005 mg/L	
Skin pigmentation	Zebrafish (juveniles)	↓ Pigmentation	-	100 mg/L	Mukhi et al., 2007
	Zebrafish (juveniles)	↓ Pigmentation	-	250 mg/L	Brown, 1997[c]
	Fathead minnow (larvae/juveniles)	↓ Pigmentation	1 mg/L	10 mg/L	Crane et al., 2005
	Threespine stickleback (adults)	↓ Pigmentation	12 mg/L	30 mg/L	Bernhardt et al., 2011[d]
	Chinese rare minnow (larvae)	↔ Pigmentation	0.05 mg/L	-	Li et al., 2011b
Swim bladder	Zebrafish (embryos)	↓ Inflation	-	200 mg/L	Jomaa et al., 2014
	Chinese rare minnow (larvae)	↓ Inflation	-	0.05 mg/L	Li et al., 2011b
Craniofacial development	Zebrafish (F1 embryos)	↓ Jaw development	-	100 mg/L	Mukhi and Patiño, 2007
Fins	Zebrafish (juveniles)	↓ Differentiation and growth of pectoral fins ↓ Differentiation and growth of pelvic fins	- -	250 mg/L 250 mg/L	Brown, 1997[c]

Endpoint	Species	Effect	NOEC	LOEC	Reference
	Threespine stickleback (adults)	↓ Length of pectoral fins	30 mg/L	60 mg/L	Bernhardt and von Hippel, 2008[d]
Scales	Zebrafish (juveniles)	↓ Development	-	250 mg/L	Brown, 1997[c]
	Fathead minnow (larvae/juveniles)	↓ Development	1 mg/L	10 mg/L	Crane et al., 2005
Eye	Zebrafish (embryos)	↔ Eye size	2000 mg/L	-	Li et al., 2012
Behaviour	Threespine stickleback (adults)	↓ Swimming performance ↓ Nest building ↓ Courtship behaviour	- - -	30 mg/L 30 mg/L 30 mg/L	Bernhardt and von Hippel, 2008[d]

[a] Mortality in the 18 mg/L group was reported as random and rare throughout the experimental period. However, signs of general toxicity and some acute mortality was observed in the 677 mg/L group.

[b] Greater mortality of females was observed in the 100 mg/L treatment.

[c] 500 mg/L was toxic to zebrafish.

[d] Threespine sticklebacks treated with ≥30 mg/L suffered higher mortality rates than the negative control fish.

4.2.1.1. Thyroid histopathology

Histopathological effects of perchlorate exposure have been examined in adult zebrafish (Liu et al., 2006; Mukhi et al., 2005; Patiño et al. 2003); in juvenile zebrafish exposed from 0-35 dpf (Schmidt et al., 2012), from 3-33 dpf (Mukhi et al., 2007), from 4-33 dpf (Sharma and Patiño, 2013), for 5 weeks (Schmidt et al., 2017) or for 10 weeks (Mukhi and Patiño, 2007); in fathead minnow larvae exposed from 0-28 dpf (Crane et al., 2005); in threespine sticklebacks exposed from fertilisation through to adulthood (Furin et al., 2015; Petersen et al., 2015; Gardell et al., 2017); in adult eastern mosquitofish (Bradford et al., 2005); and in immature rainbow trout (van Putten et al., 1983).

Exposure to perchlorate caused various changes in thyroid histology in fish. Increased follicular epithelial cell height was observed in all the fish species and stages (juveniles and adults) examined for thyroid histopathological effects of perchlorate, except for one study where follicular epithelial cell height decreased in adult sticklebacks although it was increased in juveniles (Petersen et al., 2015). Further frequently observed effects of perchlorate exposure included increased number of thyroid follicles and follicular epithelial cell hyperplasia. Moreover, perchlorate caused various degrees of depletion of colloid content in the thyroid follicles of zebrafish, fathead minnow and threespine stickleback but induced colloid in eastern mosquitofish. The sensitivity of the various histopathological endpoints differed between the studies.

4.2.1.2. TH levels

No significant effects of ammonium perchlorate on whole-body T4 concentrations in adult zebrafish were observed after 12 weeks of exposure to 11 mg/L (Mukhi et al., 2005). However, exposure to 10 or 100 mg/L perchlorate for 16 weeks greatly suppressed whole-body T4 concentrations in adult zebrafish (Mukhi and Patiño, 2007). Likewise, T4 content was decreased while T3 content was unchanged in embryos derived from the exposed parents. Significantly reduced T4 levels were also observed in zebrafish after exposure to perchlorate from 0-35 dpf (Schmidt et al., 2012). In adult medaka, T4 concentrations were significantly decreased, while T3 levels remained constant following exposure to perchlorate for 7 days (Lee et al., 2014).

Whole-body T4 content in fathead minnows exposed to perchlorate until 28 dpf was significantly elevated, but T3 content was not significantly affected (Crane et al., 2005). Consequently T3/T4 ratios were significantly decreased in fathead minnows exposed to perchlorate.

Whole-body T3 and T4 concentrations were not significantly affected by perchlorate exposure in threespine sticklebacks exposed as adults to 100 ppm (Gardell et al., 2015) or from fertilisation until 336 dpf to 10, 30 or 100 ppm (Gardell et al., 2017). Likewise, T4 and T3 concentrations in larval, juvenile and adult whole threespine stickleback chronically exposed to 10, 30 or 100 ppm perchlorate were not significantly different from controls, except for T3 at the lowest perchlorate concentration indicating a non-monotonic dose response curve (Petersen et al., 2015).

No significant effects on plasma T4 were observed in adult Chinese rare minnows (*Gobiocypris rarus*) exposed to perchlorate for 21 days (Li et al., 2011b). However, the plasma T3 level in males decreased significantly after perchlorate treatment.

Perchlorate reduced whole-body T4 concentrations in adult eastern mosquitofish exposed for 30 days, but clear dose-response relationships were less evident for T4 than for histopathological endpoints (Bradford et al., 2005).

Administration of perchlorate once a week for 8 weeks significantly elevated the TSH level in the pituitary gland of catfish (Singh et al., 1977).

An accumulation of T4 immunoreactivity at the interphase between the follicular epithelium and the colloid lumen has been observed. This colloidal T4 ring may reflect the accumulation of T4 at its site of synthesis, and thus represent the level of TH synthetic activity in the thyroid follicles (Mukhi et al., 2005; Mukhi and Patiño, 2007).

Perchlorate caused a decrease in the colloidal T4 ring intensity (optical density) of adult zebrafish thyroid follicles at 2 and 12 weeks of exposure (Mukhi et al., 2005). Colloidal ring intensity was the most sensitive of the evaluated endpoints, which also included thyroid histology and T4 concentrations. The intensity of the colloidal T4 ring was still reduced after 12 weeks of recovery of the fish.

Furthermore, perchlorate suppressed T4 immunoreactivity in thyroid follicles in an immunofluorescence quantitative test with zebrafish eleutheroembryos from 2-5 dpf (Raldúa and Babin, 2009; Thienpont et al., 2011) and in zebrafish embryos treated from 1-4 dpf (Li et al., 2012) or 0-5 dpf (Alt et al., 2006).

4.2.1.3. Gene expression

The expression of *slc5a5* (*nis*) was significantly downregulated after exposure for 7 days, significantly upregulated after exposure for 14 days, but showed no significant variation after exposure for 21 days to perchlorate in Chinese rare minnow larvae (Li et al., 2011b). In the livers of adult fish, the expression of *sls5a5* was significantly downregulated after exposure for 7 or 14 days and significantly upregulated after exposure for 21 days. The expression of *slc5a5* was generally upregulated in the brains of the adult fish.

Significant downregulation of *thra* and *thrb* transcripts and upregulation of *dio2* transcripts were observed in adult medaka exposed to perchlorate for 7 days (Lee et al., 2014). In livers of adult Chinese rare minnows, *dio3* expression levels were downregulated at 7-day exposure, while no significant effects were observed for *dio1* or *dio2* (Li et al., 2011b). After 14-day exposure, an upregulation of *dio2* expression and a downregulation of *dio1* and *dio3* expressions were observed. No significant changes in the expression of *dio1*, *dio2* or *dio3* in the livers were observed after exposure for 21 days. In the brains of the adult male fish, a general upregulation of *dio2* expression was observed after exposure for 7 or 14 days, while *dio2* expression was significantly downregulated after 21-day exposure. In the brains of the adult females, a general downregulation of *dio2* and *dio3* expression was observed. In larvae, *dio2* mRNA levels were downregulated after exposure to perchlorate for 7 days and upregulated after exposure to perchlorate for 14 or 21 days.

4.2.1.4. Skin pigmentation

Perchlorate reduced the development of skin pigmentation in zebrafish exposed from 3-33 dpf (Mukhi et al., 2007) or from 14-65 dpf (Brown, 1997); in fathead minnows exposed from 0-28 dpf (Crane et al., 2005); and in threespine sticklebacks spawned and raised to sexual maturity in perchlorate treated water (Bernhardt et al., 2011). However, no significant effects of perchlorate exposure on the skin pigmentation were observed 21 dph in Chinese rare minnows (Li et al., 2011b).

4.2.1.5. Swim bladder

Upon exposure to sodium perchlorate from 0-5 dpf, deflated swim bladders were observed in 12% of zebrafish embryos (Jomaa et al., 2014). Likewise, retarded swim bladder development was observed in larval Chinese rare minnow after perchlorate treatment for 21 days (Li et al., 2011b).

4.2.1.6. Craniofacial development

In zebrafish, offspring from parents exposed to perchlorate showed a significantly reduced forward protrusion of the Meckel's cartilage (which forms the lower jaw) and the ceratohyal cartilage (which supports the lower jaw) at 4 dpf (Mukhi and Patiño, 2007).

4.2.1.7. Fins

Perchlorate inhibited differentiation and growth of pectoral and pelvic fins in zebrafish exposed from 14-65 dpf (Brown, 1997), and likewise decreased the length of pectoral fins in threespine sticklebacks exposed throughout development (Bernhardt and von Hippel, 2008).

4.2.1.8. Scales

The development of scales was delayed in zebrafish (Brown, 1997) and fathead minnow (Crane et al., 2005) exposed to perchlorate.

4.2.1.9. Eye

Potassium perchlorate did not significantly affect eye size in zebrafish larvae 3-5 dpf (Li et al., 2012).

4.2.1.10. Behaviour

While no significant behavioural effects were observed in a stickleback parent generation exposed to perchlorate as adults, poor swimming ability, and inhibition of nest construction and courtship behaviour were observed in their offspring exposed for one year throughout development (Bernhardt and von Hippel, 2008).

4.2.1.11. General conclusions on perchlorate

Several general conclusions can be drawn from the literature survey on the effects of perchlorate on THS related endpoints in fish.

Increased follicular epithelial cell height was observed as an effect of perchlorate exposure in all the 10 studies, counting 5 different fish species, examining this histopathological endpoint. Further frequently observed effects of perchlorate exposure included increased number of thyroid follicles and follicular epithelial cell hyperplasia. Moreover, perchlorate caused various degrees of depletion of colloid content in the follicular lumen in zebrafish (6 studies), fathead minnow and threespine stickleback; but induced colloid in the single study in eastern mosquitofish.

In general, no effects were observed on whole-body T3 levels. Perchlorate either had no effect or decreased whole-body T4 levels, except from fathead minnows where increased T4 levels were observed.

Decreased swim bladder inflation, scale development and fin growth were observed in all the studies examining these endpoints. Moreover, skin pigmentation was decreased in 4 out of 5 studies. Conversely, no effect was observed on eye size in the single study examining this endpoint.

4.2.2. Propylthiouracil (PTU): TPO inhibition (and possible DIO inhibition)

PTU is a well-studied anti-thyroidal compound which inhibits the ability of TPO to incorporate iodine into tyrosyl residues of Tg. In addition, PTU is generally considered a DIO1 inhibitor in mammals. However, fish DIO1 appears to be less susceptible to inhibition by PTU (Sanders et al., 1997; Orozco et al., 2003; Orozco and Valverde, 2005; Orozco et al., 2012).

Table 3 summarises the THS related effects of PTU observed in fish and is followed by a more detailed description of effects of PTU on potential THS related endpoints.

Table 3. Summarised THS related effects of PTU in fish

Endpoint	Species	Effect	NOEC	LOEC	Reference
Thyroid histopathology	Zebrafish (juveniles)	↑ Thyrocyte height ↑ Follicle number ↑ Thyrocyte hyperplasia	2.5 mg/L - -	10 mg/L 2.5 mg/L 2.5 mg/L	Schmidt and Braunbeck, 2011
	Zebrafish (juveniles)	↑ Thyrocyte height	2.5 mg/L	10 mg/L	Schmidt et al., 2017
	Zebrafish (adults and F1 juveniles)	↑ Thyrocyte height ↓ Colloid ↑ Thyrocyte hyperplasia	1 mg/L 1 mg/L 1 mg/L	10 mg/L 10 mg/L 10 mg/L	van der Ven et al., 2006
	Zebrafish (adults)	↓ Thyrocyte height ↔ Follicle area ↔ Follicle number	- 5 mg/kg fish 5 mg/kg fish	5 mg/kg fish - -	Pinto et al., 2013
	Rainbow trout (juveniles)	↑ Thyrocyte height	-	5 mg/g food	Quesada-Garcia et al., 2016
TH levels	Zebrafish (embryos)	↓ Whole-body T4 ↔ Whole-body T3 ↓ Whole-body rT3	- 100 mg/L -	100 mg/L - 100 mg/L	Walter et al., 2019c
	Zebrafish (larvae/juveniles)	↓ Whole-body T4 ↓ Whole-body T3	- -	37 mg/L 37 mg/L	Stinckens et al., 2020
	Zebrafish (juveniles)	↓ Whole-body T4	25 mg/L	50 mg/L	Schmidt and Braunbeck, 2011

Endpoint	Species	Effect	NOEC	LOEC	Reference
	Zebrafish (adults)	↓ Plasma T4 ↓ Plasma T3	1 mg/L 10 mg/L	10 mg/L 100 mg/L	van der Ven et al., 2006
	Zebrafish (adults)	↓ Plasma T4 ↓ Plasma T3	- -	100 mg/L 100 mg/L	Liu et al., 2011a
	Tilapia (larvae)	↓ Whole-body T4 ↔ Whole-body T3	- 50 mg/L	50 mg/L -	Okimoto et al., 1993
	Tilapia (adults)	↑ Plasma T4 (low doses) ↓ Plasma T4 (high dose) ↔ Plasma T3 (low doses) ↓ Plasma T3 (high dose)	- - 5 mg/kg fish -	5 mg/kg fish 10 mg/kg fish - 10 mg/kg fish	Peter and Peter, 2009
	Sea bream (juveniles)	↔ Plasma T4 ↔ Plasma T3	0.05 mg/kg fish 0.05 mg/kg fish	- -	Morgado et al., 2009
	Sea bream (immature)	↔ Plasma T4 ↔ Plasma T3	1 mg/kg fish 1 mg/kg fish	- -	Campinho et al., 2012
	Zebrafish (embryos)	↓ T4 in thyroid follicles	10 mg/L	31 mg/L	Raldúa and Babin, 2009
	Zebrafish (embryos)	↓ Fluorescence of intrafollicular T3 ↓ Fluorescence of intrafollicular T4	2.5 mg/L 25 mg/L	10 mg/L 50 mg/L	Rehberger et al., 2018
TH distributor proteins	Sea bream (juveniles)	↑ Plasma TTR	-	0.05 mg/kg fish	Morgado et al., 2009
	Sea bream (immature)	↓ Plasma TTR	-	1 mg/kg fish	Campinho et al., 2012

Endpoint	Species	Effect	NOEC	LOEC	Reference
Gene expression	Zebrafish (embryos)	↔ *tsh* ↑ *tpo* ↔ *dio1* ↑ *dio2* ↓ *dio3* ↓ *thra* ↓ *thrb*	50 mg/L - 50 mg/L - - - 100 mg/L	- 50 mg/L - 50 mg/L 50 mg/L 50 mg/L 250 mg/L	Baumann et al., 2016
	Zebrafish (embryos)	↑ *slc5a5* (*nis*) ↑ *tg*	- -	100 mg/L 100 mg/L	Liu et al., 2013
	Zebrafish (larvae)	↑ *tshb*	-	111 mg/L	Tonyushkina et al., 2017
	Zebrafish (adults)	↔ *slc5a5* (*nis*) ↔ *tg* ↑ *tsh* ↔ *tpo*	5 mg/kg fish 5 mg/kg fish - 5 mg/kg fish	- - 5 mg/kg fish -	Pinto et al., 2013
	Zebrafish (embryos)	↓ Green opsin transcripts in eye ↓ UV opsin transcripts in eye ↓ Arrestin transcripts in eye	- - -	350 mg/L 350 mg/L 350 mg/L	Baumann et al., 2019
	Coho salmon (embryos)	↓ Blue opsin transcripts in cones of eye ↑ UV opsin transcripts in cones of eye	- -	35 mg/L 35 mg/L	Gan and Flamarique, 2010
	Sea bream (juveniles)	↓ *tsh* in brain ↓ *dio2* in brain ↓ *dio3* in brain ↓ *tsh* in pituitary ↔ *dio2* in pituitary ↓ *thrb* in pituitary	- - - - 0.05 mg/kg fish	0.05 mg/kg fish 0.05 mg/kg fish 0.05 mg/kg fish 0.05 mg/kg fish - 0.05 mg/kg fish	Morgado et al., 2009

Endpoint	Species	Effect	NOEC	LOEC	Reference
		↑ *dio2* in liver ↑ *ttr* in liver	- - -	0.05 mg/kg fish 0.05 mg/kg fish	
	Sea bream (immature)	↔ *tshβ* in pituitary ↔ *thrb* in pituitary ↔ *dio1* in liver ↔ *dio2* in liver ↔ *ttr* in liver ↔ *dio1* in kidney	1 mg/kg fish 1 mg/kg fish 1 mg/kg fish 1 mg/kg fish 1 mg/kg fish 1 mg/kg fish	- - - - - -	Campinho et al., 2012
Skin pigmentation	Zebrafish (F1 juveniles)	↓ Pigmentation	1 mg/L	10 mg/L	van der Ven et al., 2006
Swim bladder	Zebrafish (embryos)	↓ Inflation of posterior swim bladder ↓ Posterior chamber surface area	- 226.9 mg/L	EC50: 259 mg/L 259 mg/L	Stinckens et al., 2018
	Zebrafish (larvae/juveniles)	↓ Inflation of anterior swim bladder ↓ Anterior chamber surface area ↑ Posterior chamber surface area	- - -	37 mg/L 37 mg/L 37 mg/L	Stinckens et al., 2020
Scales	Zebrafish (juveniles)	↓ Scale thickness	-	1 mg/L	van der Ven et al., 2006
Eye	Zebrafish (embryos)	↓ Pigmentation ↓ RPE cell diameter ↓ Size ↓ Optokinetic response	100 mg/L 50 mg/L 50 mg/L 100 mg/L	250 mg/L 100 mg/L 100 mg/L 250 mg/L	Baumann et al., 2016
	Zebrafish (embryos)	↓ Pigmentation	-	170 mg/L	Macaulay et al., 2015
Behaviour	Zebrafish (embryos)	↑ Preference for light over dark ↓ Swimming activity	100 mg/L 50 mg/L	250 mg/L 100 mg/L	Baumann et al., 2016

Endpoint	Species	Effect	NOEC	LOEC	Reference
	Zebrafish (embryos)	↓ Swimming activity	-	10 mg/L	Walter et al., 2019b

4.2.2.1. Thyroid histopathology

Exposure of zebrafish to PTU for 35 days, beginning 3 dpf, resulted in increased numbers of thyroid follicles, and follicular epithelial cell hyperplasia and hypertrophy (Schmidt and Braunbeck, 2011; Schmidt et al. 2017). Likewise, exposure of adult zebrafish to PTU for 21 days and following exposure of the F1 generation caused hypertrophy of the follicular epithelial cells and depletion of colloid in both adults and F1 juveniles. However, the thyroid pathology appeared less sensitive in adults (van der Ven et al., 2006).

In rainbow trout, follicular epithelial cell height was significantly increased in PTU treated fish compared to controls (Quesada-Garcia et al., 2016).

In contrast to the other studies, follicular epithelial cell height was significantly lower in adult zebrafish exposed to low concentrations of PTU compared to controls (Pinto et al., 2013). Follicle area and follicle number were not significantly affected by the PTU exposure.

4.2.2.2. TH levels

In general, decreased T4 and T3 levels were observed in zebrafish exposed to PTU (van der Ven et al., 2006; Raldúa and Babin, 2009; Liu et al., 2011a; Schmidt and Braunbeck, 2011; Rehberger et al. 2018; Walter et al., 2019c; Stinckens et al., 2020). In tilapia larvae, whole-body T4 levels decreased upon exposure to PTU, while T3 levels were not significantly affected (Okimoto et al., 1993). In adult tilapia, plasma TH levels increased or decreased depending on the exposure dose of PTU (Peter and Peter, 2009). In sea bream (*Sparus auratus*), plasma T4 and T3 levels were not significantly affected by exposure to PTU (Campinho et al., 2012; Morgado et al., 2009).

Exposure of zebrafish embryos to PTU for 0-5 dpf resulted in a significant decrease in the number of follicles that stained for T3, whereas a trend for increase in follicles that stained for T4 was observed after exposure from 2-5 dpf (Rehberger et al., 2018). However, the intrafollicular content of both T3 and T4 decreased significantly in zebrafish eleutheroembryos at 5 dpf after exposure to PTU (Raldúa and Babin, 2009; Thienpont et al., 2011; Rehberger et al., 2018).

4.2.2.3. TH distributor proteins

In sea bream, 0.05 mg PTU/kg fish significantly increased (Morgado et al., 2009) while 1 mg PTU/kg fish significantly decreased (Campinho et al., 2012) plasma levels of TTR.

4.2.2.4. Gene expression

PTU exposure in zebrafish upregulated the expression of *tsh* in adults (Pinto et al., 2013) and larvae (Tonyushkina et al., 2017), whereas *tsh* expression was unaffected in embryos exposed from 2 hpf to 5 dpf (Baumann et al., 2016). In sea bream, *tsh* expression in the brain and pituitary was significantly downregulated by PTU treatment (Morgado et al., 2009) while *tshβ* expression in the pituitary was unaffected in another study (Campinho et al., 2012).

Exposure to PTU significantly increased mRNA expression of *slc5a5* (*nis*) and *tg* in zebrafish (Liu et al., 2013). However, Pinto et al. (2013) found no significant effects on the expression of these genes in zebrafish.

The expression of *tpo* was significantly upregulated in zebrafish 5 dpf upon PTU treatment (Baumann et al., 2016); whereas Pinto et al. (2013) found no significant effect of PTU exposure on *tpo* expression.

In sea bream, *ttr* expression in the liver was either significantly upregulated (Morgado et al., 2009) or unaffected (Campinho et al., 2012) by PTU treatment.

PTU treatment significantly upregulated *dio2* expression, significantly downregulated *dio3* expression but caused no significant effects on *dio1* expression in zebrafish 5 dpf (Baumann et al., 2016). In sea bream, *dio2* and *dio3* expressions were significantly downregulated in the brain, *dio2* expression was significantly upregulated in the liver, while no significant effect was observed on *dio2* expression in the pituitary after PTU exposure (Morgado et al., 2009). Another study with sea bream showed no significant effects of PTU exposure on *dio* expression in liver or kidney (Campinho et al., 2012).

PTU treatment either had no significant effect on *thrb* expression (Campinho et al., 2012) or significantly downregulated (Morgado et al., 2009) *thrb* expression in the pituitary of sea bream. In zebrafish whole-bodies, PTU treatment significantly downregulated the expression of *thra* and *thrb* (Baumann et al. 2016). No significant effects were observed on the expression of *thra* or *thrb* in the eyes, whereas opsin, crystalline and peripherin gene transcripts were mainly downregulated in the eyes of PTU exposed zebrafish 5 dpf (Baumann et al. 2019). In coho salmon, blue opsin transcripts were downregulated, while UV opsin transcripts were upregulated in the in cones of the eye (Gan and Flamarique, 2010).

4.2.2.5. Skin pigmentation

PTU reduced skin pigmentation in 28 dph juvenile F1 zebrafish (van der Ven et al., 2006).

4.2.2.6. Swim bladder

Exposure to PTU resulted in impaired posterior chamber inflation (EC50: 259 mg/L) and significantly decreased surface area at 259 mg/L in 5 dpf zebrafish in a FET test based on TG 236 (OECD, 2013b; Stinckens et al., 2018). In a FELS test based on OECD TG 210 (OECD, 2013a), exposure to 37 and 111 mg/L of PTU resulted in increased percentages of the zebrafish larvae having non-inflated anterior swim bladder chambers at 20 dpf (Stinckens et al., 2020). PTU also affected the surface area of both the anterior chamber and the posterior chamber, with the anterior chamber surface area being significantly smaller compared to control larvae, and posterior chamber surface increasing with decreasing anterior chamber surface.

4.2.2.7. Scales

A concentration-dependent decrease in scale thickness was observed after exposure to PTU in juvenile zebrafish 42 dph (van der Ven et al., 2006). Scale development appeared to be more sensitive to PTU treatment than circulating TH levels and thyroid histology.

4.2.2.8. Eye

PTU significantly reduced the eye size in zebrafish larvae at 5 dpf (Baumann et al., 2016) and caused significantly reduced pigmentation of the eyes of zebrafish at 30 hpf (Macaulay et al., 2015) or 5 dpf (Baumann et al., 2016). PTU treatment further significantly decreased the diameter of the retinal pigment epithelium (RPE) cells in the eye of zebrafish larvae (Baumann et al., 2016). Moreover, PTU exposed zebrafish showed concentration-dependent impairment of their optokinetic response (OKR) at 5 dpf (Baumann et al. 2016).

4.2.2.9. Behaviour

At 5 dpf, PTU exposed zebrafish showed significant suppression of their phototactic swimming activity, while their preference for light over dark increased significantly (Baumann et al. 2016). Additionally, PTU exposure significantly decreased the swimming activity during dark periods in visual-motor response tests with zebrafish at 4 and 5 dpf (Walter et al., 2019b).

4.2.2.10. General conclusions on PTU

In general, changes in thyroid histopathology were more sensitive indicators of THS disruptive effects of PTU than were TH levels, gene expression and behavioural endpoints.

In early life stage tests, exposure of zebrafish to PTU caused increased follicular epithelial cell height. However, in a single study using low exposure concentrations of PTU follicular epithelial cell height was significantly lower in PTU exposed adult zebrafish compared to controls. Follicle area and follicle number were not consistently affected by PTU exposure.

In general, T3 levels, T4 levels, swim bladder inflation, eye pigmentation, skin pigmentation, scale thickness, optokinetic response and swimming activity were reduced by PTU.

4.2.3. Iopanoic acid: DIO inhibition

The iodinated radiographic contrast dye, iopanoic acid, is a model competitive inhibitor of all three DIO types (St. Germain, 1988; Sharifi and St. Germain, 1992; Braga and Cooper, 2001; Renko et al., 2015).

Table 4 summarises the THS related effects of iopanoic acid observed in fish and is followed by a more detailed description of effects of iopanoic acid on potential THS related endpoints.

1 **Table 4. Summarised THS related effects of iopanoic acid in fish**

Endpoint	Species	Effect	NOEC	LOEC	Reference
Thyroid histopathology	Fathead minnow (larvae)	↔ Thyrocyte hyperplasia ↔ Thyrocyte hypertrophy ↔ Colloid quality	6 mg/L 6 mg/L 6 mg/L	- - -	Cavallin et al., 2017
TH levels	Zebrafish (larvae/juveniles)	↔ Whole-body T4 (14, 21 and 32 dpf) ↑ Whole-body T3 (21 and 32 dpf) ↔ Whole-body T3 (14 dpf)	0.35 mg/L - 0.35 mg/L	- 0.35 mg/L -	Stinckens et al., 2020
	Fathead minnow (embryos, larvae)	↑ Whole-body T4 (4, 10, 14, 18 and 21 dpf) ↔ Whole-body T4 (6 dpf) ↑ Whole-body T3 (4 and 6 dpf) ↓ Whole-body T3 (10, 14, 18 and 21 dpf)	- 0.6 mg/L 0.6 mg/L 0.6 mg/L	0.6 mg/L - 1.9 mg/L 1.9 mg/L	Cavallin et al., 2017
Gene expression	Fathead minnow (embryos, larvae)	↔ *dio1* ↑ *dio2* ↑ *dio3* (10 and 18 dpf) ↓ *tpo* (6, 10, 14 and 18 dpf) ↔ *tpo* (21 dpf)	0.6 mg/L 0.6 mg/L 0.6 mg/L - 0.6 mg/L	- 1.9 mg/L 1.9 mg/L 0.6 mg/L -	Cavallin et al., 2017
Swim bladder	Zebrafish (embryos)	↓ Inflation of posterior swim bladder	-	EC50: 2.79 mg/L	Stinckens et al., 2018
	Zebrafish (larvae/juveniles)	↓ Inflation of anterior swim bladder ↓ Anterior chamber surface area ↑ Posterior chamber surface area	- - -	0.35 mg/L 0.35 mg/L 0.35 mg/L	Stinckens et al., 2020
	Fathead minnow (embryos, larvae)	↓ Inflation of posterior swim bladder (6 and 14 dpf) ↔ Inflation of posterior swim bladder (18 dpf) ↓ Length of posterior swim bladder (6 dpf) ↑ Length of posterior swim bladder (14 and 18 dpf) ↓ Inflation of anterior swim bladder (14 dpf)	1.9 mg/L 0.6 mg/L 1.9 mg/L - - 0.6 mg/L -	6 mg/L - 6 mg/L 0.6 mg/L 0.6 mg/L - 0.6 mg/L	Cavallin et al., 2017

		↔ Inflation of anterior swim bladder (18 dpf) ↓ Length of anterior swim bladder (14, 18 and 21 dpf)			
Eye	Zebrafish (embryos)	↓ Pigmentation	-	3 mg/L	Macaulay et al., 2015

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4.2.3.1. Thyroid histopathology

Histological assessment revealed no thyroid follicle pathology related to iopanoic acid exposure in fathead minnows (Cavallin et al., 2017). No effects like epithelial cell hyperplasia, hypertrophy, or changes in gland size or colloid quality were observed in the thyroid follicles of the larvae.

4.2.3.2. TH levels

Exposure of zebrafish embryos to iopanoic acid from 1 hpf until 32 dpf in a FELS test based on OECD TG 210 (OECD, 2013a) resulted in decreased whole-body T3 (at 21 and 32 dpf, but not at 14 dpf), but unaffected whole-body T4 levels (Stinckens et al., 2020). Whole-body T3 concentrations significantly decreased and whole-body T4 concentrations increased in fathead minnow larvae upon exposure to iopanoic acid from 6-21 dpf (Cavallin et al., 2017). In a fathead minnow embryo study, T4 was significantly increased at 4 dpf; however, by 6 dpf, there were no significant differences in T4 concentrations. Whole-body T3 concentrations were significantly increased at both 4 and 6 dpf (Cavallin et al., 2017).

4.2.3.3. Gene expression

Dio2 and *dio3* expression showed an increase in fathead minnows exposed to iopanoic acid up to 21 dpf; with expression of *dio2* as the most impacted. *Dio1* mRNA expression was unaffected by iopanoic acid exposure (Cavallin et al., 2017).

Expression of *tpo* was significantly downregulated by exposure to iopanoic acid in an embryo study at 6 dpf and in a larval study at 10, 14 and 18 dpf. By 21 dpf, there were no longer any statistically significant differences between the treatments and controls (Cavallin et al., 2017).

4.2.3.4. Swim bladder

Exposure to iopanoic acid resulted in impaired posterior chamber inflation in zebrafish at 5 dpf in a FET test based on TG 236 (OECD, 2013b) with an EC50 of 2.79 mg/L (Stinckens et al., 2018). In a 32-day FELS test based on OECD TG 210 (OECD, 2013a), exposure to 0.35, 1 and 2 mg/L iopanoic acid resulted in 35, 43 and 61%, respectively, of the zebrafish larvae having non-inflated anterior swim bladder chambers at 21 dpf; and at the end of the experiment, around 25% of the iopanoic acid exposed larvae still had impaired anterior chamber inflation (Stinckens et al., 2020). Iopanoic acid also affected the surface area of both the anterior chamber and the posterior chamber. In all cases where the anterior chamber was inflated, its surface area was significantly smaller compared to control larvae, and the posterior chamber surface increased with decreasing anterior chamber surface. Furthermore, incidence and length of inflated posterior swim bladders were significantly reduced in fathead minnows 6 dpf upon iopanoic acid treatment (Cavallin et al., 2017).

4.2.3.5. Eye

Iopanoic acid caused significantly reduced pigmentation of the eyes of zebrafish 30 hpf (Macaulay et al., 2015).

4.2.3.6. General conclusions on iopanoic acid

Histological assessment revealed no thyroid follicle pathology related to iopanoic acid exposure in the single study examining this endpoint. Still, reduced eye pigmentation was observed in the single study examining this endpoint. Furthermore, decreased swim bladder inflation was observed in the three studies examining this endpoint.

4.2.4. Tetrabromobisphenol A (TBBPA): interaction with TH binding

TBBPA is a brominated flame retardant, which has THS disrupting properties by interacting with TH binding to TRs (Kitamura et al., 2002) and the TH distributor protein TTR (Meerts et al., 2000; Hamers et al., 2006; Iakovleva et al., 2016).

Table 5 summarises the THS related effects of TBBPA observed in fish and is followed by a more detailed description of effects of TBBPA on potential THS related endpoints.

Table 5. Summarised THS related effects of TBBPA in fish

Endpoint	Species	Effect	NOEC	LOEC	Reference
Thyroid histopathology	Zebrafish (juveniles, adults)	No signs of altered thyroid activity ↔ Estimated follicle number	816 μg/L 816 μg/L	- -	Kuiper et al., 2007b
	European flounder (juveniles)	No signs of altered thyroid activity	435 μg/L	-	Kuiper et al., 2007a
TH levels	Zebrafish (embryos)	↑ Whole-body T4 (1 and 3 dpf) ↓ Whole-body T4 (5 dpf) ↓ Whole-body T4 (6 dpf) ↑ Whole-body T3 (1 dpf) ↔ Whole-body T3 (3 and 5 dpf) ↓ Whole-body T3 (6 dpf)	- 100 μg/L - - 500 μg/L 100 μg/L	100 μg/L 500 μg/L 100 μg/L 100 μg/L - 500 μg/L	Pang et al., 2020
	Zebrafish (6 dpf)	↑ Whole-body T4 ↓ Whole-body T3	100 μg/L 100 μg/L	200 μg/L 200 μg/L	Zhu et al., 2018
	European flounder (juveniles)	↑ Plasma T4 ↔ Plasma T3	1.4 μg/L 193 μg/L	11 μg/L -	Kuiper et al., 2007a
Gene expression	Zebrafish (embryos)	↔ *tsh* ↓ *tpo* ↔ *dio1* ↔ *dio2* ↔ *dio3* ↑ *thra* ↔ *thrb*	100 μg/L - 100 μg/L 100 μg/L 100 μg/L - 100 μg/L	- 100 μg/L - - - 100 μg/L -	Baumann et al., 2016
	Zebrafish (embryos)	↑↔ *ttr* (depends on age) ↑↔ *dio1* (depends on age and exposure conc.)	98 μg/L 22 μg/L -	250 μg/L 98 μg/L 22 μg/L	Parsons et al., 2019

Endpoint	Species	Effect	NOEC	LOEC	Reference
		↓↔ *dio3b* (depends on age) ↔ *thraa* ↔ *thrb*	22 μg/L 22 μg/L	- -	
	Zebrafish (embryos)	↓ *thra*	-	2.67 μg/L	Liu et al., 2018a
	Zebrafish (embryos)	↔ *thraa* ↑ *thrab* ↔ *thrb*	1000 μg/L - 1000 μg/L	- 1000 μg/L -	Pang et al., 2020
	Zebrafish (embryos)	↔ *thra* in eye ↔ *thrb* in eye ↑ Opsin transcripts in eye	200 μg/L 200 μg/L -	- - 200 μg/L	Baumann et al., 2019
	Zebrafish (embryos and larvae)	↑ *tshβ* (larvae) ↓ *tshβ* (embryos) ↔ *tg* ↔ *slc5a5* (*nis*) ↔ *tpo* ↑ *ttr* (larvae) ↔ *ttr* (embryos) ↑ *thra* ↔ *thrb*	- - 530 μg/L 530 μg/L 530 μg/L 110 μg/L 110 μg/L 110 μg/L 530 μg/L	110 μg/L 530 μg/L - - - - 3950 μg/L 3950 μg/L -	Chan and Chan, 2012
	Zebrafish (larvae)	↑ *tshβ* ↑ *tg* ↓ *ttr* ↓ *thrb*	50 μg/L 50 μg/L 100 μg/L 100 μg/L	100 μg/L 100 μg/L 200 μg/L 200 μg/L	Zhu et al., 2018
	Zebrafish (larvae)	↔ *tshβ* ↔ *tpo* ↔ *thra*	13 μg/L 13 μg/L 13 μg/L	- - -	Godfrey et al., 2017

Endpoint	Species	Effect	NOEC	LOEC	Reference
		↔ *thrb*	13 μg/L	-	
Swim bladder	Zebrafish (embryos)	↔ Posterior swim bladder inflation	35 x 10^3 μg/L	-	Noyes et al., 2015
Craniofacial development	Zebrafish (embryos)	↑ Jaw malformation ↑ Snout malformation	35 μg/L 35 x 10^2 μg/L	348 μg/L 35 x 10^3 μg/L	Noyes et al., 2015
Fins	Zebrafish (embryos)	↑ Caudal fin malformation ↔ Pectoral fins malformation	348 μg/L 35 x 10^3 μg/L	35 x 10^2 μg/L -	Noyes et al., 2015
Neuro-development	Zebrafish (embryos)	↓ Motor neuron development	-	27 x 10^2 μg/L	Chen et al., 2016a
Eye	Zebrafish (embryos)	↓ Pigmentation ↔ RPE diameter ↓ Size ↓ Optokinetic response	- 200 μg/L - 200 μg/L	200 μg/L - 200 μg/L 300 μg/L	Baumann et al., 2016
Behaviour	Zebrafish (embryos)	↑↓ Swimming activity (depends on exposure conc.) ↔ Preference for light over dark	200 μg/L 100 μg/L	300 μg/L -	Baumann et al., 2016
	Zebrafish (embryos)	↓ Average activity ↓ Average speed of movement	- -	27 x 10^2 μg/L 27 x 10^2 μg/L	Chen et al., 2016a
	Zebrafish (embryos)	↑ Average swimming speed ↑ Aggression (males)	- 2.7 μg/L	2.7 μg/L 27 μg/L	Chen et al., 2016b
	Zebrafish (embryos)	↔ Locomotor activity	16 x 10^2 μg/L	-	Alzualde et al., 2018
	Zebrafish (larvae)	↓ Locomotor activity ↓ Average swimming speed	100 μg/L 50 μg/L	200 μg/L 100 μg/L	Zhu et al., 2018

4.2.4.1. Thyroid histopathology

No indications of altered thyroid activity were revealed by histological examinations in European flounder (Kuiper et al., 2007a) or zebrafish (Kuiper et al., 2007b) after exposure to TBBPA. In both juveniles and adults, thyroid tissue appeared similar in all groups, displaying variably sized colloid containing follicles lined by cubic cells, interpreted as moderately active (Kuiper et al., 2007b). There was no difference in the estimated number of follicles throughout the groups.

4.2.4.2. TH levels

TBBPA affected TH levels in a developmental stage-dependent manner in zebrafish embryos (Pang et al., 2020). At 1 dpf, before the onset of TH synthesis when low TH levels are present (due to maternal transfer), exposure to 100 or 500 µg/L TBBPA induced a significant increase in T4 and T3 levels. This effect was still observed for T4 at 3 dpf, while T3 levels were not significantly affected by TBBPA exposure. At 5 dpf, after the onset of TH synthesis when TH levels are high, exposure to 500 µg/L TBBPA significantly decreased T4 levels, while T3 levels were not significantly affected. At 6 dpf both T4 and T3 levels were significantly decreased by exposure to 500 µg/L TBBPA, while only T4 levels were significantly decreased by exposure to 100 µg/L TBBPA.

However, TBBPA exposure significantly increased T4 levels and significantly decreased T3 levels in zebrafish exposed to 200 or 400 µg/L TBBPA from 2 hpf to 6 dpf (Zhu et al., 2018). Furthermore, T4 increased with internal concentrations of TBBPA in European flounder after exposure for 105 days, while T3 levels were not affected (Kuiper et al., 2007a).

4.2.4.3. Gene expression

Expression of *tshβ* was significantly upregulated in zebrafish exposed to 100-400 µg/L TBBPA from 2 hpf to 6 dpf (Zhu et al., 2018). Furthermore, 530-3950 µg/L TBBPA significantly induced the transcription of *tshβ* in zebrafish exposed from 4-8 dpf (Chan & Chan, 2012). However, in zebrafish exposed to 820 µg/L TBBPA from 0-4 dpf, *tshβ* expression was significantly downregulated (Chan and Chan, 2012), whereas no significant effects on *tsh* expression levels were seen in zebrafish exposed to 100-400 µg/L TBBPA from 0-5 dpf (Baumann et al., 2016) or to 13 µg/L TBBPA from 0-6 dpf (Godfrey et al., 2017).

Expression of *tg* was significantly upregulated in zebrafish exposed to 100-400 µg/L TBBPA from 2 hpf to 6 dpf (Zhu et al., 2018). However, no significant effect was observed on *tg* expression in embryos and larvae after exposure to 110-820 µg/L TBBPA from 0-4 dpf or 530-3950 µg/L TBBPA from 4-8 dpf, respectively (Chan and Chan, 2012).

No significant effects were observed on *slc5a5* (*nis*) or *tpo* expression in embryos and larvae after exposure to 110-820 µg/L TBBPA from 0-4 dpf or to 530-3950 µg/L TBBPA from 4-8 dpf, respectively (Chan and Chan, 2012). Likewise, no significant effects were observed on *tpo* expression after exposure to 13 µg/L TBBPA from 0-6 dpf (Godfrey et al., 2017). However, *tpo* expression was significantly downregulated after exposure to 100-400 µg/L TBBPA from 0-5 dpf (Baumann et al., 2016).

Exposure of zebrafish to 200 or 400 µg/L TBBPA from 2 hpf to 6 dpf significantly decreased the transcription of *ttr* (Zhu et al., 2018). Conversely, exposure of zebrafish to 250 µg/L TBBPA resulted in significantly higher levels of *ttr* at 2 dpf; however, no effects were observed at 4 or 5 dpf (Parsons et al., 2019). Furthermore, no significant effects were observed on *ttr* expression in zebrafish embryos after exposure from 0-4 dpf to 111-820 µg/L TBBPA, whereas significantly induced transcription of *ttr* in zebrafish larvae was observed after exposure from 4-8 dpf to 3950 µg/L TBBPA (Chan and Chan, 2012).

Exposure of zebrafish to 100 µg/L TBBPA resulted in significantly higher levels of *dio1* transcripts at 4 dpf; however, no significant effects were observed at 20 or 250 µg/L or at 2 dpf or 5 dpf (Parsons et al., 2019).

Significantly lower levels of *dio3b* expression were observed at 4 dpf after exposure to 20-250 µg/L TBBPA compared to controls; however, no significant effects were observed at 2 or 5 dpf (Parsons et al., 2019). Furthermore, no significant effects were observed on *dio1*, *dio2* and *dio3* expression in zebrafish after exposure to 100-400 µg/L TBBPA from 0-5 dpf (Baumann et al., 2016).

In zebrafish, TBBPA exposure significantly downregulated the expression of *thra* after exposure to 160-650 µg/L from 2-122 hpf (Liu et al., 2018a) and of *thrb* after exposure to 200 or 400 µg/L TBBPA from 2 hpf to 6 dpf (Zhu et al., 2018). TBBPA significantly induced the expression of *thra* but not *thrb* in zebrafish exposed to 820 µg/L from 0-4 dpf (Chan and Chan, 2012), to 100-200 µg/L from 0-5 dpf (Baumann et al., 2016), or to 3950 µg/L from 4-8 dpf (Chan and Chan, 2012). 1000 µg/L TBBPA significantly upregulated the transcriptional level of *thrab* by 58% at 26 hpf, while transcription levels of *thraa* and *thrb* were not significantly affected (Pang et al., 2020).

TBBPA did not cause significant changes in the expression of neither *thra* nor *thrb* in whole-body homogenates of zebrafish exposed to 13 µg/L from 0-6 dpf (Godfrey et al., 2017) or to 20-250 µg/L from fertilisation to 2, 4 or 5 dpf (Parsons et al., 2019). However, whole-mount *in situ* hybridisation analyses showed tissue-specific alterations in both *thraa* and *thrb* expression in zebrafish embryo-larvae following exposure to TBBPA. Expression of *thraa* was higher in the brain and branchial arches of exposed 2 dpf embryos compared to controls, while *thrb* expression was enhanced in the liver, brain, jaw cartilage, otic vesicle and swim bladder of 96 hpf larvae (Parsons et al., 2019). No significant effects were observed on the expression of *thra* or *thrb* in the eyes, whereas opsin gene transcripts were mainly upregulated in the eyes of TBBPA exposed zebrafish 5 dpf (Baumann et al., 2019).

4.2.4.4. Neurodevelopment

TBBPA significantly inhibited motor neuron development in zebrafish exposed from 8-48 hpf (Chen et al., 2016a).

4.2.4.5. Eye

Zebrafish eye development was affected by exposure to TBBPA from 0-5 dpf (Baumann et al., 2016). TBBPA treatment resulted in pathological alterations of the eyes including reduced size and pigmentation. Visual impairments in zebrafish after TBBPA exposure were further demonstrated by significantly and concentration-dependent decreased OKR 5 dpf.

4.2.4.6. Behaviour

Phototactic swimming activity was significantly affected in TBBPA exposed zebrafish at 5 dpf (Baumann et al., 2016). The effect of TBBPA on swimming activity was stimulating at 300 µg/L and strongly inhibiting at 400 µg/L. No significant effects on the preference of the zebrafish for light or dark backgrounds were observed.

In zebrafish exposed to 100-400 µg/L TBBPA from 2 hpf to 6 dpf, decreases in visual-motor response and average swimming speed were observed (Zhu et al., 2018). Likewise, behavioural assays conducted at 5 dpf after exposure to 5 µM TBBPA from 8-48 hpf showed that the exposed zebrafish had significantly lower average activity (Chen et al., 2016a). However, no significant effects were observed on locomotor activity in zebrafish after exposure to 0.1-3 µM TBBPA from 3-5 dpf (Alzualde et al., 2018).

Higher average speed was observed in a photomotor response assay with adult zebrafish at 9 months post fertilisation after chronic exposure to 5 nM TBBPA from 1-120 dpf (Chen et al., 2016b). Furthermore, exposed males showed significantly heightened aggression in a mirror attack test.

4.2.4.7. General conclusions on TBBPA

TBBPA exposure increased T4 levels, inhibited motor neuron development, eye development and visual performance, and caused behavioural effects. Conversely, no significant effects were observed on thyroid histopathology.

4.3. Assessment of THS related endpoints

The case studies of model compounds acting through different MIEs reveal several endpoint as potential candidates for inclusion in fish tests, including thyroid histopathology, TH levels, gene expression, pigmentation, behaviour, and development of swim bladders, fins, scales and eyes. Table 6 summarises which endpoints were affected by the compounds.

Table 6. Summary of THS related endpoints affected by model compounds in fish

	Perchlorate	PTU	Iopanoic acid	TBBPA
Thyroid histopathology	✓	✓		
TH levels	✓	✓	✓	✓
Gene expression	✓	✓	✓	✓
Skin pigmentation	✓	✓		
Swim bladder	✓	✓	✓	
Scales	✓	✓		
Eye		✓	✓	✓
Behaviour		✓		✓

The various endpoints are further reviewed in sections 4.3.1- 4.3.12.

4.3.1. Thyroid histopathology

In contrast to higher vertebrates like mammals and amphibians having compact, encapsulated thyroid glands, the fish thyroid tissue shows a more variable arrangement (Blanton and Specker, 2007). Thyroid tissue in most teleosts, including the zebrafish, is scattered diffusely as vascularised follicles in the basibranchial region ventral to the pharyngeal midline predominantly along the ventral aorta. In zebrafish, thyroid follicles begin to form around 55 hpf (Alt et al., 2006). The first follicle formed remains the most anterior follicle and new follicles are added caudally (Alt et al., 2006). The thyroid follicles are lined with a single layer of epithelial cells, and the lumens of the follicles are filled with extracellular proteinaceous colloid consisting mainly of Tg. In the unstimulated thyroid tissue, the follicular epithelial cells are squamous or cuboidal in appearance, and the colloid in the lumen is usually homogeneous (Leatherland, 1994).

Thyroid tissue is readily examined histologically, and follicle morphology presents several indicators of thyroid activity (Eales and Brown, 1993).

Histological changes in the thyroid are often observed as compensatory feedback responses to disruptions of T4 synthesis and secretion which might otherwise decrease plasma T4 levels (Brown et al., 2004). Fish (and other vertebrates) can compensate for disturbances in the THS, e.g. by proliferation leading to hyperplasia of the thyroid tissue to increase the TH concentrations in the body (DeVito et al., 1999; Carr and Patiño, 2011).

Sustained stimulation by TSH due to feedback mechanisms may also cause structural changes in the thyroid epithelial cells that are related to TH synthesis and release. TSH can cause the commonly squamous follicular epithelial cells to hypertrophy and assume a columnar shape, leading to observable thickening of the follicular epithelium (Fig. 5). The enlargement of the follicular epithelial cells is to a great extent owing to an increase in rough endoplasmic reticulum (ER) and Golgi apparatus for Tg synthesis (Norris and Carr, 2013).

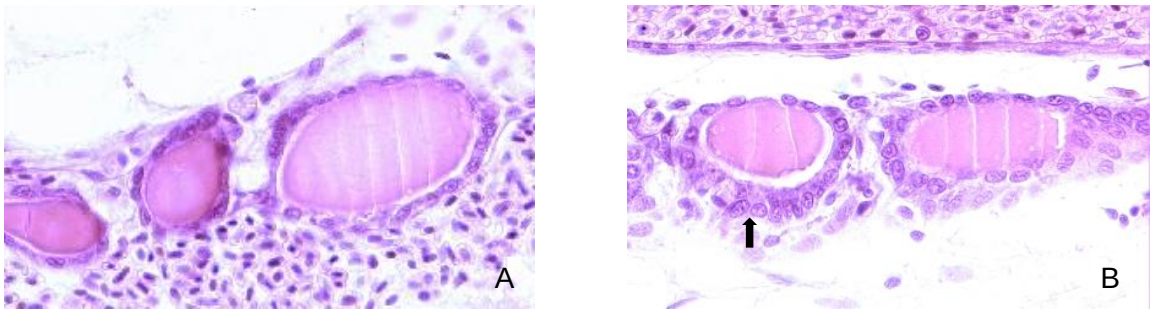

Fig. 5. Histological sections of thyroid follicles from (A) control zebrafish and (B) zebrafish treated with a potential THS disrupting chemical. The treated fish shows follicular epithelial cell hypertrophy (arrow) (Kinnberg et al., as yet unpublished).

In zebrafish, histological endpoints in the thyroid tissue following exposure to model THS disrupting chemicals include follicular epithelial cell hypertrophy (perchlorate and PTU) and hyperplasia (perchlorate and PTU), colloid depletion (perchlorate and PTU), increased follicle number (perchlorate and PTU) and altered follicle size (perchlorate).

4.3.1.1. NIS inhibition

As described in section 4.2.1.1, the model NIS inhibitor, perchlorate, generally increased follicular epithelial cell height, thyroid follicular epithelium hyperplasia and follicle number in zebrafish juveniles and adults, and in fathead minnows, threespine sticklebacks, eastern mosquitofish and rainbow trout. Furthermore, various degrees of depletion of follicular colloid content were observed in fish exposed to perchlorate. The sensitivity of the various histopathological endpoints differed between the studies.

4.3.1.2. TPO inhibition

As described in section 4.2.2.1, the model TPO inhibitor, PTU, generally increased follicular epithelial cell hyperplasia and hypertrophy, and caused depletion of colloid in the follicles. Likewise, the related TPO inhibitor, phenylthiourea, induced thyroid follicular epithelial cell hyperplasia and hypertrophy in zebrafish exposed from 52 hpf to 100 hpf, 6 dpf or 11 dpf (Trubiroha et al., 2018; Giusti et al., 2020). Similar results were observed after exposure to another model TPO inhibitor, 2-mercaptobenzothiazole. Histopathological examination identified a significantly greater prevalence of both thyroid follicular epithelial cell hypertrophy and hyperplasia, and a decrease in lumen area and depletion of colloid in fathead minnows exposed to 2-mercaptobenzothiazole for 14 or 21 days (with more pronounced effects 14 dpf) (Nelson et al., 2016).

Follicle number was not consistently affected by TPO inhibition. PTU exposure had no effect on the number of thyroid follicles in adult zebrafish (Pinto et al., 2013), whereas increased follicle number was observed in an early life stage test (Schmidt and Braunbeck, 2011). Conversely, a reduction in the number of thyroid follicles was observed in zebrafish embryos after exposure to yet another TPO inhibitor, methimazole, from 0-120 or 48-120 hpf (Jomaa et al., 2014).

In general, the thyroid pathology appeared less sensitive in adults than in juveniles.

4.3.1.3. DIO inhibition

As described in section 4.2.3.1, histological assessment revealed no thyroid follicle pathology related to iopanoic acid exposure in fathead minnows (Cavallin et al., 2017). No effects like epithelial cell hyperplasia, hypertrophy, or changes in gland size or colloid quality were observed in the thyroid follicles of the larvae.

4.3.1.4. Interaction with TH binding

As described in section 4.2.4.1, no indications of altered thyroid activity were revealed by histological examinations in zebrafish (Kuiper et al., 2007b) or European flounder (Kuiper et al., 2007a) after exposure to TBBPA.

4.3.1.5. Undetermined or mixed MIEs

One- or three-months exposure to β-hexachlorocyclohexane of medaka eggs from fertilisation or of young fish from one-month post hatching showed hypertrophy of thyroid follicular epithelial cells and diminished colloid content in the follicles (Wester et al., 1986).

Hypertrophy and hyperplasia of the follicular epithelial cells concomitant with a reduction in the cross-sectional area of the colloid were observed in zebrafish exposed to perfluorononanoate (PFNA) (Liu et al., 2011c).

4.3.1.6. General discussion and conclusions on thyroid histopathology as an endpoint for THS related disruption

Histopathology of the thyroid gland is considered as an endpoint very specific to THS disruptive MOA and is used as such in TG 231 with *Xenopus laevis* (OECD, 2009; Coady et al., 2010; 2014).

Thyroid histopathology may be valuable as an endpoint specific to thyroid disruption via NIS- and TPO inhibition. Particularly, increased follicular epithelial cell height, thyroid follicular epithelium hyperplasia, and depletion of follicular colloid content seem to be useful parameters in both juvenile and adult zebrafish, with varied relative sensitivity between studies.

No responses or changes in thyroid histopathology were observed in the single study examining effects of DIO inhibition. However, only one chemical (iopanoic acid) was examined, and it is not known whether higher test concentrations or other DIO inhibiting chemicals would induce effects. However, in the case of DIO inhibition, an excess of T4 is available, therefore, the fish do not need to compensate via increased follicular epithelial cell height or thyroid follicular epithelium hyperplasia; and colloid depletion does not occur. Likewise, interaction with TH binding will plausibly not lead to increased T4 production reflected in the thyroid histology.

Considering the high natural variability between individual zebrafish in follicular dimensions and numbers (Rehberger et al., 2018) and their possible dependence on the sectioning plane, the follicle size and number may not be as reliable parameters as follicle cell dimensions for assessing TH related disruption.

Care should be taken to minimise potential sources of variability for the histological assessments (Wolf et al., 2021), e.g. by means of computer-assisted morphometry procedures (Pickford, 2010).

4.3.2. TH levels

Many of the MIEs for THS related disruption (see section 4.1) will lead to changes in TH levels, with follow-on effects on TSH levels.

4.3.2.1. Plasma and whole-body levels

Plasma T4 level is probably the most used index of thyroidal status in vertebrates.

Only the free portion of THs, i.e. THs that are not bound to distributer proteins, are responsible for biological action. Thus, free TH levels provide a biologically more meaningful measure of thyroid function than total TH levels. However, since less than 1% of the THs have been estimated to appear in the free form in the blood of both fish and mammals (Eales and Shostak, 1985; Rabah et al., 2019), large sample volumes are required for determinations of free THs.

Another challenge concerns the methodological limitations in the measurement of free TH levels. Separation of free and protein bound THs can be achieved by using equilibrium dialysis or ultrafiltration. However, the separation methods imply technical and theoretical efforts and uncertainties (Holm et al., 2004) and many laboratories use direct measurement of free THs without prior separation from the protein bound THs (Welsh and Soldin, 2016). Free THs can be quantified by analytical chemistry methods employing chromatography separation and mass spectrometry (e.g. liquid chromatography tandem mass spectrometry (LC-MS/MS)) or by biochemical analysis methods employing immunodetection (e.g. enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA)) (van Deventer and Soldin, 2013; Welsh and Soldin, 2016; Martin et al., 2020). Chromatography may be the most sensitive method to measure THs and has the advantage that several hormones can be measured at the same time (Martin et al., 2020). However, measurement by LC-MS/MS has higher costs than measurement by immunoassays performed on automated platforms (Welsh and Soldin, 2016). Furthermore, more specialised equipment and expertise are required for LC-MS/MS analysis, which are not available in all laboratories (Martin et al., 2020). Thus, free THs are often measured by immunoassays. However, direct free TH measurement by immunoassays may be subject to interferences from TH distributer proteins (Welsh and Soldin, 2016) and thus may not be fully reliable.

Presumably because of the mentioned challenges regarding sample volume, methodological efforts and reliability of the results, total TH levels are usually measured instead of free TH levels in studies of THS disruption in fish.

The fish blood plasma volumes required to measure THs by RIA, ELISA and chemical methods have been estimated to 10, 100 and 50 µL, respectively (Martin et al., 2020); while typical plasma volumes available from individual mature zebrafish and fathead minnows amounts 5-15 and 5-60 µL, respectively (Wheeler et al., 2021).

Due to the small size of zebrafish, TH levels are usually quantified in whole-body homogenates. However, whole-body homogenates do not reflect TH depletion in specific tissues caused, for example, by interferences with membrane transporters. In addition, the analyses still require the pooling of several larvae to obtain enough homogenate for measurement of THs. For example, Stinckens et al. (2016) pooled 70-80 zebrafish larvae 5 dpf or 3-5 larvae 32 dpf for LC-MS/MS analysis of THs; Wang et al. (2014) pooled 200 zebrafish larvae 6 dpf for ELISA analysis of THs; and Mukhi et al. (2005) pooled 5 adult zebrafish for RIA analysis of THs.

4.3.2.2. NIS inhibition

As described in section 4.2.1.2, perchlorate exerted contrasting effects on the TH levels of fish.

4.3.2.3. TPO inhibition

As described in section 4.2.2.2, PTU generally decreased T4 and T3 levels in zebrafish. Likewise, 2-mercaptobenzothiazole exposure decreased T4 in zebrafish 5 dpf and 32 dpf (Stinckens et al., 2016) and in fathead minnows 6 dpf (Nelson et al., 2016). In contrast, there was no observed effect of 2-mercaptobenzothiazole on T3 in zebrafish 5 dpf or 32 dpf (Stinckens et al., 2016) or in fathead minnows 6

or 21 dpf, although T3 levels were significantly reduced at 14 dpf (Nelson et al., 2016). Similarly, exposure to methimazole for 10 days depressed plasma levels of T4 in both sexes of adult fathead minnows; while no effects were observed on T3 (Lema et al., 2009).

4.3.2.4. DIO inhibition

As described in section 4.2.3.2, no consistent trends were observed on TH levels after exposure to iopanoic acid.

4.3.2.5. Interaction with TH binding

As described in section 4.2.4.2, TBBPA exposure increased T4 levels, whereas decreased or unaffected T3 levels were observed. Conversely, T4 levels were significantly decreased, while T3 concentrations were significantly increased after exposure to TDCPP in zebrafish 6 dpf (Wang et al., 2013). In adult zebrafish, TDCPP exposure for 14 days decreased T4 and T3 levels in males, and increased T4 and T3 levels in females (Liu et al., 2019b).

4.3.2.6. Undetermined or mixed MIEs

In general, brominated flame retardants decreased T4 levels. Reduced T4 levels were observed in zebrafish 14 dpf after exposure to DE-71 (Yu et al., 2010). Likewise, exposure to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) (Lema et al., 2008) for 21 days depressed plasma levels of T4 in both sexes of adult fathead minnows, while no effects were observed on T3. Furthermore, T4 levels were significantly decreased, while T3 concentrations were significantly increased in zebrafish 14 dpf after exposure to 2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether (BDE-209), with concentration-dependent increases in the T3/T4 ratio (Chen et al., 2012b). However, T4 levels were increased in zebrafish 6 dpf upon exposure to TBP (Fu et al., 2020) and 7 dpf upon exposure to BDE-209 (Wang et al., 2014), while no significant difference was observed for T3.

Whole-body T4 and T3 levels at 5 dpf decreased in a dose-dependent manner after PFOA and PFECA exposure in zebrafish (Wang et al., 2020). Likewise, whole-body T3 and T4 levels decreased in zebrafish upon exposure to PFDoA until 4 dpf (Zhang et al., 2018a). Conversely, PFOS exposure significantly increased T3 levels in zebrafish at 15 dpf, whereas T4 content remained unchanged (Shi et al., 2009). Likewise, the related chemical, PFNA, significantly elevated plasma T3 levels in both F0 and F1 adult zebrafish (Liu et al., 2011c). Furthermore, the PFOS alternative, F-53B, increased T4 levels in both sexes of adult zebrafish, whereas T3 levels showed a decreasing trend after F-53B exposure in both sexes (Shi et al., 2019). For F1 embryos, parental exposure resulted in an increase in T4 levels, whereas T3 levels decreased. Another study reported increased T4 levels in zebrafish exposed to F-53B for 0-5 dpf, whereas no significant effects on T3 levels were found (Deng et al., 2018).

T4 levels were significantly decreased, while T3 concentrations were significantly increased after exposure to MEHP in zebrafish 7 dpf (Zhai et al., 2014). However, exposure to DEHP (Jia et al., 2016) increased both T4 and T3 concentrations in zebrafish larvae 7 dpf, with no significant change in T3/T4 ratio.

Bisphenol F (BPF) exposure from 0-5 dpf significantly increased T4 levels in zebrafish, while T3 levels were not affected (Lee et al., 2019). Conversely, significant decreased T4 contents and significant increased T3 and TSH contents, as well as T3/T4 ratios, were observed in BPF treated zebrafish 6 dpf (Huang et al., 2016). Bisphenol A (BPA) and bisphenol S (BPS) significantly increased T3 levels, while T4 levels were not affected. However, T4 and T3 levels were both significantly decreased in zebrafish exposed to BPS until 7 dpf (Zhang et al., 2017), while TSH concentrations were significantly increased by BPS exposure. Furthermore, bisphenol AP significantly decreased T4 levels but did not significantly affect T3 and TSHβ levels in 5 dpf zebrafish (Lee et al., 2020).

Zebrafish exposed to *o,p'*-DDT from 0-7 dpf showed significantly increased whole-body T4 and T3 levels, whereas the *p,p'*-DDE exposure group showed significantly decreased whole-body total T4 and T3 levels (Wu et al., 2019). The T3/T4 ratio was significantly reduced in both exposure groups.

T4 levels were significantly decreased, while T3 concentrations were significantly increased after exposure to hexaconazole and tebuconazole in zebrafish 5 dpf (Yu et al., 2013). Difenoconazole exposure significantly decreased T4 levels in zebrafish exposed from 0-5 dpf, while T3 concentrations were not affected (Liang et al., 2015). Triadimefon exposure increased T4 and reduced T3 levels in zebrafish 5 dpf (Liu et al., 2011b). Similarly, T4 levels were increased in zebrafish embryos 5 dpf upon exposure to triclocarban, however, no significant difference was observed for T3 (Dong et al, 2018). Exposure to pentachlorophenol significantly decreased T4 and TSH levels but significantly increased T3 levels in zebrafish exposed from 0-6 dpf (Lei et al., 2020).

Similarly, significantly increased T3 content and T3/T4 ratio, and significantly reduced TSH was observed in zebrafish exposed to oxytetracycline from 2-5 dpf (Yu et al., 2020). Exposure to $CoFe_2O_4$ nanoparticles increased both T4 and T3 concentrations in zebrafish larvae 7 dpf (Ahmad et al., 2015).

In a transgenerational study with parental exposure of zebrafish to tebuconazole for 60 days, T4 and T3 were significantly reduced in F0 females and F1 larvae at 10 dpf, while unchanged T3 levels and significantly diminished T4 levels were observed in F0 males and F1 embryos at 1.5 and 5 dpf (Li et al., 2019).

4.3.2.7. General discussion and conclusions on plasma and whole-body TH levels as endpoint for THS related disruption

Whole-body or plasma levels of THs have been commonly employed as indicators of THS disruption in fish. TPO inhibitors generally decreased the T4 levels of fish, while T3 levels were decreased or unaffected. Contrasting effects, i.e. increased, decreased or unaffected T4 and T3 concentrations, were observed following exposure to chemicals acting via other MIEs. These diverse patterns in TH levels may be owing to different exposure scenarios (e.g. fish age and exposure duration). However, no clear trends can be deduced from the relatively sparse number of comparable exposure scenarios.

The TH levels are a function of the momentary synthesis and degradation rates, and the cause of the varying observations may be connected to the degree of (over-)compensation in the THS. The efficient feedback control of T4 on the hypothalamus and pituitary works toward compensatory restoration of plasma TH concentrations following exposure to THS disrupting chemicals. The TH levels measured at a given time may depend on the MIE, chemical exposure concentration, timing and duration, and species sensitivities. Additionally, in fish natural diurnal, seasonal and developmental stage fluctuations in TH levels occur, which require special consideration of the optimum timing of sampling during experimentation (Blanton and Specker, 2007; Martin et al., 2020; Wheeler et al., 2021). Furthermore, the TH levels alone do not provide information on the rate of TH turnover (Eales and Brown, 1993).

Moreover, it should be noted that whole-body or plasma measurements of THs do not necessarily reflect tissue levels of hormonally active T3 and may not reveal effects that occur in specific tissues such as disruption of TH metabolism or TR-mediated effects in peripheral tissues. Conversely, changes in whole-body or plasma TH levels may occur even though T3 levels and function remain the same in peripheral tissues (Noyes et al., 2019).

Additionally, the small sample volume available for TH measurements in small fish species like zebrafish and medaka could be a concern (Martin et al., 2020; Wheeler et al., 2021). Especially for embryos and larvae, pooling of several fish samples for TH analysis is required, which may mask individual differences. It may further require a larger number of fish used in the fish early life stage TGs. To determine the required number of fish for detecting changes in TH levels, power analyses, taking into account the sensitivity of the detection method, should be conducted (Martin et al., 2020; Wheeler et al., 2021).

The above-mentioned aspects may reduce the suitability of TH concentrations as endpoints for THS disruption (particularly in early life stage TGs). It is unlikely that measurement of TH levels alone will offer adequate information to assess THS related disruption.

4.3.2.8. Intrafollicular T4 and T3 content

Intrafollicular T4 and T3 content can be evaluated by means of fluorescence-labelled antibodies in whole-mounts of zebrafish embryos and larvae (Alt et al., 2006; Thienpont et al., 2011; Raldúa et al., 2012; Jomaa et al., 2014; Rehberger et al., 2018).

In the Zebrafish Eleutheroembryo Thyroid Assay (Thienpont et al., 2011; Raldúa et al., 2012), immunofluorescence analysis of T4 concentrations in the thyroid follicles is performed in whole-mount 5 dpf zebrafish following chemical exposure. Perchlorate, PTU, methimazole, amiodarone, pyrazole, sulfamethoxazole, resorcinol, benzophenone-2, mancozeb, genistein and NaBr significantly reduced the T4 immunofluorescence signal in this assay (Raldúa and Babin, 2009; Thienpont et al., 2011).

Exposure of zebrafish embryos to PTU resulted in a significant decrease in the number of follicles that stained for T3, whereas a trend for increase in follicles that stained for T4 was observed. In contrast, the intensity of the fluorescence decreased significantly for both THs after exposure to PTU (Rehberger et al., 2018). Furthermore, while in control fish the areas staining for intrafollicular hormones appeared spherical with distinct boundaries, the follicular shape progressively changed to more irregular and tube-like forms with increasing concentrations of PTU, which might be explained by peripheral vacuolation, a phenomenon indicative of activated follicles (Rehberger et al., 2018).

The related TPO inhibitor, phenylthiourea, caused a strong reduction of the follicular T4 immunofluorescence staining of 5 dpf zebrafish (Elsalini and Rohr, 2003; Giusti et al., 2020). Moreover, methimazole exposure resulted in both lower T4 signal intensity and lower number of staining follicles in zebrafish at 5 dpf (Jomaa et al., 2014).

Exposure to potassium perchlorate, methimazole or phenylthiourea decreased T4 immunostaining in the thyroid follicles of zebrafish larvae 5 dpf (Alt et al., 2006).

An accumulation of T4 immunoreactivity has been observed at the interphase between the follicular epithelium and the colloid lumen. This colloidal T4 ring may reflect the accumulation of T4 at its site of synthesis, and thus represent the level of TH synthetic activity in the thyroid follicles (Mukhi et al., 2005; Mukhi and Patiño, 2007).

Perchlorate caused a decrease in the colloidal T4 ring intensity (optical density) of adult zebrafish thyroid follicles at 2 and 12 weeks of exposure (Mukhi et al., 2005). Colloidal ring intensity was the most sensitive of the evaluated endpoints, which also included thyroid histology and T4 concentrations. Furthermore, the intensity of the colloidal T4 ring was still reduced after 12 weeks of recovery of the fish, whereas follicular epithelial cell height changes were no longer detectable.

4.3.2.9. General discussion and conclusions on intrafollicular TH content as an endpoint for THS related disruption

Follicular T4 and T3 may be a useful endpoint to detect THS disrupting chemicals acting through various MIEs in zebrafish (eleuthero)embryos. However, this endpoint has not been assessed in juveniles or adults.

Colloidal T4 ring intensity appears to be a sensitive endpoint for NIS disruption in adult zebrafish. However, to fully assess this endpoint further work is required regarding the effects of other MIEs for THS related disruption.

4.3.3. Gene expression

The widespread actions of THs can be ascribed to the multiple genes that are regulated, positively and/or negatively, by THs. The expression profiles of several genes known to participate in thyroid development and TH synthesis, distribution, action, feedback, and metabolism in fish have been used as indicators for THS disrupting effects of various compounds.

Key genes include those coding for: **1**) CRH (*crh*, expressed in the hypothalamus) and TSHβ (*tshβ*, expressed in the pituitary) which are both involved in HPT axis mediated feedback regulation of TH levels, **2**) thyroid transcription factors (*hhex, nkx2.1a*/*nkx2.4b*, *pax2a* and *pax8*) expressed in the thyroid, **3**) NIS (*slc5a5 (nis)*), Tg (*tg*) and TPO (*tpo*) which are all expressed in the thyroid follicles and are involved in TH synthesis, **4**) TTR (*ttr*) which is expressed in the liver and functions as a TH distributor protein in the plasma, **5**) DIOs (*dio1*, *dio2* and *dio3*) which are expressed in peripheral tissues and which are involved in activation and inactivation of THs, **6**) TRs (*thra* and *thrb*) expressed in essentially all tissues and responsible for initiating TH action and **7**) UGT (*ugt1ab*) which is expressed in metabolic tissues and plays a role in the metabolism of THs.

Gene expression in zebrafish is typically quantified by real-time polymerase chain reaction (qRT-PCR) measuring mRNA in whole-body samples. The method potentially provides a sensitive screen for potential THS disrupters.

4.3.3.1. crh

In non-mammalian vertebrates, including fish, CRH released from the hypothalamus appears to stimulate TSH secretion (De Groef et al., 2006).

In general, chemical exposure has been shown to upregulate the transcription levels of *crh*.

The transcription of *crh* was significantly induced in zebrafish 4 dpf upon exposure to PFDoA (Zhang et al., 2018a), 5 dpf upon exposure to BPS (Lee et al., 2019), hexaconazole (Yu et al., 2013), tebuconazole (Yu et al., 2013), difenoconazole (Liang et al., 2015), or perfluoropolyether carboxylic acids, PFOA, PFO3OA and PFO5DoDA (Wang et al., 2020), 7 dpf after exposure to DEHP (Jia et al., 2016), BPS (Zhang et al., 2017), *o,p'*-DDT or *p,p'*-DDE (Wu et al., 2019), and 14 dpf after exposure to DE-71 (Yu et al., 2010) or BDE-209 (Chen et al., 2012b). The upregulation of *crh* observed in the embryo/larval studies may be ascribed to reduced negative feedback on the hypothalamus due to decreased levels of circulating T4. However, *crh* expression was significantly decreased in zebrafish 6 dpf upon exposure to TBP (Fu et al., 2020), and was not significantly affected by BPA or BPF exposure from 0-5 dpf (Lee et al., 2019).

In F0 female zebrafish, F-53 exposure for 180 days significantly upregulated the transcription level of *crh*, whereas the transcription level of *crh* remained unchanged in F0 male brain and F1 males, and decreased significantly in F1 female brains (Shi et al., 2019).

4.3.3.2. tshβ

The pituitary gland regulates TH synthesis via the secretion of TSH which stimulates the transcription of genes involved in TH synthesis: *slc5a5 (nis)*, *tg* and *tpo* (see Fig. 2) (Van Heuverswyn et al., 1985; Chazenbalk et al., 1987; Riedel et al., 2001). TSH secretion is stimulated by fluctuations in the concentrations of circulating THs via feedback mechanisms.

TSH is composed of an α- and a β-subunit (MacKenzie et al., 2009). The α-subunit of TSH is nearly identical to that of FSH and LH. The β-subunits are structurally distinct and thus defines the functional specificity of the hormone (Pierce and Parsons, 1981; Maugars et al., 2014).

Chemical exposure has been shown to generally upregulate the transcription levels of *tshβ*.

The transcription of *tshβ* was significantly upregulated in zebrafish 80 hpf after treatment with phenylthiourea (Opitz et al., 2011), 5 dpf upon exposure to triadimefon (Liu et al., 2011b), PFOA (Wang et al., 2020), difenoconazole (Liang et al., 2015), phenylthiourea (Opitz et al., 2011), or hexaconazole or tebuconazole (Yu et al., 2013), 6 dpf upon exposure to TDCPP (Wang et al., 2013) or PFOA (Godfrey et al., 2017), 7 dpf after exposure to DEHP (Jia et al., 2016), MEHP (Zhai et al., 2014), *o,p'*-DDT or *p,p'*-DDE (Wu et al., 2019), and 14 dpf upon exposure to DE-71 (Yu et al., 2010) or BDE-209 (Chen et al., 2012b). The upregulation of *tshβ* observed after exposure to various compounds may be ascribed to reduced negative feedback on the pituitary due to decreased levels of circulating T4.

PTU exposure in zebrafish likewise significantly upregulated the expression of *tsh* in larvae (Tonyushkina et al., 2017) and adults (Pinto et al., 2013), whereas *tsh* expression was unaffected in embryos exposed from 0-5 dpf (Baumann et al., 2016). BDE-47 exposure significantly decreased the expression of *tshβ*, whereas BPA significantly induced the transcription of *tshβ* in zebrafish exposed from 4-8 dpf (Chan and Chan, 2012). However, *tshβ* expression was not affected in zebrafish exposed form 0-4 dpf to BDE-47, BDE-209 or 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) (Zezza et al., 2019), or from 0-5 dpf to BPA or BPF, whereas BPS increased the expression of *tshβ* (Lee et al., 2019).

Expression of *tshβ* was also significantly upregulated in zebrafish exposed to 100-400 µg/L TBBPA from 2 hpf to 6 dpf (Zhu et al., 2018) or to 530-3950 µg/L TBBPA from 4-8 dpf (Chan and Chan, 2012). However, in zebrafish exposed to 820 µg/L TBBPA from 0-4 dpf, *tshβ* expression was significantly downregulated (Chan and Chan, 2012), whereas no significant effects on *tsh* expression levels were seen in zebrafish exposed to 100-400 µg/L TBBPA from 0-5 dpf (Baumann et al., 2016) or to 13 µg/L TBBPA from 0-6 dpf (Godfrey et al., 2017).

Significantly reduced *tshβ* expression was observed in zebrafish after exposure to PFDoA from 0-4 dpf (Zhang et al., 2018a), TBP from 0-6 days (Fu et al., 2020), the antifouling biocide tralopyril from 0-5 dpf (Chen et al., 2020) or PFOS from 0-15 dpf (Shi et al., 2009). Furthermore, significant decreases in *tshβ* mRNA were observed in the F1 larvae of zebrafish exposed to tebuconazole from 60-120 dpf (Li et al., 2019).

Sex-specific differences in *tshβ* expression responses have been observed. Exposure to PFOA caused an increase in *tshβ* mRNA expression in 10 dpf Japanese medaka females but not in males (Godfrey et al., 2019). Conversely, *tshβ* expression was significantly reduced in the brain of zebrafish F0 males, whereas no significant effects were observed on the transcription level of *tshβ* in F0 female fish after treatment with F-53B (Shi et al., 2019). In F1 female brains, the transcriptional level of *tshβ* was significantly reduced after parental exposure to F-53B, which was likely a negative feedback reaction to elevated T4 levels. Furthermore, the expression of *tshβ* was significantly increased in both female and male livers of zebrafish exposed to tebuconazole for 60 days (Li et al., 2019).

Tissue-specific expressions have been examined in fathead minnows and sea bream. Elevated *tshβ* mRNA abundance in the pituitary glands of adult fathead minnows were observed upon exposure to methimazole for 10 days (Lema et al., 2009) or BDE-47 for 21 days (Lema et al., 2008). In sea bream, *tsh* expression in the brain and pituitary was significantly downregulated by PTU exposure (Morgado et al., 2009) while *tshβ* expression in the pituitary was unaffected in another study (Campinho et al., 2012).

4.3.3.3. hhex, nkx2.1a/nkx2.4b and pax8

The thyroid transcription factors are involved in the development of the thyroid primordium (Porazzi et al., 2009; Wendl et al., 2002) and regulate the transcription of *slc5a5* and *tg* (Zoeller et al., 2007). Zebrafish thyroid precursor cell markers hhex, nkx2.1a/nkx2.4b and pax2a can be detected as soon as 24 hpf (Rohr and Concha, 2000; Wendl et al., 2002; Opitz et al., 2013), while pax8 is expressed from about 28 hpf (Wendl et al., 2002).

In general, chemical exposure has been shown to upregulate the transcription levels of thyroid transcription factor genes.

Treatment with TDCPP significantly increased the transcription of *hhex*, *nkx2.1a* (RefSeq NM_131589.1; also named *nkx2.4b* (Manoli and Driever, 2014)) and *pax8* in zebrafish 6 dpf (Wang et al., 2013). Likewise, *nkx2.1a* (*nkx2.4b*) and *pax8* were significantly upregulated in zebrafish 7 dpf upon exposure to MEHP (Zhai et al., 2014), *o,p'*-DDT or *p,p'*-DDE (Wu et al., 2019), and 14 dpf upon exposure to DE-71 (Yu et al., 2010) or BDE-209 (Chen et al., 2012b). Benzophenone-4 significantly increased the expression of *hhex* and *pax8* in zebrafish exposed for approximately 5 days from 2-4 hpf (Zucchi et al., 2011). BPS exposure from 0-7 dpf also significantly increased *pax8* expression (Zhang et al., 2017). However, exposure to BPS, BPA or BPF in zebrafish until 5 dpf caused no significant effects on the expression of *pax8*, while the treatments significantly increased the expression of *hhex* (Lee et al., 2019). The transcripts of *nkx2.1* (*nkx2.4b*) were significantly increased after DEHP exposure of zebrafish from 2 hpf to 7 dpf, whereas expression of *pax8* showed no significant change (Jia et al., 2016). Likewise, the expression levels of *pax8* were not affected by exposure to TBP in zebrafish 6 dpf (Fu et al., 2020). Chen et al. (2020) found that the *pax8* expression response in zebrafish 5 dpf depended on the exposure concentrations of tralopyril. While 3 µg/L significantly increased the expression, 1 µg/L significantly decreased the expression, while no effects were observed at 2 µg/L. Du et al. (2013a; 2013b) found that exposure to PFOA and PFOS resulted in significantly higher expression levels of *hhex* and *pax8* in a short-term zebrafish assay from 0-5 dpf. Likewise, PFOS increased the expression levels of *hhex* and *pax8* in zebrafish exposed from 0-5.5 dpf (Shi et al., 2008).

4.3.3.4. slc5a5 (nis)

Slc5a5 is expressed in the thyroid (Alt et al., 2006) and its product, the sodium/iodide symporter (NIS), is responsible for the iodine uptake from the blood to the thyroid follicles (Zoeller et al., 2007). The transcription of *slc5a5* is regulated by thyroid transcription factors stimulated by TSH (Zoeller et al., 2007).

Chemical exposure has been shown to generally upregulate the transcription levels of *slc5a5* in embryos/larvae.

The transcript levels of *slc5a5* were significantly upregulated in zebrafish embryos 80 hpf after treatment with phenylthiourea (Opitz et al., 2011), 4 dpf following exposure to BDE-47 (Chan and Chan, 2012), 5 dpf after triclocarban (Dong et al., 2018), hexaconazole (Yu et al., 2013), tebuconazole (Yu et al., 2013) or phenylthiourea (Opitz et al., 2011) treatment, 6 dpf upon treatment with TDCPP (Wang et al., 2013), 7 dpf upon exposure to MEHP (Zhai et al., 2014), BPS (Zhang et al., 2017), *o,p'*-DDT or *p,p'*-DDE (Wu et al., 2019), 14 dpf upon DE-71 (Yu et al., 2010) or BDE-209 exposure (Chen et al., 2012b) and 15 dpf upon PFOS exposure (Shi et al., 2009). Furthermore, triclosan caused a significant upregulation of *slc5a5* in adult zebrafish exposed for 21 days (Pinto et al., 2013). The upregulation of *slc5a5* mRNA levels observed in zebrafish after exposure to various compounds may reflect feedback mechanisms in the HPT axis to keep TH homeostasis.

Exposure to PTU also significantly increased mRNA expression of *slc5a5* in zebrafish from 0-5 dpf (Liu et al., 2013), whereas Pinto et al. (2013) found no significant effects in adult zebrafish. Likewise, no significant effects were observed on *slc5a5* expression in embryos or larvae after exposure to TBBPA from 0-4 dpf or from 4-8 dpf (Chan and Chan, 2012). Similarly, the transcripts of *slc5a5* showed no significant changes after DEHP exposure of zebrafish from 2 hpf to 7 dpf (Jia et al., 2016). Furthermore, exposure to difenoconazole from 0-5 dpf caused no significant changes in *slc5a5* expression in zebrafish (Liang et al., 2015). PFDoA on the other hand significantly decreased the levels of *slc5a5* in a concentration-dependent manner after exposure of zebrafish from 0-4 dpf (Zhang et al., 2018a).

A study in Chinese rare minnow showed time- and tissue-dependent effects of exposure to perchlorate on *slc5a5* expression (Li et al., 2011b). In larvae, the expression of *slc5a5* was significantly downregulated after 7-day exposure, significantly upregulated after 14-day exposure and showed no significant difference

after 21-day exposure to perchlorate. The expression of *slc5a5* was generally upregulated in the brains of the adult fish. However, in the livers of adult fish the expression of *slc5a5* was significantly downregulated after 7- or 14-day exposure and significantly upregulated after 21-day exposure.

4.3.3.5. tg

Tg is the substrate upon which THs are synthesised (Zoeller et al., 2007), and Tg synthesis is regulated by thyroid transcription factors.

Generally, *tg* expression is upregulated in fish following exposure to THS disrupting chemicals.

The transcription of *tg* was significantly increased in zebrafish 5 dpf upon phenylthiourea (Opitz et al., 2011) or BPA exposure (Lee et al., 2019), 6 dpf upon treatment with TDCPP (Wang et al., 2013), 7 dpf upon exposure to DEHP (Jia et al., 2016), MEHP (Zhai et al., 2014), BPS (Zhang et al., 2017), *o,p'*-DDT or *p,p'*-DDE (Wu et al., 2019), and 14 dpf upon exposure to DE-71 (Yu et al., 2010) or BDE-209 (Chen et al., 2012b). Furthermore, BDE-47 significantly induced *tg* expression in zebrafish exposed from 4-8 dpf (Chan and Chan, 2012). Moreover, mRNA levels of *tg* were significantly increased in F1 larvae following parental exposure to F-53B (Shi et al., 2019).

Expression of *tg* was significantly upregulated in zebrafish exposed to 100-400 µg/L TBBPA from 2 hpf to 6 dpf (Zhu et al., 2018), whereas no significant effect was observed on *tg* expression in embryos and larvae after exposure to 110-820 µg/L TBBPA from 0-4 dpf or to 530-3950 µg/L TBBPA from 4-8 dpf (Chan and Chan, 2012). Likewise, the expression levels of *tg* in zebrafish were not significantly affected by exposure from 0-5 dpf to BPS (Lee et al., 2019) or difenoconazole (Liang et al., 2015), or to TBP from 0-6 dpf (Fu et al., 2020).

Exposure to PTU from 0-5 dpf significantly increased mRNA expression of *tg* in zebrafish (Liu et al., 2013). However, Pinto et al. (2013) found no significant effects of PTU on the expression of *tg* in adult zebrafish. Likewise, triclosan exposure of adult zebrafish for 21 days did not significantly modify the expression of *tg* (Pinto et al., 2013). Furthermore, exposure to hexaconazole or tebuconazole did not significantly affect the transcription of *tg* in zebrafish 5 dpf (Yu et al., 2013). In zebrafish exposed from 60-120 dpf, *tg* transcripts were significantly increased in the livers of females, while no significant effects were observed on the expression of *tg* in the livers of males or in the F1 generation (Li et al, 2019).

Finally, *tg* expression was significantly downregulated in zebrafish 15 dpf upon PFOS exposure (Shi et al., 2009) and 4 dpf upon exposure to PFDoA (Zhang et al., 2018a).

4.3.3.6. tpo

The TPO enzyme catalyses the oxidation of iodide ions and the addition of these onto tyrosine residues on Tg to produce T4 (Zoeller et al., 2007).

Various effects of chemical exposure on the expression of *tpo* have been observed.

The expression of *tpo* was significantly upregulated in zebrafish 5 dpf upon PTU (Baumann et al., 2016), phenylthiourea (Opitz et al., 2011) or BPS (Lee et al., 2019) exposure, 7 dpf after exposure to *o,p'*-DDT or *p,p'*-DDE (Wu et al., 2019), 6 and 28 dpf after exposure to TDCPP, PFOA, PFBA and DOPO (Godfrey et al., 2017), or following exposure to BDE-47 from 4-8 dpf (Chan and Chan, 2012); and in fathead minnows 14, 21 and 22 dpf upon exposure to 2-mercaptobenzothiazole (Nelson et al., 2016). The increased expression levels may be part of a compensatory feedback response to TPO inhibition caused by the chemical exposure.

However, no significant effects were observed upon exposure to BPA or BPF in zebrafish from 0-5 dpf (Lee et al., 2019). Pinto et al. (2013) likewise found no significant effect of PTU exposure on *tpo* expression

in adults. Furthermore, no significant effect on *tpo* expression was observed in fathead minnows 2 or 6 dpf upon exposure to 2-mercaptobenzothiazole (Nelson et al., 2016).

Additionally, no significant effects were observed on *tpo* expression in zebrafish embryos and larvae after exposure to 110-820 µg/L TBBPA from 0-4 dpf or to 530-3950 µg/L TBBPA from 4-8 dpf (Chan and Chan, 2012), or to 13 µg/L TBBPA from 0-6 dpf (Godfrey et al., 2017). However, *tpo* expression was significantly downregulated in zebrafish after exposure to 100-400 µg/L TBBPA from 0-5 dpf (Baumann et al., 2016).

Furthermore, exposure to methimazole decreased the expression of *tpo* in zebrafish 28 dpf (Godfrey et al., 2017). The transcription level of *tpo* was also decreased in F1 and F2 zebrafish after F0 exposure to F-53B (Shi et al., 2019). Moreover, expression of *tpo* was significantly downregulated in fathead minnows by exposure to iopanoic acid in an embryo study at 6 dpf and in a larval study at 10, 14 and 18 dpf. However, by 21 dpf there were no longer any statistically significant differences between the treatments and controls (Cavallin et al., 2017). Finally, *tpo* expression was downregulated in zebrafish 5 dpf after exposure to triclocarban (Dong et al., 2018), whereas triclosan exposure of adult zebrafish for 21 days did not modify the expression of *tpo* (Pinto et al., 2013).

4.3.3.7. ttr

TTR is a key TH distributor protein in fish plasma (McLean et al., 2017; Power et al., 2000).

Effects of chemical exposure on the expression of *ttr* have been examined in numerous studies.

The transcription of *ttr* was significantly induced in zebrafish upon exposure to PFOA (Wang et al., 2020), BPA or BPS (Lee et al., 2019), difenoconazole (Liang et al., 2015), or hexaconazole or tebuconazole (Yu et al., 2013) until 5 dpf, to TBP until 6 dpf (Fu et al., 2020), and to DEHP from 2 hpf to 7 dpf (Jia et al., 2016). Likewise, BDE-47 induced the transcription of *ttr* in zebrafish exposed from 4-8 dpf (Chan and Chan, 2012). Furthermore, the abundance of *ttr* transcripts was induced in F0 adult male zebrafish exposed to PFNA, and in their offspring (Liu et al., 2011c).

Exposure of zebrafish to 250 µg/L TBBPA resulted in significantly higher levels of *ttr* at 2 dpf; however, no effects were observed at 4 or 5 dpf (Parsons et al., 2019). Furthermore, no effects were observed on *ttr* expression in zebrafish embryos after exposure from 0-4 dpf to 111-820 µg/L TBBPA, whereas induced transcription of *ttr* in zebrafish larvae was observed after exposure from 4-8 dpf to 3950 µg/L TBBPA (Chan and Chan, 2012).

Exposure of zebrafish to 200 or 400 µg/L TBBPA from 2 hpf to 6 dpf decreased the transcription of *ttr* (Zhu et al., 2018). Likewise, *ttr* expression was downregulated in zebrafish 5 dpf after exposure to tralopyril (Chen et al., 2020), 7 dpf after exposure to MEHP (Zhai et al., 2014) or BPS (Zhang et al., 2017), 14 dpf upon exposure to DE-71 (Yu et al., 2010) or BDE-209 (Chen et al., 2012b) and 15 dpf upon exposure to PFOS (Shi et al., 2009). However, *ttr* expression was not affected in zebrafish exposed form 0-4 dpf to BDE-47, BDE-209 or BDE-99 (Zezza et al., 2019).

The transcription of *ttr* showed different expression pattern in female and male zebrafish upon exposure to microcystin-LR (Cheng et al., 2017). However, the transcriptional levels of *ttr* were increased in both sexes of zebrafish after F-53B exposure (Shi et al., 2019).

In sea bream, *ttr* expression in the liver was either upregulated (Morgado et al., 2009) or unaffected (Campinho et al., 2012) by PTU exposure in spite of similar exposure scenarios.

4.3.3.8. dio1, dio2 and dio3

Fish express three isotypes of deiodinases: DIO1, DIO2 and DIO3, which are responsible for the peripheral regulation of concentrations of active and inactive THs (see Fig. 4) (Orozco and Valverde, 2005; Darras and Van Herck, 2012). These enzymes are expressed in a tissue- and developmental-specific manner and

thus, at the precise time and site, tightly regulate the concentrations of active and inactive THs (Orozco and Valverde, 2005).

All vertebrates have a single gene for each of the DIO1 and DIO2 enzymes. However, due to a whole-genome duplication, fish have two paralog genes, *dio3a* and *dio3b*, coding for different isoforms of DIO3; with DIO3b seemingly having a predominant role over DIO3a in zebrafish (Guo et al., 2014; Heijlen et al., 2014).

While being similar to mammalian DIOs in their molecular and functional properties, fish DIOs show distinctive characteristics, including the relative insensitivity of teleostean DIO1 to PTU inhibition (Orozco and Valverde, 2005).

Numerous chemicals have been shown to affect the expression of DIO genes in different fish species, life stages and tissues.

dio1

The expression of *dio1* was significantly upregulated in zebrafish 5 dpf upon exposure to PFO4DA (Wang et al., 2020), BPA (Lee et al., 2019), hexaconazole (Yu et al., 2013), difenoconazole (Liang et al., 2015), or triclocarban (Dong et al., 2018), 6 dpf upon TDCCP exposure (Wang et al., 2013), 7 dpf upon exposure to MEHP (Zhai et al., 2014), BPS (Zhang et al., 2017), *o,p'*-DDT or *p,p'*-DDE (Wu et al., 2019), 14 dpf upon exposure to DE-71 (Yu et al., 2010) or BDE-209 (Chen et al., 2012b), and 15 dpf upon PFOS exposure (Shi et al., 2009).

Although *dio2* expression was significantly increased, no significant effects on *dio1* expression were observed in zebrafish exposed until 5 dpf to PTU (Baumann et al., 2016), PFO3OA or PFO5DoDA (Wang et al., 2020), to DEHP from 2 hpf to 7 dpf (Jia et al., 2016), or to F-53B as adults (Shi et al, 2019). Likewise, *dio2* and *dio3* expression showed an increase in fathead minnows exposed to iopanoic acid up to 21 dpf, while *dio1* expression was unaffected (Cavallin et al., 2017). Furthermore, no effects were observed on *dio1* expression in zebrafish exposed to PBDE/OH-BDE 12, 23 or 45 dpf (Macaulay et al., 2017). Likewise, *dio1* expression was not significantly affected in zebrafish exposed form 0-4 dpf to BDE-47, BDE-209 or BDE-99 (Zezza et al., 2019).

On the other hand, *dio1* expression was significantly decreased in zebrafish 5 dpf after triadimefon exposure, while the levels of *dio2* transcripts were not affected (Liu et al., 2011b). The expression of *dio1* was furthermore significantly decreased in zebrafish by exposure to tralopyril from 0-5 dpf (Chen et al., 2020) and to TBP from 0-6 dpf (Fu et al., 2020).

dio2

The expression of *dio2* was significantly upregulated in zebrafish exposed to PFDoA from 0-4 dpf (Zhang et al., 2018a), to PTU (Baumann et al., 2016), hexaconazole (Yu et al., 2013), difenoconazole (Liang et al., 2015), PFOA, PFO3OA or PFO5DoDA (Wang et al., 2020) or to the herbicide metabolite metolachlor ethanesulfonic acid (Rozmánková et al., 2020) until 5 dpf, to MEHP (Zhai et al., 2014), DEHP (Jia et al., 2016) or BPS (Zhang et al., 2017) until 7 dpf, and to DE-71 (Yu et al., 2010) or BDE-209 (Chen et al., 2012b) until 14 dpf. Additionally, upregulation of *dio2* transcripts was observed in adult medaka exposed to perchlorate for 7 days (Lee et al., 2014). In fathead minnow, *dio2* and *dio3* expression showed an increase after exposure to iopanoic acid up to 21 dpf; with expression of *dio2* as the most impacted (Cavallin et al., 2017). *p,p'*-DDE exposure significantly downregulated the expression of *dio2* in zebrafish exposed from 0-7 dpf (Wu et al., 2019).

Although *dio1* expression was significantly increased, no significant effects on *dio2* expression were observed in zebrafish exposed until 5 dpf to PFO4DA (Wang et al, 2020), BPA (Lee et al., 2019), or

triclocarban (Dong et al., 2018), to TDCCP until 6 dpf (Wang et al., 2013), or to PFOS until 15 dpf (Shi et al., 2009). Triadimefon exposure did not significantly affect the levels of *dio2* transcripts in zebrafish 5 dpf even though *dio1* expression was significantly decreased (Liu et al., 2011b). Furthermore, no significant effects were observed on *dio1* or *dio2* expression in zebrafish exposed to PBDE/OH-BDE 12, 23 or 45 dpf (Macaulay et al., 2017).

dio3

Iopanoic acid increased *dio3* expression in fathead minnows 21 dpf (Cavallin et al., 2017). *p,p'*-DDE likewise upregulated *dio3a* and *dio3b* expression in zebrafish exposed from 0-7 dpf; whereas *o,p'*-DDT downregulated *dio3a* and *dio3b* expression (Wu et al., 2019). PTU treatment also significantly downregulated *dio3* expression in zebrafish 5 dpf (Baumann et al., 2016). Likewise, TBP exposure significantly decreased *dio3a* and *dio3b* expression in zebrafish 6 dpf (Fu et al., 2020). However, the expression of *dio3* was not significantly affected by exposure to BPS from 0-7 dpf (Zhang et al., 2017).

Time-, tissue- and sex-specific responses in dio expression

In Chinese rare minnow larvae, *dio2* mRNA levels were downregulated by perchlorate exposure at 7 dph and upregulated at 14 or 21 dph (Li et al., 2011b). *Dio1* expression was not detected until 21 dph, whereas *dio3* mRNA was not detected at high levels in larvae even by 21 dph. In adults, at 7-day exposure only *dio3* was downregulated in the liver. However, after 14-day exposure, an upregulation of *dio2* and a downregulation of *dio1* and *dio3* were observed. No significant changes in the expression of *dio1*, *dio2* or *dio3* in the livers were observed after 21-day exposure. In the brains of the adult male fish, a general upregulation of *dio2* expression was observed after 7- and 14-day exposure, while *dio2* expression was downregulated after 21-day exposure. In the brains of the adult females, a general downregulation of *dio2* and *dio3* expression was observed. Expression of *dio1* was not detected at high levels in the brains of rare minnow.

In juvenile sea bream, *dio2* expression was significantly upregulated in the liver, *dio2* and *dio3* expression was significantly downregulated in the brain, while no significant effect was observed on *dio2* expression in the pituitary after PTU treatment for 21 days (Morgado et al., 2009). Another study with sea bream using a similar treatment scenario showed no significant effects of PTU exposure on *dio* expression in the liver or kidney (Campinho et al., 2012).

In zebrafish, a significant decrease in *dio1* mRNA and a significant increase in *dio2* expression was observed in the livers of females exposed to tebuconazole for 60 days, (Li, 2019). However, no significant effects on *dio1* or *dio2* expression were observed in the male livers, while the expression levels of *dio1* and *dio2* were decreased in F1 larvae. Furthermore, the transcription of *dio2* was increased in both sexes of F0 zebrafish exposed to F-53B, and in F1 larvae from the exposed parents (Shi et al., 2019).

In general, *dio1* and *dio2* expression was increased after exposure to chemicals. However, *dio3* expression showed more variable effects in the relatively few studies examining this endpoint. The overall changes in deiodinase expression activity may serve as a compensatory response mechanism to sustain the homeostasis of T3.

4.3.3.9. thra and thrb

TRs function as ligand-induced transcription factors that bind T3 (and with a lower affinity T4) and stimulates the transcription of genes containing TREs (Darras et al., 2011). Zebrafish express various isoforms of TRs encoded by two *thra* gene variants (*thraa* and *thrab*) and a single *thrb* gene with differing tissue distribution (Marelli and Persani, 2018).

Numerous chemicals have been shown to affect the expression of *thra* and *thrb*.

TBBPA significantly induced the expression of *thra* but not *thrb* in zebrafish exposed to 820 µg/L from 0-4 dpf (Chan and Chan, 2012), to 100-200 µg/L from 0-5 dpf (Baumann et al., 2016) or to 3950 µg/L from 4-8 dpf (Chan and Chan, 2012). Conversely, TBBPA exposure significantly downregulated the expression of *thra* after exposure to 160-650 µg/L from 2-122 hpf (Liu et al., 2018a) and of *thrb* after exposure to 200 or 400 µg/L TBBPA from 2 hpf to 6 dpf (Zhu et al., 2018). TBBPA did not cause significant changes in the expression of neither *thra* nor *thrb* in zebrafish exposed to 13 µg/L from 0-6 dpf (Godfrey et al., 2017) or to 20-250 µg/L from fertilisation to 2, 4 or 5 dpf (Parsons et al., 2019). Likewise, no significant effects were observed on the expression of *thra* or *thrb* in the eyes of TBBPA exposed zebrafish 5 dpf (Baumann et al., 2019). Similarly, the expression of *thra* and *thrb* was not significantly affected by exposure to BPF or BPS from 0-5 dpf (Lee et al, 2019) or BPS from 0-7 dpf (Zhang et al., 2017). However, BPA significantly increased the expression of *thra* in zebrafish exposed from 0-5 dpf, while *thrb* expression was unaffected (Lee et al., 2019). TBP exposure significantly decreased both *thra* and *thrb* expression in zebrafish 6 dpf (Fu et al., 2020).

PFOA upregulated the expression of *thra*, while no significant effects were observed on *thrb* in zebrafish 6 dpf (Godfrey et al., 2017). PFOS significantly downregulated the expression of *thra* but significantly upregulated *thrb* expression in zebrafish larvae 15 dpf (Shi et al., 2009). PFDoA exposure of zebrafish until 4 dpf significantly downregulated both *thra* and *thrb* expression (Zhang et al., 2018a). DOPO exposure caused a significant upregulation of the expression of *thrb* with no significant effect on *thra* in zebrafish 6 dpf (Godfrey et al., 2017).

The triazole fungicides hexaconazole and tebuconazole also significantly upregulated the transcription of *thrb* in larval zebrafish 5 dpf; and hexaconazole further significantly upregulated the transcription of *thra* (Yu et al., 2013). However, the expression of *thrb* was not significantly affected by difenoconazole exposure in zebrafish from 0-5 dph (Liang et al., 2015). The S-metolachlor metabolites metolachlor oxanilic acid and metolachlor ethanesulfonic acid increased the expression levels of *thrb*; and metolachlor oxanilic acid furthermore increased the expression levels of *thraa* in zebrafish exposed from 3-4 hpf (Rozmánková et al., 2020).

BDE-47 significantly induced the expression of *thra* and reduced *thrb* expression in zebrafish exposed from 4-8 dpf (Chan and Chan, 2012). Expression of *thra* was also significantly increased at 12 dpf in zebrafish exposed to a PBDE/OH-BDE mixture, however, with no significant effects at 23 or 45 dpf (Macaulay et al., 2017). An increase in *thrb* expression was observed at 45 dpf in zebrafish exposed to 6-OH-BDE-47, with no significant effects at 12 or 23 dpf (Macaulay et al., 2017).

The levels of *thrb* mRNA were significantly downregulated in zebrafish 5 dpf after exposure to triadimefon, while no significant changes were observed in the mRNA expression of *thra* (Liu et al., 2011b). Both *thra* and *thrb* expression were significantly downregulated in zebrafish exposed until 5 dpf to PTU (Baumann et al., 2016) or to tralopyril (Chen et al., 2020).

Expression levels of *thra* and *thrb* remained unchanged in zebrafish exposed to DEHP from 2 hpf to 7 dpf (Jia et al., 2016) or DE-71 from 0-14 dpf (Yu et al., 2010).

Sex-specific expression of *thr* has been examined in Japanese medaka, fathead minnow and zebrafish. Exposure to PFOA caused a significant increase in *thrb* mRNA expression in medaka females 10 dpf but not in males (Godfrey et al., 2019). TCCPP significantly upregulated *thra* expression in males, whereas *thra* expression was unaffected in females. In fathead minnows, BDE-47 significantly decreased the *thrb* mRNA levels in the brain of both sexes and elevated transcripts for *thra* in the brain of females (Lema et al., 2008). Conversely, in F0 zebrafish *thrb* increased significantly in males in the lowest exposure group, whereas the transcription of *thra* was unchanged in male brains but was significantly increased in female brains after 180 days of exposure to F-53B (Shi et al., 2019). The transcriptional profiles of *thr* in the liver were different to those observed in the brain. In the male liver, the transcriptional level of *thrb* was

significantly downregulated after F-53B exposure, whereas no significant change was found in *thra* expression. In the female liver, the transcription of *thrb* was significantly increased in the lowest exposure group. In the brains and livers of F1 females and in F2 larvae, the transcriptional level of *thra* decreased significantly after F0 exposure to F-53B, whereas *thrb* showed no significant change. Transcripts of *thra* and *thrb* were significantly downregulated in the liver of female zebrafish exposed to tebuconazole, while in male zebrafish livers tebuconazole significantly upregulated mRNA expression of *thrb* but caused no significant effects on *thra* expression (Li et al., 2019). Furthermore, transcripts of *thra* and *thrb* were significantly downregulated in the F1 larvae.

Tissue-specific expression of *thr* has been further examined in adult fathead minnows. Neither *thra* nor *thrb* were significantly affected in the brain, liver or gonads of adult fathead minnows by treatment with methimazole for 10 days (Lema et al., 2009). Likewise, dietary intake of BDE-47 did not significantly affect the *thr* transcript levels in the liver of fathead minnows (Lema et al., 2008).

4.3.3.10. ugt1ab

UGT plays a role in the metabolism of THs (Hood and Klaassen, 2000). Among UGT family proteins, UGT1AB participates in the glucuronidation of THs (Shi et al., 2019).

Various compounds have been shown to affect the transcription of *ugt1ab* in zebrafish.

The transcription of *ugt1ab* was significantly induced in zebrafish 5 dpf upon exposure to perfluoropolyether carboxylic acids (Wang et al., 2020), BPA, BPS or BPF (Lee et al., 2019), difenoconazole (Liang et al., 2015), or hexaconazole or tebuconazole (Yu et al., 2013), 6 dpf upon exposure to TDCPP (Wang et al., 2013), 7 dpf upon exposure to MEHP (Zhai et al., 2014) or BPS (Zhang et al., 2017), and 14 dpf upon exposure to DE-71 (Yu et al., 2010). Likewise, significantly increased expression of *ugt1ab* was observed in both the F0 (liver) and F1 generation after parental exposure of zebrafish to tebuconazole for 60 days (Li et al., 2019).

Conversely, *ugt1ab* was transcriptionally significantly downregulated in zebrafish 4 dpf after exposure to PFDoA (Zhang et al., 2018a), 5 dpf upon exposure to tralopyril (Chen et al., 2020), 7 dpf upon exposure to DEHP (Jia et al., 2016), *p,p'*-DDE or *o,p'*-DDT (Wu et al., 2019), and 14 dpf upon exposure to BDE-209 (Chen et al., 2012b). However, the expression levels of *ugt1ab* were not significantly affected by exposure to TBP in zebrafish 6 dpf (Fu et al., 2020).

The transcription of *ugt1ab* showed different expression pattern in female and male zebrafish upon exposure to microcystin-LR (Cheng et al., 2017). However, exposure to F-53B significantly increased the transcription of *ugt1ab* in both sexes of F0 adult fish, whereas transcriptional levels of *ugt1ab* significantly decreased in the F1 female liver (Shi et al., 2019).

4.3.3.11. General discussion and conclusions on gene expression levels as endpoint for THS related disruption

Various effects of exposure to chemicals on THS related gene expression in fish have been observed. Table 7 provides a summary of the effects in zebrafish embryos/larvae.

Table 7. Summary of effects of chemical exposure on whole-body expression of THS related genes in zebrafish embryos/larvae

Gene	*crh*	*tshβ*	*hhex*	*nkx2*	*pax8*	*slc5a5*	*tg*	*tpo*	*ttr*	*dio1*	*dio2*	*dio3*	*thra*	*thrb*	*ugt1ab*
Effect	↑ (↓↔)	↑ (↓↔)	↑	↑	↑ (↓↔)	↑ (↓↔)	↑ (↓↔)	↑↓↔	↑↓↔	↑ (↓↔)	↑ (↓↔)	↑↓ (↔)	↑↔ (↓)	↑↓↔	↑ (↓ ↔)

↑: upregulation and ↓: downregulation and ↔: no effect. The predominant effects are indicated without parentheses, while less prevalent effects are indicated in parentheses.

The effects on gene expression integrates different MIEs of THS related disruption, which probably explain much of the divergent effects on gene expression seen for most genes in the THS upon exposure to various chemicals (Table 7). Although much of the variation in gene expression may be due to the differences in MIEs between chemicals, variations in gene response patterns are also observed for the same chemical. For several compounds, both up- and downregulation is observed for the same gene. In these cases, clear exposure concentration dependence patterns cannot always be established. This suggests that the expression response may also be dependent on the timing of exposure to the chemicals. Indeed, Jarque et al. (2019) have shown that, at least, some THS related genes show different expression responses depending on the exposure window. Furthermore, expression patterns of key genes in the THS may vary across species, sexes and (for some genes) tissues. However, particularly in embryos of small fish species the genes are measured in (pools of) whole animals, which precludes information on tissue-specific expression and may decrease the sensitivity of the endpoint because significant local effects may be masked or diluted (Dong et al., 2013). Inclusion of gene expression analyses in fish TGs may require a larger number of fish used, especially in early life stage tests, which should be clarified by the conduction of power analyses.

The most consistent expression pattern after exposure to chemicals was observed for *hhex* and *nkx2.1a/nkx2.4b*, each members of the homeodomain family (Fernandez et al., 2015). However, their expression was primarily examined in studies with embryos, in which *hhex* and *nkx2.1a/nkx2.4b* are known to function as thyroid transcription factors involved in the development of the thyroid primordium. Yet, the role of the thyroid transcription factors in the differentiated thyroid is less well known, and *hhex* and *nkx2.1a/nkx2.4b* is also expressed in other tissues than the thyroid (Fernandez et al., 2015; Villasenor et al., 2020). This calls in to question the applicability of expression patterns of these genes as thyroid specific endpoints in whole-body homogenates.

Although the gene response pattern varies to different degrees for the other genes, in general an upregulation of genes leading to increased T4 (*crh*, *tshβ*, *pax8*, *slc5a5 and tg* (yet not *tpo*)) and T3 (*dio2* (and *dio1*)) levels is observed. The observed upregulation of expression of these genes may be interpreted as a compensatory response due to decreased TH levels caused by the chemical exposure.

Less specific response patterns were observed for *tpo*, *ttr*, *dio3*, *thra* and *thrb*, whereas *ugt1ab* expression was mostly upregulated.

Although gene expression levels can provide valuable information by themselves, the complexity of gene response patterns in the THS renders it difficult to pinpoint specific genes as endpoints for THS disruption.

The term ‘gene expression’ is often used synonymously with mRNA measurements (Buccitelli and Selbach, 2020). However, changes in gene expression can be studied at the level of both the mRNAs and their products, proteins, which are the effectors of essentially all biological functions and thus more relevant functionally. Yet it has been common practice to use mRNA levels as indicators for the levels and activities of the corresponding proteins, thus assuming that mRNA levels are the main determinant of protein levels (Vogel and Marcotte, 2012). However, mRNA level changes are not always directly related to protein level changes due to regulatory mechanisms that control mRNA half-life, translational efficiency, post-translational modification and protein half-life (Schwanhäusser et al., 2011; Vogel and Marcotte, 2012; Buccitelli and Selbach, 2020). Assessing actual protein levels, rather than mRNA levels could, thus, be considered to provide more relevant information to characterise the effects of THS disrupters. Yet relatively few of the studies on THS disruption in fish examine levels of proteins involved in the THS. These studies employ Western blotting, mostly for the analysis of TTR or TG protein expression (Chen et al., 2012b; Deng et al., 2018; Chen et al., 2020; Fu et al., 2020). Plausible explanations for the less common use of

protein assessment, compared to mRNA assessment, may be the added experimental complexity and limited sensitivity since, unlike mRNAs, proteins cannot be amplified. Compared to qRT-PCR, Western blotting is more time consuming, requires more initial biological material and is not considered a particularly accurate quantitative procedure (Mahmood and Yang, 2012; Rocha-Martins et al., 2012; Butler et al, 2019; Pillai-Kastoori et al., 2020). However, the Western blot procedure is continually being refined to overcome these weaknesses (e.g. Butler et al., 2019; Pillai-Kastoori et al., 2020). Besides the refinement of conventional methods for protein analysis, considerable progress has been made in the development of new proteomics technologies (Aslam et al., 2016; Timp and Timp, 2020), which may open novel perspectives for the discovery and development of applicable protein endpoints for THS related disruption.

4.3.4. Skin pigmentation

Skin pigmentation in fish is a complex process that involves a series of cellular, genetic and physiological factors that influence the external appearance of a fish at a given developmental stage (Colihueque, 2010).

Pigment cell types in zebrafish include melanophores (black), xanthophores (yellowish) and iridophores (silvery) (Kelsh et al., 1996; Lister et al., 2002; Hirata et al., 2003).

In zebrafish, pigmentation of the skin begins at approximately 24 hpf in the melanophores located dorsolaterally (Kimmel et al., 1995). The pigment cells quickly expand, and within a few hours they constitute a prominent characteristic of the embryo (Kimmel et al., 1995). By around 4 dpf, an embryonic/early larval pattern has formed with melanophore stripes and widespread xanthophores (McMenamin et al., 2014). Between 3 and 5 weeks of age, the pattern starts changing into that of the adult with dark stripes, containing black melanophores and silvery iridophores, and light inter-stripes containing xanthophores and iridophores (McClure, 1999; McMenamin et al., 2014).

Fig. 6. Adult zebrafish

Development of pigmentation appears to be partly regulated by THs, which coordinate the development of various pigment cell types and regulate the transcriptional activity of genes responsible for pigment synthesis (Walpita et al., 2009; McMenamin et al., 2014; Guillot et al., 2016; Saunders et al., 2019; Prazdnikov, 2020). For example, in zebrafish TH drives melanophores into a terminally differentiated state (Saunders et al., 2019) and possibly regulates mRNA expression of tyrosinase, the key enzyme in melanin synthesis (Korner and Pawelek, 1982; Camp and Lardelli, 2001; Walpita et al., 2009; Guillot et al., 2016). The effects of T3 treatment of zebrafish melanogenesis appear to vary with age. While T3 causes increased melanisation at early life stages (Walpita et al., 2007), it induces skin paling in adult zebrafish (Guillot et al., 2016). Furthermore, THs have been shown to regulate zebrafish melanogenesis in a gender-specific manner (Guillot et al., 2016).

Melanophore skin pigmentation is an easily observable trait, which can be assessed by image analysis in all life stages.

Exposure to THS disrupting chemicals has been shown to generally reduce melanophore skin pigmentation in fish.

4.3.4.1. NIS inhibition

As described in section 4.2.1.4, perchlorate reduced the development of skin pigmentation in juvenile zebrafish exposed from 3-33 dpf (Mukhi et al., 2007) or from 14-65 dpf (Brown, 1997); in fathead minnows exposed from 0-28 dpf (Crane et al., 2005); and in threespine sticklebacks spawned and raised to sexual maturity in perchlorate treated water (Bernhardt et al., 2011). However, no effects on the skin pigmentation were observed in larval Chinese rare minnows (Li et al., 2011b) exposed to perchlorate from 3-24 dpf.

4.3.4.2. TPO inhibition

PTU reduced skin pigmentation in 28 dph juvenile F1 zebrafish (van der Ven et al., 2006). Likewise, 2-mercaptobenzothiazole inhibited the skin pigmentation of zebrafish embryos at 35 and 55 hpf (Choi et al., 2007) and 5 dpf (Stinckens et al., 2016). Furthermore, phenylthiourea reduced pigmentation in zebrafish embryos at 35 and 55 hpf (Choi et al., 2007) and 3 dpf (Li et al., 2012). The action of phenylthiourea on skin pigmentation in early life stages of zebrafish has been proposed to be due to its ability of tyrosinase inhibition (Choi et al., 2007; Li et al., 2012).

4.3.4.3. Interaction with TH binding

Exposure to 6-OH-BDE-47 reduced pigmentation in 30 hpf and 6 dpf zebrafish (Macaulay et al., 2015). However, exposure at a later stage, from 9-23 dpf, to 6-OH-BDE-47 did not significantly affect skin pigmentation (Macaulay et al., 2017).

4.3.4.4. Undetermined or mixed MIEs

BPF exposure caused a strong reduction in pigmentation in zebrafish at 2 dpf, while exposure to BPA, BPS and bisphenol AF did not have significant effects on pigmentation (Mu et al., 2018). However, parental BPS exposure significantly reduced lateral stripe melanocyte pigmentation in F1 zebrafish at 4 dpf (Wei et al., 2018).

4.3.4.5. General discussion and conclusions on skin pigmentation as endpoint for THS related disruption

Skin pigmentation functions in kin recognition, shoaling, mate choice, UV radiation protection and camouflage (Engeszer et al., 2008; Mueller and Neuhauss, 2014; Singh and Nüsslein-Volhard, 2015), and disturbed skin pigmentation may thus cause population-relevant adverse effects.

Skin pigmentation appears to be a responsive THS related endpoint in zebrafish. Reduced pigmentation was observed by exposure to chemicals acting through NIS inhibition, TPO inhibition and interaction with TH binding in zebrafish. However, skin pigmentation showed differing responses to exposure to bisphenols.

Though skin pigmentation may be responsive to THS disruption, it may not be specific to THS disruption. Other signalling systems, such as those of insulin, melanocyte-stimulating hormone and melanin-concentrating hormone (Kawauchi and Baker, 2004; Logan et al., 2006; Zhang et al., 2018b; Patterson and Parichy, 2019), as well as many non-endocrine factors (Fujii, 2000; Vissio et al., 2021), have also been shown to affect skin pigmentation in fish.

4.3.5. Swim bladder development and inflation

At around 3-7 dpf (Liu and Chan, 2002; Verbueken et al., 2018; Vergauwen et al., 2018), depending on rearing conditions (Wilson, 2012), zebrafish undergo an embryonic-to-larval transition. In zebrafish, this transition includes inflation of the posterior swim bladder chamber. The transition depends on TH activity (Liu and Chan, 2002; Chang et al, 2012), and may be particularly vulnerable to THS disrupting chemicals (Walter et al., 2019c).

The swim bladder is a flexible gas-filled sac which in most fish species, including the zebrafish, is divided into two connected chambers, a posterior chamber and an anterior chamber. In zebrafish, the posterior chamber is inflated 4-5 dpf and the anterior chamber is developed around 20-21 dpf as an evagination from the posterior chamber (Robertson et al., 2007; Winata et al., 2009; Stinckens et al., 2020). Active control of the swim bladder in zebrafish occurs at around 30 dpf (Robertson et al., 2007). Both chambers are responsible for buoyancy; and in ostariophysians (including zebrafish) the anterior chamber furthermore functions as a resonance chamber thus playing a role in sound detection. Vibrations from the swim bladder are transmitted to the inner ear, through a chain of bones (the Weberian ossicles) acting as an amplifier of sound waves and thus enhancing hearing (Bird et al., 2020). By regulating the volume of gas in the swim bladder, fish can attain neutral buoyancy at any depth, thereby minimising the swimming effort expended to keep the vertical position in the water (Lindsey et al., 2010). Impaired swim bladder inflation is, therefore, likely to affect swimming performance and hearing, which may influence reproduction, feeding and predator avoidance, consequently reducing survival (Czesny et al., 2005; Stinckens et al., 2020).

The inflation of the swim bladder in fish is controlled by THs. Disruption of TH levels during embryonic development by combined knockdown of *dio1* and *dio2* or knockdown of *dio3b* in zebrafish interfered with normal inflation of the posterior swim bladder chamber in embryonic zebrafish 4 dpf (Bagci et al., 2015). Furthermore, in both zebrafish and fathead minnow, a peak was observed in the expression levels of *dio1*, and in zebrafish a peak of *dio2* and *dio3* expression was additionally observed, at the time of posterior swim bladder inflation (Vergauwen et al., 2018). Reports of expression of *dio2* (Dong et al., 2013), *thraa* and *thrb* (Marelli et al., 2016) specifically in the zebrafish swim bladder tissue around the time of posterior chamber inflation further corroborate the role of THs in swim bladder inflation.

The swim bladder is relatively easy to observe in the semi-transparent fish larvae, and the size of the swim bladder can be derived from photographs of live fish using digital image analysis. In zebrafish, this has been done up to 32 dpf (Stinckens et al., 2020). However, in fathead minnows at 21 dpf the musculature is developed to an extent at which it is no longer possible to examine the swim bladders externally (Cavallin et al., 2017). Therefore, swim bladder size may be derived from digital image analysis of histological sections in juvenile and adult fish, although ruptures during histological processing may complicate this. Alternatively, an early non-lethal subsampling in which the fish are digitally photographed and put back into the exposure tanks may be employed in the chronic tests.

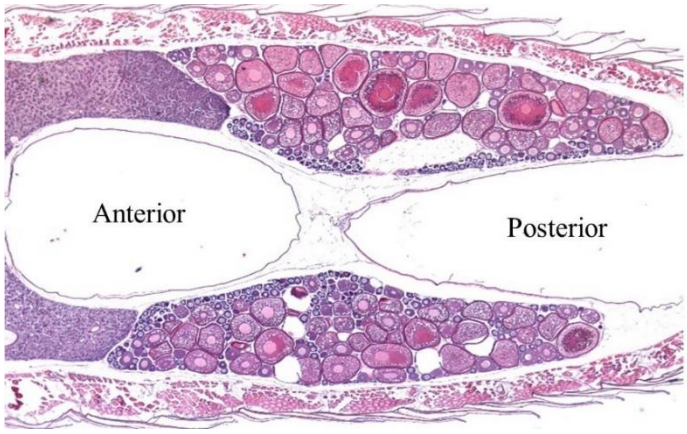

Fig. 7. Transverse section of zebrafish showing the anterior and posterior swim bladder chambers

4.3.5.1. Posterior swim bladder

In zebrafish, the posterior swim bladder is inflated 4-5 dpf and has a key function in the earliest coordinated behaviours of larvae (Lindsey et al, 2010).

Various chemicals have been shown to affect the development and inflation of posterior swim bladders in fish.

Embryo tests

Effects on the posterior chamber in short-term embryo tests are dependent on the underlying MIEs of THS disruption, probably because of the presence of maternally derived T4.

4.3.5.2. NIS inhibition

Upon exposure to sodium perchlorate from 0-5 dpf, deflated swim bladders were observed in 12% of zebrafish embryos (Jomaa et al., 2014).

4.3.5.3. TPO inhibition

The TPO inhibitor methimazole did not significantly affect the surface area of the posterior swim bladder chamber in zebrafish at 5 dpf (Liu and Chan, 2002; Stinckens et al., 2020), at 6 dpf (Godfrey et al., 2017) or at 7 dpf (Stinckens et al., 2018). Likewise, exposure to 2-mercaptobenzothiazole did not impair posterior chamber inflation in zebrafish 5 dpf (Stinckens et al., 2016; 2020) or in fathead minnow at 6 dpf (Nelson et al., 2016). These results indicate that embryonic TPO activity may not be required for inflation of the posterior chamber of the swim bladder. This is probably owing to the presence of sufficient maternally derived T4 during early development.

At 5 dpf, the posterior swim bladder chamber surface area was not affected after exposure to 37 or 111 mg/L PTU (Stinckens et al., 2020). However, exposure to PTU resulted in impaired posterior chamber inflation (EC50: 259 mg/L) and significantly decreased surface area at 259 mg/L in zebrafish in a FET test based on TG 236 (OECD, 2013b; Stinckens et al., 2018). These results may indicate that the combined inhibitory effects of PTU on TPO and DIO are strong enough to cause effects on the posterior swim bladder at this concentration.

Fetter et al. (2015) observed non-inflated swim bladders in zebrafish exposed to methimazole or phenylthiourea from 2-5 dpf. However, the malformations were observed relatively close to concentrations causing lethality and hence may also represent secondary effects caused by an unspecific toxicity.

4.3.5.4. DIO inhibition

Exposure to iopanoic acid, 2,4,6-tribromophenol, or salicylic acid resulted in impaired posterior chamber inflation in zebrafish 5 dpf in a FET test based on TG 236 (OECD, 2013b; Stinckens et al., 2018). If posterior chambers were inflated, the surface area was smaller when exposed to 2,4,6-tribromophenol, or salicylic acid (Stinckens et al., 2018). Likewise, the relative posterior chamber surface area was significantly reduced in zebrafish exposed to iopanoic acid from 0-5 dpf (Stinckens et al., 2020). Although 8-anilino-1-naphthalene sulfonic acid, a DIO inhibitor, did not completely prevent the posterior chamber to inflate, it likewise significantly reduced the posterior chamber surface area (Stinckens et al., 2018).

In fathead minnow, iopanoic acid treatment caused significant reductions in both incidence and length of inflated posterior swim bladders at 6 dpf (Cavallin et al., 2017).

4.3.5.5. Interaction with TH binding

A significant decrease in the surface area of the posterior swim bladder chamber was observed after exposure of zebrafish to T3, TDCPP or PFOA from 0-6 dpf (Godfrey et al., 2017).

4.3.5.6. Undetermined or mixed MIEs

Amiodarone hydrochloride led to deflated swim bladders in 35% of zebrafish embryos exposed from 0-5 dpf (Jomaa et al., 2014). Similarly, exposure to S-metolachlor from 0-5 dpf significantly caused non-inflated swim bladders in a zebrafish embryo test (Rozmánková et al., 2020). Likewise, zebrafish exposed to triadimefon from 0-5 days exhibited non-inflated swim bladders (Liu et al., 2011b). Additionally, zebrafish exposed to the flame-retardant tris(2,3-dibromopropyl)isocyanurate significantly showed non-inflated posterior swim bladder chambers 7 dpf (Li et al., 2011a). Moreover, exposure to triclocarban delayed swim bladder development in 90% of zebrafish 5 dpf (Dong et al., 2018).

Non-inflated posterior swim bladder chambers were also observed in several studies following exposure to PFOS for different time periods (up to 7 dpf) during zebrafish embryonic development (Shi et al., 2008; Huang et al., 2010; Hagenaars et al., 2011; 2014; Chen et al., 2014; Sant et al., 2017). Likewise, the percentages of embryos with non-inflated swim bladders significantly increased in zebrafish at 5 dpf after exposure to PFOA, PFO3OA, PFO4DA and PFO5DoDA (Wang et al., 2020). Furthermore, exposure to PFOA or BPA resulted in impaired posterior chamber inflation in zebrafish at 7 dpf (Stinckens et al., 2018). If posterior chambers were inflated, the surface area was smaller. Likewise, a significant decrease in the surface area of the posterior swim bladder chamber was observed after exposure of zebrafish to PFBA from 0-6 dpf (Godfrey et al., 2017).

Peng et al. (2020) showed that embryonic exposure to the emerging flame retardant pentabromobenzene in various exposure windows up to 4.5 dpf significantly inhibited the inflation of posterior swim bladders in zebrafish. The underlying mechanisms among thyroid and adrenocortical (glucocorticoid) disruptions as well as the prolactin pathway were examined. Reduced T3 levels were found in pentabromobenzene exposed zebrafish larvae at 5 dpf. The mRNA levels of *crh*, which in fish appears to stimulate TSH secretion (De Groef et al., 2006), were upregulated, indicating a feedback regulation by reduced T3 levels. However, the transcriptional profile of other genes involved in the synthesis (*tsh*), biological conversion (*ugt1ab, dio2*) and function (*thra*, *thrb*) of THs showed no significant changes (Peng et al., 2020). Pikulkaew et al. (2011) showed that the glucocorticoid pathway may play a role in swim bladder inflation, since the knockdown of maternal glucocorticoid receptor mRNA significantly increased the number of zebrafish progeny lacking an inflated gas bladder at 5 dpf. This is supported by the observation of reduced swim

bladder inflation in zebrafish embryos treated with a glucocorticoid receptor antagonist (RU486) (Wilson et al., 2013). However, the transcription of the glucocorticoid receptor gene showed no significant changes in the study by Peng et al. (2020), indicating that glucocorticoid and its receptor was not involved in pentabromobenzene induced inhibition of swim bladder inflation. Conversely, the results suggested that pentabromobenzene exposure significantly changed the expression of genes involved in prolactin receptor pathway and in ion transport, indicating a role of these pathways in pentabromobenzene induced posterior bladder deflation in zebrafish. Supporting this, knockout of the prolactin gene caused failure in swim bladder inflation and changes of ion transporter expression in zebrafish larvae; with recovery of both phenotypes by prolactin mRNA injection (Shu et al., 2016). These findings could indicate that the effects of pentabromobenzene on the swim bladder may not be primarily THS related.

Long-term tests

Whereas the size of the posterior swim bladder was generally reduced by chemical exposure in embryos, the size of the posterior swim bladder is generally increased in long-term tests. For example, the lengths of the posterior swim bladders were significantly increased in zebrafish 32 dpf after treatment with 2-mercaptobenzothiazole (Stinckens et al., 2016), and in fathead minnows 14 and 18 dpf after iopanoic acid treatment (Cavallin et al., 2017); or 14, 18 or 22 dpf after 2-mercaptobenzothiazole treatment (Nelson et al., 2016). A plausible explanation of the increased posterior swim bladder length is disrupted evagination of the anterior swim bladder from the posterior swim bladder (Cavallin et al., 2017). This theory is supported by the results from a FELS test based on OECD TG 210 (OECD, 2013a), which showed increasing surface area of the posterior chamber with decreasing anterior chamber surface in zebrafish exposed to methimazole, iopanoic acid or PTU (Stinckens et al., 2020). This suggests a possible compensatory mechanism, and the sum of both swim bladder surfaces was found to be equal to controls for most treatments. However, when the anterior chamber remained non-inflated, the total surface of both chambers did, for most treatments, not reach control levels, although the posterior chamber surface area increased significantly. Further studies are needed to confirm the association between posterior and anterior swim bladder chamber size, ascertain causality and elucidate the underlying mechanisms.

Transgenerational effects

Shi et al. (2019) examined transgenerational effects of F-53B, an alternative to PFOS, on the swim bladders of zebrafish. Parental exposure to F-53B for 180 days caused an increase in the occurrence of non-inflated posterior swim bladders in F1 larvae at 5 dpf. Although no significant change was found in TH levels, the percentage of non-inflated posterior swim bladders was also significantly increased in F2 larvae. Furthermore, the percentage of F1 zebrafish with fully inflated swim bladders at 4 dpf significantly decreased after parental exposure to BPS from 2 hpf to 120 dpf (Wei et al., 2018).

4.3.5.7. Anterior swim bladder

In zebrafish, the anterior swim bladder is developed around 20-21 dpf as an evagination from the posterior chamber.

Various chemicals have been shown to affect the development and inflation of anterior swim bladders in fish.

4.3.5.8. NIS inhibition

Retarded swim bladder inflation was observed in larval Chinese rare minnow after perchlorate treatment for 21 days (Li et al., 2011b).

4.3.5.9. TPO inhibition

The effects of PTU, methimazole and 2-mercaptobenzothiazole on the inflation of the anterior swim bladder in zebrafish have been examined in FELS tests based on OECD TG 210 (OECD, 2013a).

Exposure to PTU resulted in significantly increased percentages (approximately 25-50%) of the zebrafish larvae having non-inflated anterior swim bladder chambers at 18 dpf (Stinckens et al., 2020). However, at the end of the experiment at 32 dpf, the percentages of fish with inflated anterior swim bladders were not significantly different between the control and treated fish. PTU furthermore significantly decreased the surface area of the anterior chambers. This effect persisted throughout the experiment.

Exposure to methimazole resulted in none of the zebrafish larvae having inflated anterior swim bladder chambers at 23 dpf compared to 100% of the control larvae having inflated anterior swim bladders (Stinckens et al., 2020). At the end of the experiment at 32 dpf, over 80% of the methimazole exposed larvae still had non-inflated anterior chambers. Methimazole also significantly decreased the surface area of the anterior chambers at 30 and 32 dpf. In another experiment, exposure to methimazole from 0-28 dpf, resulted in approximately 60% of the zebrafish not developing anterior swim bladder chambers (Godfrey et al., 2017).

Exposure to 2-mercaptobenzothiazole, resulted in significantly impaired anterior swim bladder inflation in zebrafish 20-32 dpf (Stinckens et al., 2016). At 21 dpf, 91% of the control larvae had an inflated anterior swim bladder, while only 34% of the larvae exposed to 0.35 mg/L 2-mercaptobenzothiazole had inflated anterior swim bladders. At 32 dpf, 22% of the larvae in this exposure group had non-inflated anterior swim bladders. Anterior swim bladder inflation in zebrafish was affected at lower 2-mercaptobenzothiazole concentrations than those required to impact anterior inflation in fathead minnows, where exposure to 0.5 mg/L 2-mercaptobenzothiazole did not significantly affect anterior swim bladder inflation at 6, 14 or 21 dpf (Nelson et al., 2016). By contrast, approximately 50% of the fish exposed to 1 mg/L 2-mercaptobenzothiazole did not have an inflated anterior swim bladder at 14 dpf. Yet at 21 dpf, all fish had inflated anterior swim bladders. However, even though inflation could occur, exposure to 2-mercaptobenzothiazole consistently reduced relative anterior chamber size throughout the experiments with both zebrafish and fathead minnows. Thus, it appears that although compensation may cause the fathead minnows to ultimately inflate their anterior swim bladders, these do not develop as normal.

4.3.5.10. DIO inhibition

In a 32-day FELS test based on OECD TG 210 (OECD, 2013a), exposure to 0.35, 1 and 2 mg/L iopanoic acid resulted in 35, 43 and 61%, respectively, of the zebrafish larvae having non-inflated anterior swim bladder chambers at 21 dpf, and at the end of the experiment approximately 25% of the larvae in each iopanoic acid exposure group still had impaired anterior chamber inflation (Stinckens et al., 2020). Iopanoic acid also affected the surface area of both the anterior chamber and the posterior chamber. In all cases where the anterior chamber was inflated, its surface area was significantly smaller compared to control larvae, and the posterior chamber surface increased with decreasing anterior chamber surface.

Both incidence of inflation and length of anterior swim bladder were significantly reduced in fathead minnows at 14 dpf after exposure to iopanoic acid, but inflation recovered by 18 dpf (Cavallin et al., 2017). However, anterior swim bladder length was still significantly decreased in the exposed fish at 18 and 21 dpf. Inflation of the anterior chamber was at least 10 times more sensitive to iopanoic acid than inflation of the posterior chamber (Cavallin et al., 2017).

4.3.5.11. Interaction with TH binding

After exposure from 0-28 dpf, approximately 30-50% of the zebrafish exposed to TDCPP, PFOA or PFBA did not develop anterior swim bladder chambers (Godfrey et al., 2017). However, the surface areas of the anterior swim bladders that did develop in exposed zebrafish were similar to controls.

Furthermore, a significant reduction in swim bladder score (based on the presence of one or two chambers) was observed 23 dpf for zebrafish treated with the TR agonist 6-OH-BDE-47 (Macaulay et al., 2017).

4.3.5.12. Single swim bladder in medaka

The swim bladder of the medaka is a single chamber, that begins to develop at 3 dpf (Godfrey et al., 2019). Sex-specific effects on the swim bladder were observed after exposure to T3, methimazole, TDCPP, PFOA and PFBA (Godfrey et al., 2019). T3, methimazole, TDCPP and PFBA resulted in an increase (approximately 30-50%) in females failing to inflate a swim bladder at 10 dpf, whereas all control and PFOA exposed females developed a swim bladder. For those females that developed a swim bladder, exposure to methimazole, TDCPP and PFOA caused a significantly increased surface area of the swim bladders, whereas T3 caused a significant decrease in the surface area of the swim bladders. Like in females, all control males developed swim bladders. However, exposure to all the tested chemicals, i.e. T3, methimazole, TDCPP, PFOA and PFBA, resulted in an increase in the percentage of fish (10-60%) that did not inflate a swim bladder. In contrast to females, none of the chemicals significantly affected the surface areas of the swim bladders in males. Thus, in medaka females appear more susceptible to THS related swim bladder disruption than males.

4.3.5.13. Genes related to swim bladder inflation

Proteins that prevent the swim bladder from deflating by lowering the surface tension include *surfactant protein a* (*sp-a*) also known as *lectin mannose binding 2* (*lman2*), *surfactant protein b* (*sp-b*) also known as *prosaposin* (*psap*) and *surfactant protein C* (*sp-C*) also known as *tendomodulin* (*tnmd*) (Godfrey et al., 2017). A few studies have examined the effects of chemicals on expression of surfactant protein genes. However, no clear response patterns to THS disrupters can be deduced from these studies.

In zebrafish 6 dpf, the expression of *sp-a* was significantly upregulated after exposure to TDCPP, PFOA, PFBA, or DOPO, while no significant effect on *sp-a* expression were observed for methimazole or TBBPA (Godfrey et al., 2017). Similarly, the expression of *sp-c* was significantly upregulated after exposure to TDCPP, PFOA or PFBA, while no significant effects were observed on *sp-c* expression for the other chemicals. A significant downregulation of the expression of *sp-b* was observed after exposure to DOPO, while no significant effects on *sp-b* expression were observed for the other chemicals. Similar effects on the expression of *sp-b* were observed at 28 dpf. The expression of *sp-a* was still significantly upregulated at 28 dpf after exposure to PFOA or DOPO, while it was significantly downregulated by exposure to methimazole, and no significant effects were observed after exposure to the other chemicals. Like at 6 dpf, the expression of *sp-c* was still significantly upregulated after exposure to TDCPP or PFOA at 28 dpf, while no significant effects were observed on *sp-c* expression for the other chemicals. The authors suggested that the observed general upregulation of the surfactant genes in the swim bladders was a compensatory response to the non-inflated swim bladders observed in the exposed fish.

Additionally, changes in the expression of genes encoding surfactant proteins were observed in F1 zebrafish larvae derived from parents exposed to BPS from 2 hpf to 120 dpf (Wei et al., 2018). Expression levels of *sp-a* and *sp-c* were significantly decreased in F1 larvae at 4 dpf, while those of *sp-b* were not significantly affected.

4.3.5.14. Swim bladder histopathology

Examination of the epithelium of the swim bladder did not reveal any significant pathology in fathead minnows exposed to 2- mercaptobenzothiazole for 14 or 21 days (Nelson et al., 2016).

Likewise, no pathologies were detected in the structure and organisation of the cellular layers of the swim bladder in fathead minnows exposed to iopanoic acid (Cavallin et al., 2017).

4.3.5.15. Adverse outcome pathways (AOPs) linking THS disruption to impaired swim bladder inflation

An adverse outcome pathway (AOP) is a conceptual construct that describes existing scientific information linking a direct MIE and an adverse effect at the individual level (for human health) or population level (for ecological health) via one or more biological KE(s) (Ankley et al., 2010; OECD, 2018). The AOP Wiki serves as the primary repository of qualitative information for the international AOP development effort coordinated by the OECD (http://aopwiki.org/). AOPs under development are continuously included and updated in the AOP Wiki, which already includes a number of proposals of AOPs linking THS disrupting MIEs to adverse outcomes. For fish, especially AOPs linking THS disrupting MIEs to impaired swim bladder inflation are well described and their further development has been included in the OECD Work Plan (AOP ID 155–159, http://aopwiki.org/aops). The AOPs include MIEs (e.g. DIO or TPO inhibition), main intermediate KEs (reduced TH levels and swim bladder inflation) and reduced swimming performance as the adverse outcome (Knapen et al., 2020). The individual AOPs can be combined into an AOP network that links the different MIEs to a common population-relevant adverse outcome through shared KEs as shown in Fig. 8.

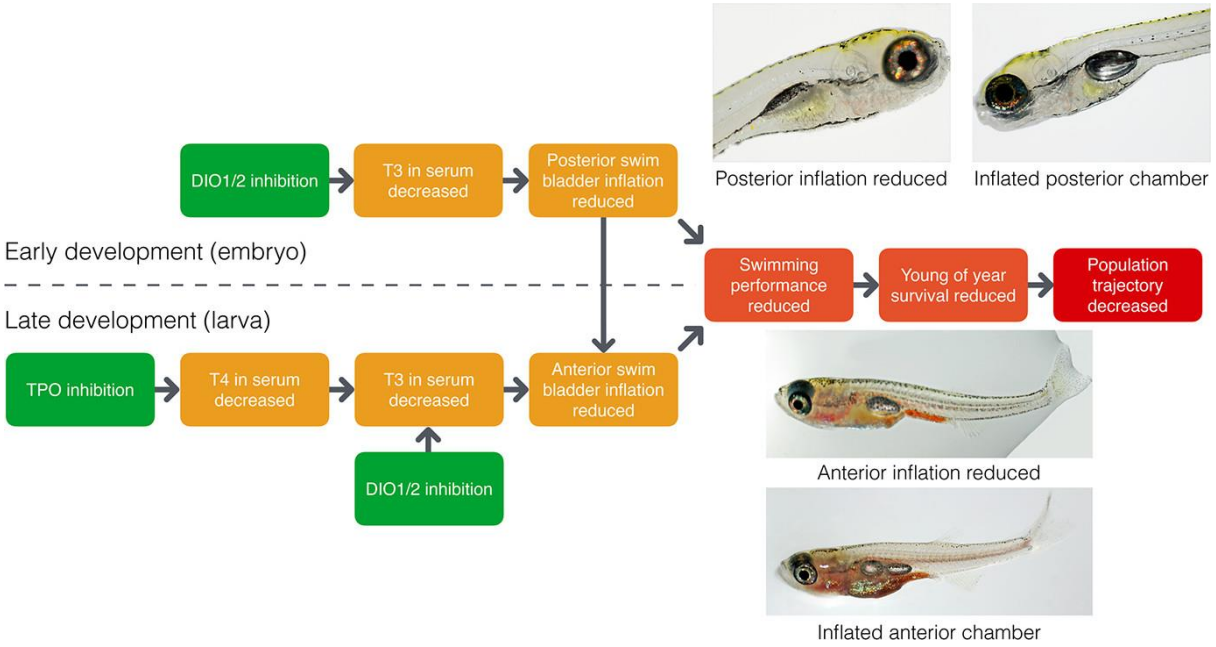

Fig. 8. AOP network related to swim bladder inflation in fish (Knapen et al., 2020)

4.3.5.16. General discussion and conclusions on swim bladder development and inflation as endpoint for THS related disruption

The effects of THS disrupting chemicals on swim bladder development depends on the underlying MIEs. TPO inhibition appears relevant only during late swim bladder development, after depletion of maternally derived T4. Thus, usually no effects are observed of TPO inhibition on the development of the posterior chamber in embryos, while the development of the larval anterior chamber is impaired by TPO inhibition. In contrast, DIO inhibition already impairs posterior chamber inflation during early development (since

conversion of T4 to T3 would still be required although maternally derived T4 is present), and consequently, additionally prevents anterior chamber inflation at later life stage. Furthermore, the NIS inhibitor, perchlorate, as well as chemicals suggested to interfere with TH binding also reduce anterior swim bladder inflation. Thus, anterior swim bladder inflation is affected by the four key MIEs NIS inhibition, TPO inhibition, DIO inhibition and interaction with TH binding, and could, therefore, be a promising endpoint for detection of THS disruption in fish. However, various degrees of recovery of the incidence of inflation of the anterior swim bladder are seen during 32-days FELS tests with zebrafish. This could reduce the applicability of this endpoint in OECD TG 234 which runs for 60 days. On the other hand, the size of the anterior swim bladder was still reduced at 32 dpf in the FELS tests, which could hold promise for the applicability of this endpoint in OECD TG 234. However, it remains to be examined if the effects on this endpoint persist beyond 32 dpf.

A potential drawback in the use of swim bladder inflation as an endpoint for THS disruption is that other chemical MOAs as well as biological perturbations may also affect swim bladder inflation (Trotter et al., 2003a; 2003b; Woolley and Qin, 2010; Villeneuve et al., 2014; Price et al., 2020). Studies suggest that effects on swim bladder inflation may occur through additional regulators, including the glucocorticoid receptor pathway (Pikulkaew et al., 2011; Wilson et al., 2013), the growth hormone and prolactin superfamily (Shu et al., 2016; Peng et al., 2020), and the IGF-1 signalling pathway (Molla et al., 2019). Moreover, effects on swim bladder inflation may result from general toxicity, secondary to heart failure (Yue et al., 2015) or from mechanical pressure from the yolk due to embryonic malabsorption syndrome with very little yolk consumption (Raldúa et al., 2008). Swim bladder inflation may thus not be a thyroid specific endpoint. Considering the influence of other types of toxicity on swim bladder inflation, dose level selection and the potency of the chemical to cause toxic effects other than THS disruption may confound the outcome and affect reliability of the results. However, swim bladder inflation could be used as an indicator of potential THS disruption and may be used in combination with other endpoints to assess THS disrupting chemicals. In this context, AOP networks potentially present a valuable tool.

4.3.6. Skeletal development

The zebrafish skeleton consists of an exoskeleton (dermal skeleton) and an endoskeleton. Scales and fin rays are part of the exoskeleton, while the endoskeleton consists of cranial, axial and appendicular skeletal elements (Tonelli et al., 2020). The appendicular skeleton includes the pectoral and pelvic girdles and the endoskeleton of the fins (Johanson, 2010). The axial skeleton includes the vertebral column and median fins skeleton (Bird and Mabee, 2003). The four most anterior vertebrae form part of the Weberian apparatus (Bird and Mabee, 2003).

THs are essential for normal skeletal development and regulates proper morphogenesis and ossification in most zebrafish bones. Numerous skeletal structures have been shown to be affected in hyperthyroid and particularly in hypothyroid fish compared to wildtypes (Keer et al., 2019). THs have been shown to influence the development of craniofacial structures, some elements of the Weberian apparatus, fins and scales.

Tight regulation of TH levels during embryogenesis is critical for proper craniofacial development since TH regulates the expression of genes important in the development of craniofacial structures (Bohnsack and Kahana, 2013).

THS disrupting chemicals have been shown to cause craniofacial malformations in fish. Zebrafish larvae derived from parents exposed to perchlorate showed significantly affected craniofacial development at 4 dpf (Mukhi and Patiño, 2007). Craniofacial malformations were also observed in zebrafish exposed from 2-5 dpf to methimazole or phenylthiourea (Fetter et al., 2015). Likewise, significant effects on craniofacial development were observed in 5 dpf zebrafish co-treated with methimazole and amiodarone (Liu and Chan, 2002). Furthermore, both TBBPA and BDE-47 exposure caused craniofacial deformities at 4 dpf (Parsons et al., 2019). Moreover, significant craniofacial abnormalities were observed in 6 dpf zebrafish

following 6-OH-BDE-47 treatment (Macaulay et al., 2015). Craniofacial abnormalities may lead to impaired foraging efficiency which could limit growth and survival during critical life stages.

However, craniofacial malformations are not specific to THS disruption but could also be caused by other MOA. THs have been reported to work alongside retinoic acid to regulate cranial neural crest development, which is critical for craniofacial development (Bohnsack and Kahana, 2013). Furthermore, T3 and T4 treatment improved, but did not completely rescue the craniofacial defects induced by phenylthiourea, suggesting that the effects may be only partly due to THS disruption (Bohnsack et al., 2011). Craniofacial malformations may additionally arise from unspecific toxicity and non-endocrine MOA (Raterman et al., 2020).

The Weberian apparatus includes the four most anterior vertebrae with their modified elements, i.e. the Weberian ossicles and the skeletal elements supporting them (Kapitanova and Shkil, 2014; Keer et al., 2019). The Weberian apparatus connects the swim bladder to the inner ear in otophysan fish, including zebrafish, thus transmitting sound/vibration and enhancing hearing (Bird et al., 2020). In zebrafish, the initial formation of the Weberian ossicles is seen histologically in the early larval stages (Bird et al., 2020), while ossification of the Weberian ossicles occurs about 22 dpf (Grande and Young, 2004).

The size, shape and ossification of the Weberian ossicles and other elements of the Weberian apparatus have been shown to be highly sensitive to abnormal developmental TH levels (Kapitanova and Shkil, 2014; Keer et al., 2019).

Since the size of the ossicles (and swim bladders) have been proposed to influence hearing ability (Lechner and Ladich, 2008), THS disruption may lead to hearing impairment in fish, which may leave them vulnerable to predators and thus reduce their survival.

Effect on the development of fins and scales are discussed separately in section 4.3.7 and section 4.3.8, respectively.

4.3.7. Fin development

Zebrafish have two paired fin types (pectoral and pelvic) and three median fin types (caudal, anal and dorsal). During zebrafish embryogenesis, a fin fold develops at approximately 22 hpf (van Eeden et al., 1996), and pectoral fin buds appear at approximately 28 hpf (Murata et al., 2010). While the pectoral fins are maintained throughout development, the fin fold disappears and is replaced by three distinct median fins, the caudal, anal and dorsal fins, during the larval-to-juvenile transition (van Eeden et al., 1996; Monnot et al., 1999). Among the median fins, the caudal fin develops first, followed by the anal and lastly the dorsal fin (Bird and Mabee, 2003). In addition, the paired pelvic fin buds begin to develop during the third wpf (Grandel and Schulte-Merker, 1998; Monnot et al., 1999; Shkil et al., 2010). The development of the fins is completed by the formation of multi-segmented fin rays, which define the adult fin shape (Goldsmith et al., 2006; Singleman and Holtzman, 2014).

Fin development and growth are at least partially dependent on THs (Brown, 1997; Shkil et al., 2012; Sharma et al., 2016), and have been shown to be affected by THS disrupting chemicals in larval and juvenile fish.

Compromised fin development may lead to decreased swimming performance and prey capture, along with increased risk of predation and mortality.

The analysis of fin development can be made from digital photos taken of anesthetised or preserved fish, whole-mounts, or histological sections using microscopy. Samples can be stained with alcian blue which stains cartilage and alizarin red which stains bone.

4.3.7.1. Caudal fin

The caudal fin is the first of the unpaired median fins to develop in zebrafish (Bird and Mabee, 2003). The development of the caudal fin begins at approximately 2 wpf and continues through the larval-to-adult transition (Goldsmith et al., 2006). The caudal fin begins development as a rounded paddle with no defined fin rays (Singleman and Holtzman, 2014). As the fin rays develop, the shape of the caudal fin transforms to a forked, bi-lobed shape, resulting from shorter fin rays in the cleft and longer fin rays in the dorsal and ventral lobes (Goldsmith et al., 2006; Singleman and Holtzman, 2014). The individual fin rays begin to bifurcate at the larval-to-juvenile transition (Singleman and Holtzman, 2014). As the fish grows to adulthood, the caudal fin rays bifurcate once more as they continue to lengthen (Singleman and Holtzman, 2014). The zebrafish caudal fin has 18-24 (Singleman and Holtzman, 2014) segmented primary fin rays in which segment length decreases from the proximal to the distal positions (Iovine and Johnson, 2000; Christou et al., 2018). A series of short, unsegmented and unbranched procurrent rays flank the primary rays on the dorsal and ventral edges of the caudal fin. The zebrafish usually develop 6 dorsal procurrent rays and 5 ventral procurrent rays in the caudal fin (Bird and Mabee, 2003).

The development of the caudal fin in zebrafish is affected by changes in TH levels. T3 exposure was shown to cause a reduction in primary and procurrent fin ray numbers in zebrafish and the large hexaploid African barb (*Labeobarbus intermedicus*) (Shkil et al., 2012). In studies with euthyroid, hyperthyroid and hypothyroid zebrafish, both the number of procurrent rays and the procurrent ray density appeared to scale inversely with developmental TH level; i.e. hyperthyroid fish appeared to have fewer procurrent rays, while the procurrent rays in hypothyroid fish appeared to be more numerous and more robust (Hu et al., 2020). TH was furthermore proposed to regulate fin morphology in zebrafish by modulating the position of fin ray branching and the fin ray segment length (Hu et al., 2020). The total ray length at the primary bifurcation and the ratio of distal segment length to proximal segment length were decreased in hyperthyroid and increased in hypothyroid compared to euthyroid fish. However, the study used different zebrafish lines for the euthyroid, hypothyroid and hyperthyroid condition, and it remains to be examined if similar results can be obtained by exposure to THs and THS disrupting chemicals using a single zebrafish line.

Exposure to 6-OH-BDE-47 from 9-23 dpf significantly reduced caudal fin area (by 26-38%) in zebrafish (Macaulay et al., 2017). In addition, 15% of the zebrafish evaluated for morphology after exposure to a 6PBDE/OH-BDE mixture from 9-23 dpf followed by a 22-day depuration period exhibited vastly stunted caudal fin ray development at 45 dpf. This phenotype appeared to be specific to the caudal fin, as no malformations were noted in the anal, dorsal, pectoral or pelvic fins.

4.3.7.2. Anal fin

The anal fin is an unpaired median fin positioned on the ventral surface of the body, posterior to the anus (Bird and Mabee, 2003). Anal fin development begins with anal fin condensation followed by anal fin ray appearance (Parichy et al., 2009). As the fish grows, the anal fin lengthens, and the fin rays thicken and bifurcate at the larval-to-juvenile transition (Singleman and Holtzman, 2014). A second anal fin ray bifurcation occurs as the fish grows to adulthood. The zebrafish has 12-14 anal fin rays (Singleman and Holtzman, 2014).

After T3 treatment of zebrafish from 0-25 dpf, the total number of rays in the anal fin was reduced from 13 to 11 in some of the fish (Shkil et al., 2019). Conversely, T4 treatment from 3 dpf stimulated the differentiation of anal fins and the formation of anal fin rays in goldfish (*Carassius auratus*) (Reddy and Lam, 1992). After 8 days of treatment, the anal fins were differentiated from the fin fold in all the treated fish, whereas in the control group the fin fold remained continuous without any differentiation of fins in the majority of the fish. After 15 days of treatment, the number of anal fin rays was significantly higher in all T4 treated groups compared to the controls. Furthermore, fin rays in the anal fin of the large African barb (*Barbus intermedius sensu*) appeared earlier in T3 exposed fish than in controls (Shkil et al., 2010). T3

exposure further resulted in drastic curving and sometimes distal fusion of anal fin rays in the large haxaploid African barb at 53 dpf (Shkil et al., 2012). In contrast, no pronounced abnormalities were found in the anal fins of fish exposed to thiourea.

4.3.7.3. Dorsal fin

The dorsal fin is an unpaired median fin positioned on the dorsal surface of the body (Bird and Mabee, 2003). In zebrafish, the dorsal fin develops slightly later than the anal fin, first evident by dorsal fin condensation within the dorsal fin fold and followed by dorsal fin ray appearance (Parichy et al., 2009). Juveniles show single bifurcations of some of the dorsal fin rays, while second bifurcations appear as the fish grow to adulthood (Singleman and Holtzman, 2014). The zebrafish has 8-10 dorsal fin rays (Singleman and Holtzman, 2014).

T4 treatment from 3 dpf stimulated the differentiation of dorsal fins and the formation of dorsal fin rays in goldfish (*Carassius auratus*) (Reddy and Lam, 1992). After 8 days of treatment, the dorsal fins were differentiated from the fin fold in all the treated fish, whereas in the control group the fin fold remained continuous without any differentiation of fins in the majority of the fish. After 15 days of treatment, the number of dorsal fin rays was significantly higher (~15-17) in all T4 treated groups compared to the controls (~12).

The dorsal fin of African barbs responded to T3 exposure by earlier appearance of fin rays (Shkil et al., 2010; 2012), accelerated growth resulting in curving of fin rays and deformations of the fins as a whole (Shkil et al., 2010; 2012), occasional fusion of fin rays in their distal part (Shkil et al., 2012) and occasional reduction in the number of fin rays (Shkil et al., 2010; 2012). In contrast, exposure to thiourea did not cause abnormalities in the development and adult morphology of the dorsal fin.

4.3.7.4. Pectoral fins

In zebrafish, the paired pectoral fins develop in two phases (Grandel and Schulte-Merker, 1998). Vertical larval pectoral fins develop at 2-3 dpf. During the third wpf, the transition to the adult fin structure begins and pectoral fins gradually rotate into a near horizontal position.

In barbs, which have a pectoral fin development pattern similar to zebrafish, high TH levels were shown to cause premature transition of pectoral fins from the larval to the adult state resulting in decreased numbers of fin rays (Shkil and Smirnov, 2015).

Likewise, exposure to THS disrupting chemicals have been shown to affect pectoral fin development.

Inhibited differentiation and growth of pectoral fins was observed in zebrafish exposed to perchlorate from 14-65 dpf or to methimazole from 4-33 dpf (Brown, 1997). Similarly, significant reductions in the lengths of pectoral fins were observed in both female and male zebrafish upon exposure to methimazole from 3-33 dpf (Sharma et al., 2016). Furthermore, while no sex-linked differences in pectoral fin length were observed in the control fish, males had significantly longer fins than females in the methimazole and T4 groups. On average, pectoral fins were ~7-10% longer in males relative to females of these two groups.

Perchlorate furthermore decreased the length of pectoral fins in threespine sticklebacks exposed throughout development (Bernhardt and von Hippel, 2008).

4.3.7.5. Pelvic fins

Unlike pectoral fins, the zebrafish paired pelvic fins develop to adult state without an intermediate larval stage (Grandel and Schulte-Merker, 1998). The pelvic fin buds begin to develop around 18 dpf and the fin skeleton has formed by around 29 dpf (Grandel and Schulte-Merker, 1998).

The development of the pelvic fin and girdle in zebrafish has been shown to be affected by increased TH levels (Shkil et al., 2019). Entire absence of pelvic fins was observed in T3 treated zebrafish.

Likewise, exposure to THS disrupting chemicals have been shown to affect pelvic fin development.

Inhibited differentiation and growth of the pelvic fins were observed in zebrafish exposed to perchlorate from 14-65 dpf or to methimazole from 4-33 dpf (Brown, 1997). In addition, alterations in the position of pelvic fins were observed in zebrafish upon exposure to methimazole from 3-33 dpf (Sharma et al., 2016). Furthermore, pupfish (*Cyprinodon diabolis*) treated with perchlorate or methimazole from 15-68 dpf showed significantly inhibited development of pelvic fins (Lema and Nevitt, 2006).

4.3.7.6. General discussion and conclusions on fin development as endpoint for THS related disruption

TH appears to have a regulatory role in the morphogenesis of all median and paired fins. In general THS disrupting chemicals appear to reduce fin development and growth. However, studies of effect of THS disrupting chemicals on fin development in fish are scarce, and more studies need to be performed to explore the sensitivity of fin development as an endpoint for THS related disruption. The endpoint seems to be non-specific to THS related disruption, since blocking retinoic acid signalling has also been shown to inhibit fin development in zebrafish (Gibert et al., 2006).

4.3.8. Scale development

In zebrafish, at approximately 30 dpf, scales start to develop from fibroblasts (Sire et al., 1997; Sire and Aakimenko, 2004). Zebrafish possess scales of the elasmoid type, which are thin, lamellar, collagenous plates located within the upper region of the dermis (Sire and Aakimenko, 2004). Scales emerge first in the posterior region of the body, near the lateral line, and the squamation subsequently expands anteriorly (Sire and Akimenko, 2004). In adult zebrafish, the body is covered by several hundreds of scales, arranged in longitudinal and vertical rows (Sire and Aakimenko, 2004).

The assessment of scale development can be made by morphometric quantification from photos taken of live fish, from external examination of preserved fish under a stereo microscope, or from histological sections.

Studies suggest, that THs are directly or indirectly involved in fish scale development (Smirnov et al., 2006; Levin, 2010; 2011; Levin et al., 2012). TH treatment induces accelerated squamation, which leads to increased scale sizes and reduced numbers of scales (Smirnov et al., 2006; Levin, 2010; Shkil et al., 2010). On the contrary, THS disrupters can delay squamation, which leads to decreased scale sizes and increased numbers of scales (Smirnov et al., 2006; Levin, 2010).

4.3.8.1. NIS inhibition

The development of scales was delayed by exposure to perchlorate from 14-65 dpf in zebrafish (Brown, 1997) and from 0-28 dpf in fathead minnow (Crane et al., 2005).

4.3.8.2. TPO inhibition

A concentration-dependent decrease in the scale thickness was observed after exposure to PTU in juvenile zebrafish 42 dph (van der Ven et al., 2006). In that study, scale development appeared to be more sensitive to PTU treatment than circulating TH levels and thyroid histology.

Furthermore, scale development was delayed in zebrafish exposed to methimazole from 4-33 dpf (Brown, 1997).

Thiourea reduced the size of the scales and increased the number of scales in the lateral line of the African barbel (*Barbus intermedicus*) (Smirnov et al., 2006). Likewise, thiourea treatment significantly increased the number of scales in the lateral line of roach (*Rutilus rutilus*) (Levin, 2010) and bream (*Abramis brama*) (Levin et al., 2012).

4.3.8.3. General discussion and conclusions on scale development as endpoint for THS related disruption

Scales serve for physical protection against potential predatory injury, which may be critical for survival. The level of THs has been considered the most influential factor on the number of scales in fish (Levin, 2010; 2011; Levin et al., 2012), and external environmental factors (temperature and salinity) probably indirectly alter scale development via alteration of TH status (Levin et al., 2011). Furthermore, estrogen may be involved in the regulation of scale mineral contents (Armour et al.,1997).

Scale development appears to be a sensitive endpoint for THS disruption in fish. In general THS disrupting chemicals appear to delay scale development, decrease the size of the scales and increase the number of scales. However, only few studies have assessed the effects of THS disrupting chemicals on scale development. This is probably due to the late appearance of scales in fish ontogeny, which means that this endpoint cannot be assessed in zebrafish early life stage tests. Furthermore, it should be noted that scale development and morphology may be influenced by other types of toxicity (Sultana et al., 2017).

4.3.9. Neurodevelopment

In zebrafish, the development of the nervous system begins at a very early developmental stage, during gastrulation at 6-10 hpf (Woo and Fraser, 1995). Neurodevelopment proceeds rapidly in zebrafish and most of the adult neuronal cell types and circuits are formed and functional around the time of hatching at 3 dpf and continue to develop at further larval stages and into adulthood (Kaslin and Ganz, 2020).

THs regulate numerous neurodevelopmental processes in vertebrates (Gothie et al., 2017). For example, blocking of TH uptake by knockdown of MCT8 in zebrafish causes disrupted neuron development and myelination and following disrupted development of the brain and spinal cord (Vatine et al., 2013; Campinho et al., 2014; de Vrieze et al., 2014). Time-lapse imaging of single axons and synapses in ZFN mediated MCT8 mutant zebrafish further revealed reduced axon branching in sensory neurons and decreased synaptic density of motor neurons (Zada et al., 2014). In addition, altered expressions of myelin related genes were observed, i.e. downregulation of *myelin basic protein* (*mbp*) and *myelin protein zero* (*p0*), and upregulation of *oligodendrocyte lineage transcription factor 2* (*olig2*). Moreover, reduced baseline locomotor activity and altered behavioural response to light/dark transitions were observed in MCT8 deficient fish (Campinho et al., 2014; de Vrieze et al., 2014; Zada et al., 2014).

Neurodevelopmental processes may also be affected by THS disrupting chemicals.

TBBPA significantly inhibited motor neuron development in zebrafish exposed from 8 hpf to 2 dpf (Chen et al., 2016a). Behavioural assays conducted at 5 dpf showed that the exposed larvae had lower average activity. Similarly, significantly downregulated transcription of genes related to the central nervous system development (e.g. *mbp*, *α1-tubulin* and *shha*), and reduced locomotor activity and swimming speed were observed in zebrafish exposed to TBBPA from 2-6 dpf (Zhu et al., 2018). The studies further demonstrated that treatment with T3 could reverse or eliminate the TBBPA-induced effects on neurodevelopmental parameters, which indicates that these effects were caused by TH related disruption.

BDE-47 was also found to significantly affect motor neuron growth and swimming behaviour in zebrafish (Chen et al., 2012c). Furthermore, mRNA and protein expression of factors associated with neuronal development (e.g. *mbp*, *α1-tubulin* and *synapsin IIa*) were significantly downregulated in F1 offspring of zebrafish exposed to TDCPP for 3 months, as were the levels of the neurotransmitters dopamine, serotonin, gamma amino butyric acid, and histamine (Wang et al., 2015). In addition, larval locomotion was

significantly decreased in the progeny of exposed fish. Likewise, *mbp*, *α1-tubulin* and *synapsin IIa* expressions were significantly reduced in larvae of zebrafish exposed to DE-71 for 150 days, as were protein levels of α1-tubulin and synapsin IIa and locomotion activity (Chen et al., 2012a). Finally, expression of mRNA associated with neuronal development (*mbp*, *α1-tubulin*, *synapsin IIa* and *gap43*) was significantly downregulated in F1 larvae after parental exposure to tebuconazole (Li et al., 2019).

In addition to locomotor abilities, neurodevelopmental effects may affect cognitive abilities and social behaviours, which may have adverse effects on reproduction and survival.

While it is well documented that THs and THS disrupters influence neurodevelopment in fish, it needs to be clarified to what extent other endocrine and non-endocrine factors may affect this endpoint.

4.3.10. Development of sensory systems

The sensory systems form the coupling between the environment and the brain (Roth, 2013). The sensory organs of fish comprise photoreceptors (visual system), chemoreceptors (olfactory and gustatory systems), mechanoreceptors (auditory system, vestibular system, lateral line and somatosensory system), and, in some fish, electroreceptors (electrosensory system). The development of various sensory structures is influenced by TH (Besson et al., 2020). Several studies have documented the role of TH in the visual system and retinal development, which is discussed separately in section 4.3.11. However, fewer studies have examined the role of THs in the development of other sensory systems, including olfactory and mechanosensory systems.

In fish, the olfactory organ is a chamber containing ciliated olfactory receptors in the sensory epithelium, which forms a rosette comprised of several folds, i.e. lamellae (Besson et al, 2020).

Studies indicate that THs are implicated in the development of the olfactory organ and in olfactory processes related to homing migrations of salmons. TH promotes the development of olfactory organ lamellae, thereby increasing the sensory surface area, in surgeonfish (*Acanthurus triostegus*) (Besson et al., 2020). In addition, fluctuations in TH levels are reflected in changes in cell proliferation in the olfactory epithelium of salmons, which is suggested to facilitate olfactory imprinting (Nevitt et al., 1994; Dittman and Quinn, 1996; Lema and Nevitt, 2004).

Sound detection in zebrafish involves the inner ear, the Weberian apparatus and the swim bladder. The adult zebrafish inner ear is a closed epithelial organ with three semicircular canals and three otoliths, one associated with each of the hair cell patches (maculae) in the utricle, saccule and lagena (Haddon and Lewis, 1996; Popper and Lu, 2000). In the embryo, the utricular and saccular otoliths are formed initially, while the lagenar otolith develops around 12 dpf (Roberts et al., 2017). Patches of sensory epithelium, consisting of mechanosensory hair cell and supporting cells, provide the basis of all ear functions. The sensory patches associated with the semicircular canals detect rotational accelerations, while the sensory patches of the otolith organs have both vestibular and auditory functions and respond to linear acceleration, gravity and sound (Popper and Fay, 1993; Haddon and Lewis, 1996; Popper and Lu, 2000; Stooke-Vaughan et al., 2012). The presence of otoliths formed by deposition of calcium carbonate overlying the sensory patches in the three otolith organs enable the detection of linear acceleration and gravity through the weight of the otolith, and sound waves through vibrational movements (Haldon and Lewis, 1996). In otophysan fish, the Weberian apparatus further enhances sound detection by conveying sound related vibrations from the swim bladder to the otolith organs.

THs have been shown to influence the development of the auditory and vestibular systems at multiple sites. THs mediate growth of the otoliths (Shiao and Hwang, 2004; Shiao et al., 2008; Schreiber at al., 2010; Coffin et al., 2012; Marelli et al., 2016). In addition, incomplete development of the semicircular canals were observed in *thrb* morphant zebrafish (Marelli et al., 2016). Furthermore, as previously discussed, THs influence the development of the Weberian apparatus (section 4.3.6) and the swim bladder (section 4.3.5).

The mechanosensory lateral line system allows detection of unsteady water flows, thus mediating prey detection, predator avoidance, navigation and communication (Webb, 2014a). The lateral line is composed of neuromasts distributed in specific patterns on the head, trunk and tail of fish (Webb and Shirey, 2003; Hu et al., 2019). Neuromasts are small epithelial receptor organs consisting of mechanosensory hair cells and non-sensory support cells (Webb and Shirey, 2003; Webb, 2014b). In adult fish, neuromasts are either superficial neuromasts located on the surface of the fish or canal neuromasts associated with fluid-filled canals and separated by canal pores (Webb, 2014a; 2014b).

Studies indicate that TH inhibits proliferation of neuromasts on the head (Hu et al., 2019) but promotes expansion of neuromasts on the trunk of zebrafish (Hu et al., 2019). In surgeonfish, T3 treatment induced an increased density of trunk canal pores, which also indicates neuromast proliferation on the trunk (Besson et al., 2020).

In embryonic and larval zebrafish, mechanoreceptive Rohon-Beard neurons play an important role in the sense of touch in the somatosensory system (Katz et al., 2020); and studies suggest that TH is required for the normal development of the Rohon-Beard mediated touch response 2 dpf (Yonkers and Ribera, 2008).

Disrupted development of the sensory systems could have damaging effects on behavioural activities which play a crucial role in fish growth, reproduction and survival (section 4.3.12).

Although it is clear, that THs influence the development of the olfactory, auditory, vestibular and lateral line systems, examinations are needed to clarify to what extent THS disrupting chemicals affect these endpoints. On the other hand, several studies have documented the influence of THS disrupting chemicals on the development of the visual system, which is discussed separately in section 4.3.11.

4.3.11. Eye development and functioning

In zebrafish, eye development begins around 12 hpf (Houbrechts et al., 2016b) and at 72 hpf, the retinal stratification is well developed (Malicki et al., 2016). Functional sight is established around 4-5 dpf (Brockerhoff, 2006; Chhetri et al., 2014).

To achieve colour vision, cone photoreceptors with different wavelength sensitivities develop from retinal progenitor cells (Deveau et al., 2020). The cone types are defined by the opsin protein expressed (Mackin et al., 2019). The zebrafish retina possess four different cone photoreceptor types with two of the types arranged in pairs and with each cone type expressing different opsins: short single cones, which express UV opsin (short-wavelength sensitive 1, SWS1); long single cones, which express blue opsin (short-wavelength sensitive 2, SWS2); and double cone pairs, in which one cone expresses red opsin (long-wavelength sensitive, LWS) and the other cone expresses green opsin (rhodopsin-like, RH2) (Viets et al., 2016). In adult zebrafish, the cone photoreceptors, together with one type of rod photoreceptors, which express rhodopsin (Ebrey and Koutalos, 2001), are arranged in a mosaic pattern with interchanging rows of UV/blue sensitive single cones and red/green sensitive double cones (Fadool, 2003; Li et al., 2009; Viets et al., 2016). The rods mediate dim-light vision, and the cones mediate daylight and colour vision (Takechi and Kawamura, 2005).

The photoreceptor cell bodies are located in the outer nuclear layer (ONL) of the retina (Fig.9). The retina is composed of three distinct nuclear layers separated by two plexiform layers (Bilotta and Saszik, 2001; Stenkamp, 2015). The inner nuclear layer (INL) contains the cell bodies of the horizontal, bipolar and amacrine cells, and the ganglion cell layer (GCL) contains the ganglion cell bodies. The outer plexiform layer (OPL) consists of the connections among photoreceptors, horizontal and bipolar cells; and the inner plexiform layer (IPL) consists of the connections among bipolar, amacrine and ganglion cells.

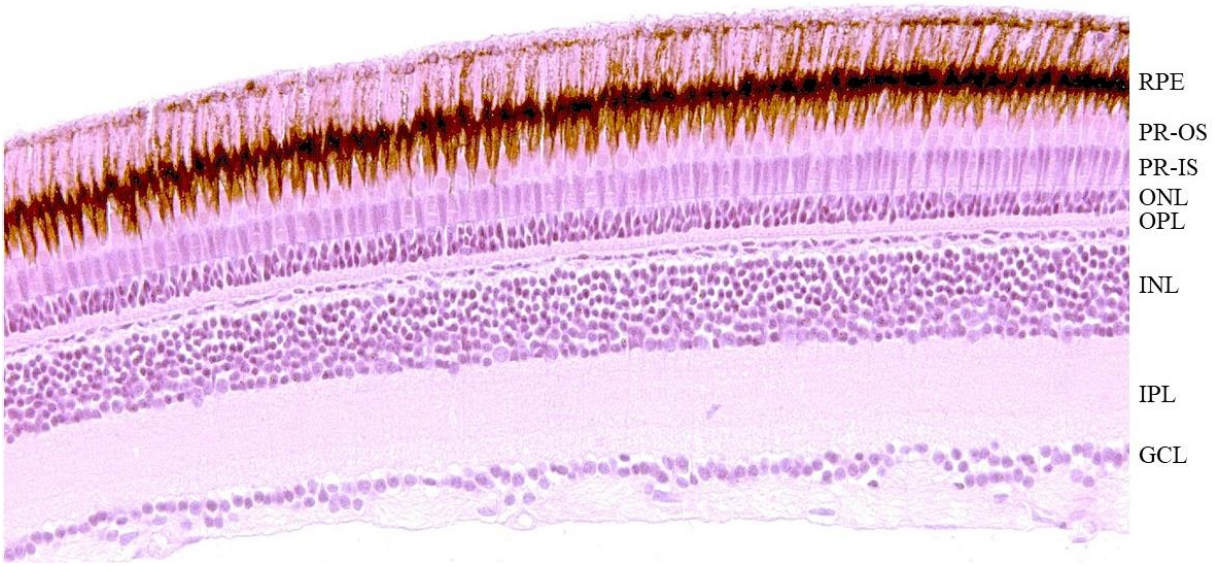

Fig. 9. The zebrafish retina. RPE: retinal pigmented epithelium; PR-OS: photoreceptor outer segments; PR-IS: photoreceptor inner segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer

Eye development and visual performance is influenced by THs as demonstrated in *thrb* and *dio* knockdown and knockout zebrafish (Suzuki et al., 2013; Heijlen et al. 2014; Bagci et al., 2015; Houbrechts et al., 2016b; Marelli et al. 2016; Deveau et al., 2020). Specifically, TRβ2 and DIOs appear to be key regulators of cone photoreceptor development in the retina (Ng et al., 2010; Suzuki et al., 2013; Deveau et al., 2019; 2020).

Proper differentiation of the cones, and subsequent proper colour perception, relies on specific transcription factors (Deveau et al., 2020). One of these is the TRβ2, an isoform encoded by *thrb*, which is expressed in the retina, and has been shown to play an important role in the structural development of the cone layer. Specifically, TRβ is essential for the development of red cones in zebrafish (Suzuki et al., 2013; Deveau et al., 2020), which become red colour blind when TRβ2 is lost by genome editing (Deveau et al., 2019; 2020). TRβ determines the fate of red cone precursors, which in the absence of TRβ transdifferentiate into UV cones and horizontal cells (Volkov et al., 2020). Conversely, transgenic overexpression of TRβ2 leads to an increase in red cones and a decrease in UV cones (Suzuki et al., 2013). These observations may indicate a negative regulation of UV cones by TRβ2.

Moreover, knockdown or knockout of the activating as well as the inactivating DIO genes in zebrafish reduces cone photoreceptor number (Heijlen et al., 2014; Bagci et al., 2015; Houbrechts et al., 2016b), which further substantiates the significance of proper TH signalling in eye development.

In addition to the cones, TRβ2 is also expressed in cells that give rise to horizontal cells (Suzuki et al., 2013).

Disruption of photoreceptor and horizontal cell development during zebrafish development may result in aberrant retinal layer organisation and reduced eye size.

TH signalling plays a crucial role in several aspects of visual functioning in zebrafish, particularly in photoreceptors. The first step of light signal reception in the photoreceptors occurs in the light-sensitive visual pigment, which is comprised of an opsin protein and a covalently bound vitamin A-derived chromophore, either 11-cis retinal or 11-cis 3,4-dehydroretinal (vitamin A1 or A2 aldehyde, respectively) (Takechi and Kawamura, 2005; Isken et al., 2007; Ward et al, 2018; Volkow et al., 2020). In the retina, TRβ2 is required for the expression of long-wavelength sensitive (LWS) opsin in red cone photoreceptors,

while in the retinal pigment epithelium (RPE), TH regulates the expression of a cytochrome P450 enzyme, cyp27c1, which converts the chromophore 11-cis retinal into the red-shifted chromophore 11-cis 3,4-dehydroretinal (Volkov et al., 2020). TH has been shown to act through all three zebrafish nuclear TRs (encoded by *thraa*, *thrab*, and *thrb*) in the regulation of cyp27c1 expression (Volkov et al., 2020).

Moreover, TRβ2 has been suggested to be required for the expression of arrestin (*arr3*) (Deveau et al, 2019; 2020), which is expressed in zebrafish double cones, and which quenches the activated state of opsins (Renninger et al, 2011).

T3 may further mediate the spatiotemporal changes in the expression pattern of red and green opsin subtypes observed over the zebrafish lifespan (Takechi and Kawamura, 2005; Mackin et al., 2019).

The significance of TRβ2 in visual performance has been demonstrated in mutant zebrafish lines created by use of CRISPR/Cas9 technology targeted at the TRβ2 isoform expressed by *thrb* (Deveau et al., 2020). Two visual performance responses were tested in the *thrb* mutants, the optomotor response (OMR) and the optokinetic response (OKR). The OMR is a reflex tendency to swim with perceived motion (Orger et al., 2000), and the OKR is a reflex where the eyes track rotating patterns and then snap back to reset (Deveau et al., 2020). The *thrb* mutants did not show an OMR to red and black striped stimulus, which indicated that they were unable to perceive red light, thus eliminating their ability to discern red from black. Furthermore, the *thrb* mutants showed a significantly lower OKR with red/black stimulus and green/black stimulus. The effect on the perception of green light might be a consequence of arrestin deficit (Deveau et al., 2020).

Since retinal development and functioning relies extensively on THs, several aspects of eye structure and functioning may be affected by THS related disruption, including expression of vision related genes, number of specific photoreceptor cells, retinal layer structure and organisation, retinal pigmentation, eye size and vision related behaviours of fish.

4.3.11.1. Genes related to vision

Exposure to THS disrupting chemicals can disrupt the expression of vision related genes. Particularly the expressions of the phototransduction related photoreceptor opsins and arrestin, which are under the influence of THs, have been shown to be responsive to chemicals, resulting in up- or downregulation. Disruption of the expression of phototransduction related genes may result in disrupted development of the photoreceptors in the retina.

4.3.11.2. *TPO inhibition*

Green opsin, UV opsin and arrestin gene transcript levels were downregulated in the eyes of PTU exposed zebrafish 5 dpf (Baumann et al., 2019). In coho salmon, blue opsin transcripts were downregulated, while UV opsin transcripts were upregulated in the in cones of the eyes of PTU treated embryos (Gan and Flamarique, 2010).

4.3.11.3. Interaction with TH binding

Red opsin gene transcript levels were upregulated, while arrestin gene transcript levels were downregulated in the eyes of TBBPA exposed zebrafish 5 dpf (Baumann et al., 2019).

4.3.11.4. Undetermined or mixed MIEs

Blue opsin, UV opsin and rhodopsin gene transcript levels were downregulated, while red opsin gene transcript levels were upregulated in zebrafish exposed to BDE-47 until 6 dpf (Xu et al., 2015; 2017).

Exposure of zebrafish to DE-71 significantly upregulated the expression of all five opsin genes at 5 dpf (Xu et al., 2013). However, at 15 dpf only green opsin and rhodopsin gene transcription were significantly increased, while red, blue and UV opsin gene transcriptions were not significantly affected (Chen et al., 2013).

BPS exposure significantly increased the transcriptional levels of red opsin, green opsin, UV opsin and rhodopsin in male zebrafish exposed until 120 dpf, while blue opsin gene transcription was not significantly affected (Liu et al., 2018b).

4.3.11.5. Retinal histology

Histological and morphometric analyses have revealed interruption of retinal development after exposure to various THS disrupting chemicals.

4.3.11.6. *TPO inhibition*

PTU exposure significantly decreased the size and pigmentation of the RPE layer but had no significant effects on the other retinal layers in the eyes of zebrafish 5 dpf (Baumann et al., 2016). However, the PTU-related compound, phenylthiourea, caused a general reduction in lens size and dorsal retinal size as a result of tighter packaging of the retinal cells of zebrafish at 3-5 dpf (Li et al., 2012).

A significant decrease in the thickness of the GCL was observed at 66 hpf, while a significant increase in the GCL thickness was observed at 70 hpf and a temperature-dependent decrease in the INL thickness was observed at 72 hpf as a response to methimazole exposure in zebrafish (Reider and Connaughton, 2014).

4.3.11.7. Interaction with TH binding

Histological analysis of the eyes of zebrafish embryos at 22 hpf revealed that exposure to 6-OH-BDE-47, but not BDE-47, was associated with a diminished proliferation of cells in the outer layers of the developing retina (Dong et al., 2014). In addition, the 6-OH-BDE-47 exposed fish revealed abundant signs of cell alteration including swelling and more numerous apoptotic cells in the developing retina, and spaces between the ONL and the RPE were observed. Furthermore, decreased numbers of cells in the lens were observed. MO gene knockdown and overexpression of TRβ mRNA suggested that the effects of 6-OH-BDE-47 on the eyes may be mediated by downregulation of TRβ.

4.3.11.8. Undetermined or mixed MIEs

In 6 dpf zebrafish, exposure to BDE-47 caused disordered photoreceptor arrangement and thickened INL in the retina (Xu et al., 2015).

Likewise, exposure to DE-71 caused disorganised retinal histology characterised by significantly increased area of the INL, significantly decreased area of the IPL and significantly decreased density of ganglion cells in zebrafish 15 dpf (Chen et al., 2013).

Structural defects were also observed in the retinas of zebrafish exposed to BPS until 120 dpf (Liu et al., 2018b). Effects included significantly decreased thickness of the GCL, IPL and total retina, as well as an irregular arrangement of photoreceptors in the ONL.

4.3.11.9. Pigmentation of the retinal pigment epithelium (RPE)

The RPE is a monolayer of pigmented cells located just outside the retina and forming part of the blood-retina barrier (Istrate et al., 2020; Lakkaraju et al., 2020). A common characteristic of the RPE cells is the presence of melanin pigment granules (melanosomes), which absorb light that passes the photoreceptors,

thus protecting the photoreceptors from scattered light (Boulton et al., 2001). Furthermore, RPE melanin has antioxidant properties and may thus contribute to cellular protection against oxidative damage (Sarna, 1992).

The pigmentation of the RPE can be assessed using microscopy, and quantification can be performed by measuring the grey values from images (Baumann et al., 2016)

In zebrafish, pigment begins to develop in the retina approximately 24 hpf (Kimmel et al., 1995). The development of pigmentation in the RPE appears to be mediated by TRβ (Kawakami et al., 2008) possibly by regulation of mRNA expression of tyrosinase, the key enzyme in melanin synthesis (Korner and Pawelek, 1982; Camp and Lardelli, 2001; Walpita et al., 2009). RPE pigmentation has been shown to be affected by several THS disrupting chemicals.

4.3.11.10. *TPO inhibition*

PTU exposure caused adverse effects on the development of the RPE layer in the eyes of zebrafish 5 dpf (Baumann et al., 2016). Histological analyses with measuring of grey values revealed a significant concentration-dependent decrease in the size and pigmentation of the RPE in PTU treated fish. Likewise, PTU caused significantly reduced pigmentation of the eye of zebrafish 30 hpf (Macaulay et al., 2015). Furthermore, both phenylthiourea and 2-mercaptobenzothiazole inhibited the RPE pigmentation of zebrafish at 35 and 55 hpf (Choi et al., 2007). Likewise, 2-mercaptobenzothiazole reduced eye pigmentation of zebrafish at 3 dpf (Li et al., 2012) and at 5 dpf (Stinckens et al., 2016). Moreover, methimazole exposure led to retardation of RPE pigmentation 3 dph in Pacific bluefin tuna (*Thunnus orientalis*) (Kawakami et al., 2008).

4.3.11.11. DIO inhibition

Exposure to iopanoic acid reduced eye pigmentation by 51% in zebrafish 30 hpf (Macaulay et al., 2015).

4.3.11.12. Interaction with TH binding

Histological examinations showed that TBBPA significantly reduced eye pigmentation in zebrafish 5 dpf as confirmed by measuring of grey values (Baumann et al., 2016). Furthermore, a decrease in eye pigmentation was observed in zebrafish at 30 hpf (42% reduction), 4 dpf and 6 dpf following exposure to 6-OH-BDE-47 (Macaulay et al., 2015). Similarly, 6-OH-BDE-47 (but not BDE-47) significantly reduced eye pigmentation in zebrafish 22 hpf (Dong et al., 2014). The pigment layers in the exposed embryos were thinner and showed non-connected pigment patterns.

4.3.11.13. Undetermined or mixed MIEs

The pigmentation of the eyes of zebrafish exposed to BPF was significantly reduced at 2 dpf (Mu et al., 2018; 2019), whereas the eyes were normally pigmented at 3 and 4 dpf (Mu et al., 2019). No significant effects on eye pigmentation in zebrafish 2-4 dpf were observed following exposure to 0.5-5 mg/L BPA or 2.5-25 mg/L BPS (Mu et al., 2018). However, exposure to 1-1000 µg/L BPS induced empty areas in the RPE in a dose-dependent manner in zebrafish at 5 dpf (Liu et al., 2018b). In contrast, exposure to 200 µg/L BPA significantly increased eye pigmentation in the Japanese medaka at 4 dpf, but not at 2 or 3 dpf (Inagaki et al., 2016).

4.3.11.14. Eye size

The eye size can be measured externally using a stereomicroscope; or from histological slides, which can also provide information on the underlying cause of any changes in eye size.

Exposure to various THS disrupting chemicals has been shown to cause reduced eye size in fish larvae.

4.3.11.15. *NIS inhibition*

Potassium perchlorate or potassium thiocyanate did not significantly affect eye size in zebrafish larvae 3-5 dpf (Li et al., 2012).

4.3.11.16. *TPO inhibition*

PTU (Baumann et al., 2016), phenylthiourea (Li et al., 2012), methimazole (Li et al., 2012; Fetter et al., 2015; Reider and Connaughton, 2014), 3-dihydroxypyridine (Li et al., 2012), resorcinol (Li et al., 2012) or 2-mercaptobenzothiazole (Li et al., 2012; Stinckens et al., 2016) reduced eye size in zebrafish larvae at 3-5 dpf.

4.3.11.17. Interaction with TH binding

TBBPA significantly reduced eye size in a concentration-dependent manner in zebrafish 5 dpf (Baumann et al., 2016).

4.3.11.18. Undetermined or mixed MIEs

The eye size was significantly decreased in zebrafish 5 dpf after exposure to BPF but not in zebrafish exposed to BPA, BPS or bisphenol Z (Lee et al., 2019). Furthermore, Senegalese sole (*Solea senegalensis*) larvae exposed to malathion displayed a significant reduction of the eye size in terms of longest eye diameter at 20 and 30 dph (Ortiz-Delgado et al., 2019).

4.3.11.19. Visual performance

As described above, THS disrupters may cause multiple disruptions in the eyes, which may consequently impair visual performance.

Visual performance can be assessed by testing the optokinetic response (OKR) which is a reflex where the eyes track rotating patterns and then snap back to reset (Deveau et al., 2020). In the OKR test, the fish are immobilised leaving only the eyes movable to track a rotating pattern. In the other visual behaviour tests, fish swim freely in response to ambient stimuli (motion, light or colour). Therefore, the OKR is regarded to be more specific to visual performance than the other behavioural responses, which may also result from a compromised locomotor capability (Chen, 2020). Results from OKR tests are discussed in this section, while result from the other visual behaviour tests are discussed in section 4.3.12.

Various THS disrupting chemicals have been documented to affect the OKR in zebrafish.

4.3.11.20. *TPO inhibition*

PTU exposed zebrafish showed concentration-dependent impairment of their OKR at 5 dpf (Baumann et al. 2016). While fish exposed to 50 and 100 mg/L PTU only showed little deficiency in their OKR, fish from the highest concentration (250 mg/L) were significantly impaired.

4.3.11.21. Interaction with TH binding

Visual impairments in zebrafish after TBBPA exposure were demonstrated by concentration-dependent decreased OKR 5 dpf (Baumann et al., 2016). While fish exposed to the lowest concentration of TBBPA (100 µg/L) showed no significant impairment of response to different contrast stimuli, those at 300 and 400 µg/L were clearly not able to follow the visual signals with their eyes, which was statistically significant.

4.3.11.22. Undetermined or mixed MIEs

BDE-47 exposure significantly reduced the OKR under blue light stimuli in zebrafish 6 dpf (Xu et al., 2017). BPS exposure until 120 dpf also significantly decreased the tracking capability of male zebrafish in OKR tests (Liu et al., 2018b). However, increased OKR was observed in zebrafish larvae exposed to DE-71 until 15 dpf (Chen et al., 2013).

4.3.11.23. General discussion and conclusions on eye development as endpoint for THS related disruption

THS disrupters affect multiple aspects of eye development in fish. As is the case for the other sensory systems (section 4.3.10), this may have detrimental effects on fish growth, reproduction and survival.

No clear trends can be deduced in the expression of opsin genes or retinal histology as response to exposure to THS disrupting chemicals. The differing trends in responses may be caused by compensatory mechanisms.

Retinal pigmentation in zebrafish showed a decrease in response to known TPO inhibitors, DIO inhibitors and chemicals interacting with TH binding. This indicates that retinal pigmentation could serve as a promising THS related endpoint in TGs using zebrafish. However, as for skin pigmentation, retinal pigmentation showed differing responses to exposure to bisphenols.

The eye size was reduced by exposure to TPO inhibitors and by interaction with TH binding, whereas no effects on eye size were observed following exposure to NIS inhibitors.

The OKR was decreased by PTU, TBBPA and BDE-47 at embryonic stages. However, OKR was increased by DE-71 at the larval stage. Since the OKR is regarded to be more specific to visual performance than the other behavioural responses, which may also be affected by a compromised locomotor capability, the OKR may be the most promising endpoint for THS related disruption of visual performance in fish. However, no studies were found on the effects of NIS inhibitors or DIO inhibitors on this endpoint.

The studies show that eye development endpoints, particularly retinal pigmentation, are affected by THS disrupting chemicals and thus could be promising THS related endpoints for inclusion in OECD TGs with fish. Due to the early onset of eye development, this endpoint can be analysed from early embryo stages. However, it remains to be examined if the effects on eye development persist into juvenile and adult stages. Furthermore, the thyroid specificity of the eye development endpoints should be examined, since other signalling pathways, such as the estrogen, retinoid, IGF-1 and aryl hydrocarbon receptor, as well as non-endocrine MOA, may also affect eye development (Hamad et al., 2007; Fu et al., 2017; Molla et al., 2019; Chen et al., 2020).

4.3.12. Behaviour

Behavioural changes may be employed as adverse outcomes integrating effects on multiple of the discussed endpoints, including development of the swim bladder, craniofacial features, fins, nervous system, olfactory system, acoustic system (including the Weberian apparatus), vestibular system, lateral line and eyes.

Behavioural endpoints in fish varies with life stage from fundamental behaviours such as spontaneous locomotor activity to more complex behaviours such as foraging (Mora-Zamorano et al., 2018). Disrupted behavioural activities such as prey localisation, navigation, habitat selection, migration, social interactions and predator avoidance may have crucial effects on fish growth, reproduction and survival.

Various behavioural assays have been designed to target specific modes of alteration in fish (Mora-Zamorano et al., 2018). Especially a large battery of visual behaviour tests exists, in which different visual properties like motion, light and colour detection and discrimination are assessed (Schuster et al., 2011).

The visual behaviour tests include the OKR (section 4.3.11.19), OMR (a reflex tendency to swim with perceived motion), visual-motor response (swimming activity in response to sudden changes between light and dark conditions), phototactic swimming activity (from dark to light areas), short- and long-term habituation to visual stimuli and prey capture (Emran et al., 2008; Roberts et al., 2013; Chen, 2020; Oldfield et al., 2020). Behavioural response to acoustic stimuli can be measured by the acoustic startle response, which is muscular activity produced reflexively in response to a sudden loud noise (Liu et al., 2018c). Prepulse inhibition tests assess effects on the reduction of the startle response when a weak non-startling stimulus is presented shortly before the startling stimulus (Banono and Esguerra, 2020). Olfaction behavioural tests include attraction to food extract odorants or avoidance of predatory odorants like taurocholic acid (Tierney et al., 2010). Behaviours involving other or multi-modal sensory stimuli include social preference, shoaling, novel tank diving, thigmotaxis in open field tests, Y-maze memory performance, sleep behaviour and swimming endurance performance in swim tunnel (Egan et al., 2009; Marit and Weber, 2011; Ahmad and Richardson, 2013; Roberts et al., 2013; Li et al., 2017; Dreosti et al., 2018, Cleal et al., 2021; Fontana et al., 2021). However, tests that rely on swimming activity may also be sensitive to morphological effects, including swim bladder and fin malformations. Likewise, foraging efficiency assays may be affected by both neurological, sensory and morphological alterations, including craniofacial features associated with gape (Wirt et al., 2018).

Exposure to THS disrupting chemicals has been shown to cause effects in multiple visual behaviour tests. However, studies examining the effect of THS disrupting chemicals on behavioural tests directed towards effects on other sensory systems are lacking.

4.3.12.1. TPO inhibition

PTU exposed zebrafish 5 dpf showed concentration-dependent impairment of their OKR and suppression of their phototactic swimming activity, while their preference for light over dark increased significantly (Baumann et al. 2016). Additionally, PTU exposure significantly decreased the swimming activity during dark periods in visual-motor response tests with zebrafish at 4 and 5 dpf (Walter et al., 2019b).

4.3.12.2. Interaction with TH binding

Visual impairments in zebrafish after TBBPA exposure were demonstrated by significantly and concentration-dependent decreased OKR 5 dpf (Baumann et al., 2016). Furthermore, phototactic swimming activity was significantly affected in the TBBPA exposed larvae. The effect of TBBPA on swimming activity was stimulating at 300 µg/L and strongly inhibiting at 400 µg/L. No significant effects on the preference of the zebrafish for light or dark backgrounds were observed.

In zebrafish exposed to 100-400 µg/L TBBPA from 2 hpf to 6 dpf, decreased visual-motor response and average swimming speed was observed (Zhu et al., 2018). Likewise, behavioural assays conducted at 120 hpf indicated that zebrafish exposed to TBBPA from 8-48 hpf had lower average activity (Chen et al., 2016a). However, no significant effects were observed on locomotor activity in zebrafish after exposure to 0.1-3 µM TBBPA from 3-5 dpf (Alzualde et al., 2018).

Chronic exposure to 5 nM TBBPA from 1-120 dpf, lead to higher average speed in a photomotor response assay at 9 months post fertilisation (Chen et al., 2016b). Furthermore, exposed males showed significantly heightened aggression in a mirror attack test.

4.3.12.3. Undetermined or mixed MIEs

BDE-47 exposure significantly reduced the OKR under blue light stimuli in zebrafish 6 dpf (Xu et al., 2017) and significantly affected spontaneous movement, decreased touch response and free-swimming speed, and altered swimming behaviour in response to light stimulation in developing zebrafish (Chen et al., 2012c).

In addition, average speed was significantly decreased in the progeny of TDCPP (Wang et al., 2015) and DE-71 (Chen et al., 2012a) exposed zebrafish.

However, increased OKR and phototactic swimming activity was observed in larval zebrafish exposed to DE-71 until 15 dpf (Chen et al., 2013).

4.3.12.4. General discussion and conclusions on behaviour as endpoint for THS related disruption

Visual behavioural parameters have been shown to be sensitive to THS disrupters. However, behavioural changes are not specific to THS disruption as they may also arise from disruption of other endocrine and non-endocrine factors, including unspecific toxicity.

4.3.13. Growth, sexual development and reproduction

In addition to the described developmental parameters, growth, sexual development and reproduction parameters are population-relevant endpoints, which have also been shown to be responsive to chemicals interfering with the THS in fish (Mukhi and Patiño, 2007; Mukhi et al., 2007; Carr and Patiño, 2011; Sharma and Patiño, 2013; Tovo-Neto et al., 2018; Houbrechts et al., 2019). These endpoints are already included in OECD fish TGs. Growth parameters are measured in TGs 236 (OECD, 2011), 210 (OECD, 2013a) and 240 (OECD, 2015a), sexual development is assessed in TG 234 (OECD, 2011) and optionally in TG 240 (OECD, 2015a), while fecundity is included in TG 229 (OECD, 2012) and 240 (OECD, 2015a). In fish, THs regulate various aspects of growth, sexual development and reproduction (Melamed et al., 1995; Mukhi and Patiño, 2007; Deal and Volkoff, 2020). However, growth, sexual development and reproduction are heavily influenced by other endocrine axes, i.e. GH/IGF-1 and HPG, and complex interactions between the THS and these axes exist (Cyr and Eales, 1996; Duarte-Guterman, 2014; Yu et al., 2015; Deal and Volkoff, 2020). Furthermore, growth and reproduction may be influenced by generalised non-endocrine toxicity. These factors make growth, sexual development and reproduction non-specific endpoints for THS disruption. Since growth, sexual development and reproduction are already included in OECD fish TGs - and since it is an aim to increase the diagnostic value of the existing OECD fish TGs by adding new endpoints more specifically related to THS disruption - growth, sexual development and reproduction are not further discussed in this DRP.

5. Concluding remarks

Several potential endpoints for THS disruption in fish have been described in the scientific literature. The endpoints include thyroid histopathology, TH levels, gene expression, swim bladder development and inflation, skin pigmentation, skeletal development including fin and scale development, neurodevelopment, development of sensory systems particularly eye development, and behaviour.

Varying amounts of data exist for the different endpoints, which complicates the attempt to make a comprehensive assessment on the applicability of the various endpoints.

The effort is further complicated by the lack of standardised methods, which may lead to seeming discrepancies in the sensitivity of the endpoints to THS disrupting chemicals. Non-monotonic dose-response curves, often U-shaped or inverted U-shaped due to overcompensations in response to disruptions in homeostasis (Calabrese and Baldwin, 2001), are frequently observed after chemical exposure. This may explain some of the seeming discrepancies observed between studies using different exposure concentrations and may complicate comparison of the sensitivity of the different endpoints.

The sensitivity of the various endpoints may also vary depending on the MIEs caused by the THS disrupting chemical; and an endpoint may not be responsive to all MIEs (Table 8). Because chemicals can disrupt TH signalling through several MIEs other than receptor activation, evaluation of THS disrupting chemicals may require a more comprehensive multi-endpoint approach, compared to other endocrine pathways.

Table 8. Summary of the responsiveness of the various endpoints to disruption via the various THS relevant MIEs, the consistency in response directionality (increase/decrease) and the endpoints specificity to THS disruptive MOA (based on the available information in this DRP).

Endpoint	Responsive via MIE				Consistent in response directionality	Specific to THS disruptive MOA
	NIS inhibition	TPO inhibition	DIO inhibition	TH binding interaction		
Thyroid histopathology	✓	✓	✗	✗	✓	✓
TH levels	✓	✓	✓	✓	✗	✓
Expression of specific genes	✓	✓	✓	✓	✗	✓
Skin pigmentation	✓	✓	?	✓	✓	✗
Posterior swim bladder inflation	✓	✗	✓	✓	✓	✗
Anterior swim bladder inflation	✓	✓	✓	✓	✓	✗
Fin development	✓[a]	✓[a]	?	✓[b]	✓	✗
Scale development	✓	✓	?	?	✓	✗
Retinal histology	?	✓	?	✓	✗	✗
Retinal pigmentation	?	✓	✓	✓	✓	✗

Eye size	✗	✓	?	✓	✓	✗

✓: Endpoint responsive/consistent in response directionality/specific

✗: Endpoint not responsive/not consistent in response directionality/not specific

?: No studies

[a] Pectoral and pelvic fins

[b] Caudal fin

As for frogs (Fort et al., 2008), the potentially useful THS related endpoints can be divided into four main classes: **1**) thyroid histopathology endpoints, **2**) biochemical endpoints (THs), **3**) molecular endpoints (gene expression) and **4**) morphological endpoints.

The biochemical and molecular endpoints have the potential to render the tests more mechanistically informative, by providing useful information on MOA and aiding to distinguish between thyroid and non-thyroid mediated responses. The endpoints are responsive to disruption via all THS relevant MIEs. However, since the HPT axis strives to maintain TH homeostasis through compensatory mechanisms in response to THS disruption, a snapshot of the TH levels may show inconsistency in response directionality (up- or downregulation) between studies, which may reduce the applicability of TH levels as an endpoint. Additionally, the differences between TH blood and tissue concentrations that may prevail due to the activity of (compensatory) deiodases and transmembrane transporters could contribute to a reduced applicability of TH plasma levels as an endpoint. Studies suggest that alterations of THS related gene transcriptions are more sensitive than TH levels (Morgado et al., 2009; Zhu et al., 2018; Shi et al., 2019). However, the complexity of gene response patterns in the THS, i.e. once again the inconsistency in response directionality (up- or downregulation), as well as non-genomic interactions initiated by THs, renders it difficult to pinpoint specific genes as endpoints for THS disruption.

Like biochemical and molecular endpoints, histological analysis of the thyroid confers specificity to the tests. Histological analyses of thyroid follicle structures have been suggested to be more sensitive to NIS inhibition than TH levels (Mukhi et al., 2005). However, thyroid histopathology does not appear to be responsive to DIO inhibition and interaction with TH binding and can thus not stand alone as an endpoint for THS disruption.

Several potential population-relevant morphological endpoints for THS related disruption in fish have been proposed, including the development of skin pigmentation, swim bladders, fins, scales and eyes. Based on the available information, it is currently not possible to decisively rank these endpoints in terms of their usefulness considering sensitivity and specificity.

Swim bladder inflation may be the most well studied morphological endpoint. While the anterior swim bladder inflation has been shown to be affected by each of the four key MIEs, NIS inhibition, TPO inhibition, DIO inhibition and interaction with TH binding, the development of the posterior swim bladder chamber is not responsive to TPO inhibition.

Among the sensory systems, eye development is by far the most studied regarding effects of THS related disruption. Particularly retinal pigmentation appears to be sensitive to THS disrupting chemicals in zebrafish early life stages. Likewise, skin pigmentation appears to be responsive to THS related disruption in zebrafish, although with a seemingly lower sensitivity than the other morphological endpoints.

Skeletal development - including fin and scale development -, neurodevelopment and behaviour are further potential endpoints, which are affected by THs. However, studies of effect of THS disrupting chemicals on these endpoints are scarce, and more studies need to be performed to explore the sensitivity and specificity

of these endpoints. Still, from the few available studies scale and fin development appear to be sensitive morphological THS related endpoints in juvenile zebrafish.

Although the morphological endpoints are responsive to THS related disruption, they may not be mechanistically specific to THS disruption, since most THS related morphological endpoints can be altered by other endocrine modalities, signalling pathways or general toxicity.

From the data compiled in this DRP it appears that no single endpoint covers all demands to assess potential THS disrupting chemicals in fish. This was also concluded in a literature study of the effects of 25 THS disrupters on zebrafish embryos up to 7 dpf (Spaan et al., 2019). An integrated AOP-based approach including both morphological endpoint(s) and mechanistically specific endpoint(s) seems to offer the most useful approach to detect THS disrupting chemicals in OECD fish TGs, which can be used in combination with other data in a weight of evidence approach. However, when revising currently existing *in vivo* test methods, potentially increasing the complexity of the experiments, the overall practicability of performance of the experiments for regulatory purposes should be considered.

The applicability of the various endpoints for inclusion in the different OECD fish TGs depends on the life stages covered by the TGs. Endpoints occurring at the larval-to-juvenile transition can be assessed in OECD TGs 240, 234 and 210 but not in OECD TG 236 (Fig.10).

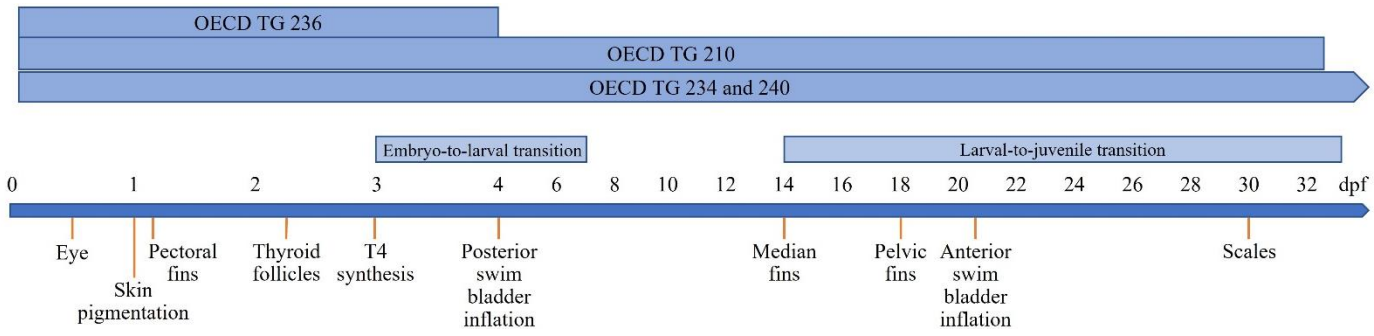

Fig. 10. Timeline showing the approximate onset time of development of various THS related endpoints in zebrafish. The embryonic-to-larval and larval-to-juvenile transition periods in zebrafish as well as the durations of OECD fish TGs are indicated in boxes. Note that the timeline is not drawn to scale.

Based on the available data, inflation of the anterior swim bladder may be a suitable endpoint for identifying THS disrupting chemicals in OECD TGs 234 and 210. The anterior swim bladder inflation has been shown to be affected by each of the four key MIEs, NIS inhibition, TPO inhibition, DIO inhibition and interaction with TH binding, which may make this endpoint superior to the other studied endpoints. The assessment of swim bladder inflation may though require an additional early non-lethal subsampling in which the fish are digitally photographed and put back into the exposure tanks. The anterior swim bladder cannot be assessed in embryo tests. In addition, the inflation of the posterior swim bladder is not affected by TPO inhibition. For OECD TG 236, eye development, particularly pigmentation of the RPE, may, therefore, be a more suitable endpoint than swim bladder inflation.

Effects on the morphological endpoints are indicative of, but may not be specific to, thyroid modality and they should, therefore, be combined with evaluation of an endpoint providing additional evidence of a THS related MOA of the chemical. Thyroid histopathology is considered as an endpoint very specific to THS disruptive MOA (OECD, 2009; Coady et al., 2010; 2014), and may thus be valuable as an endpoint for THS disruption via NIS inhibition and TPO inhibition. Thyroid histopathology seems more informative than

TH and gene analysis due to the profound inconsistency in response directionality of the latter endpoints (upregulation, downregulation or no effect). Particularly, increased follicular epithelial cell height, thyroid follicular epithelium hyperplasia, and depletion of follicular colloid content seem to be useful parameters in juvenile zebrafish. However, DIO inhibition and interaction with TH binding may not be reflected in thyroid histopathology. Thus, TH analysis and/or specific gene expression analyses could further be included as supporting evidence to increase the plausibility that observed effects are caused by a thyroid mediated MOA. This may, however, require a larger number of fish used in the fish early life stage TGs.

Although histology in general is a laborious procedure, histological sections of the gonads are already being made in the OECD TG 234 and 240, and it can be assumed that inclusion of histological analyses of the thyroid tissue, and possibly the eyes as well, in these TGs will require limited additional effort, even if it may require additional sectioning since the organs have differing optimal sectioning levels (Menke et al., 2011). Standardised training of personnel may also be needed to ensure the accuracy of histopathological analyses of new endpoints. Regarding OECD TG 236 and TG 210, the inclusion of histopathology will conceivably make these tests significantly more time-consuming compared to the existing protocols. However, as thyroid histopathology (unlike anterior swim bladder inflation) is not sensitive to all thyroid related MIEs, it could be used as a tiered endpoint, and only investigated when triggered. For example, if there is an effort to non-destructively sample fish during the OECD 210 to collect images for analysis of the anterior swim bladder during larval-to-juvenile transition, and significant effects on swim bladder inflation are noted relative to controls, then thyroid histopathology could also be targeted at study termination. The additional measurement of thyroid histopathology in these cases would help to inform if the swim bladder effects are specific to the thyroid hormone pathway. If no effects are noted on the anterior swim bladder inflation, it is possible that it would not be necessary to extend the effort to assess the thyroid histopathology endpoint.

Much ongoing research focuses on these THS related endpoints, and (pre-)validation using standardised OECD fish test procedures should be the next step to explore the robustness of these endpoints in relation to sensitivity, specificity, variability, reversibility, species differences etc. Focus should be on the endpoints, fish TGs and species that are, at this time, considered to be most useful.

Since most studies concerning histological effects of THS disrupting chemicals in fish, as well as ongoing work with many of the described THS related endpoints (eye development, swim bladder inflation etc.), are made with zebrafish, this would be the preferred species example for the (pre-)validation studies. If some of the suggested endpoints go further into validation more species, like the fathead minnow and Japanese medaka, should be included.

It has recently been decided by the lead countries of Project 2.64 in the OECD Work Plan for the Test Guidelines Programme and supported by OECD VMG-Eco to start the (pre-)validation procedures with focus on two OECD fish TGs - TG 236 representing embryonic (alternative to vertebrate) testing and TG 210 representing developmental in vivo testing. From the data compiled in this DRP, histology appears to have the potential of providing valuable endpoints for THS disruption. New histological endpoints, like thyroid, eyes and scales could be added to already existing histological endpoints in fish TGs. (Pre-)validation studies should proceed with at least the thyroid histology, eye histology and swim bladder inflation endpoints to better characterise the sensitivity and specificity of these endpoints in response to THS activity in fish. The potential endpoints should be validated with several chemicals representing all key MIEs for THS disruption as well as negative control chemicals.

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