AOP report : thyroperoxidase inhibition leading to altered visual function in fish via altered retinal layer structure

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1. Introduction and Background

Thyroid hormones (THs) are involved in the regulation of many important physiological and developmental processes, including vertebrate eye development. TH system disrupting chemicals (THSDCs) may have severe consequences, as proper functioning of the visual system is a key factor for survival in wildlife. However, the sequence of events leading from TH system disruption (THSD) to altered eye development in fish has not been fully described yet. The development of this adverse outcome pathway (AOP) was based on an intensive literature review of studies that focused on THSD and impacts on eye development, mainly in fish. In total, about 120 studies (until end of 2021) were used in the development of this AOP linking inhibition of the key enzyme for TH synthesis, thyroperoxidase (TPO), to effects on retinal layer structure and visual function in fish (AOP-Wiki, AOP #363). In a weight-of-evidence (WoE) evaluation, the confidence levels were overall moderate with ample studies showing the link between reduced TH levels and altered retinal layer structure. However, some uncertainties about underlying mechanism(s) remain. While the current WoE evaluation is based on fish, the AOP is plausibly applicable to other vertebrate classes. Through the re-use of several building blocks, this AOP is connected to the AOPs leading from TPO and deiodinase inhibition to impaired swim bladder inflation in fish (AOPs #155-159), together forming an AOP network describing THSD in fish. This AOP network addresses the lack of thyroid-related endpoints in existing fish test guidelines for the evaluation of THSDCs.

Keywords: adverse outcome pathway, endocrine disrupting compounds, ecotoxicology, thyroid hormones, eye development, zebrafish

To large extent, the thyroid hormone (TH) system is conserved across vertebrates including mammals, amphibians and fish. Although the molecular machinery required for TH synthesis, as well as distribution of the hormones to various tissues and action on nuclear receptors is largely consistent in all vertebrates (Zoeller et al., 2007, LaLone et al., 2017), it should be noted

that there are also some differences in these proteins and their functions across vertebrates, as well as differences in the metabolizing enzymes that activate or inactivate particular toxicants. Therefore, comparative research across taxa is necessary to fully understand these differences (McArdle et al., 2020; Walter et al. 2019). It is well known that THs are essential for the regulation of diverse developmental processes such as neurodevelopment and metamorphosis in vertebrates (Campinho, 2019; Darras et al., 2015), and consequently, disturbances of this system are highly relevant for organismal health and fitness. Field studies, epidemiological studies as well as laboratory experiments provide ample evidence that altered TH synthesis, metabolism and transport are associated with exposure to environmental pollutants in fish, amphibians (Carr & Patiño, 2011), birds (Brouwer et al., 1998) and mammals including humans (Patrick, 2009).

Here, we present an adverse outcome pathway (AOP) linking reduced TH synthesis to altered retinal structure and consequently to altered visual function and increased mortality in fish. The structures of the vertebrate eye and retina are well characterized and known to be also highly conserved among taxa. THs are essential for normal eye and retinal development (Lamb et al., 2007; Mc Nerney and Johnston, 2021; Raine & Hawryshyn, 2009). The retina consists of three distinct nuclear layers separated by two plexiform layers (Fig. 1). Each of these layers has a specific function in the visual system (Bilotta & Saszik, 2001; Stenkamp, 2015). For color vision, photoreceptors with different wavelength sensitivities develop from retinal progenitor cells (Deveau et al., 2020). The rods mediate vision at low light intensity, and the cones mediate daylight and color vision (Takechi & Kawamura, 2005). Consequently, the photoreceptor layer represents the essential structure of the retina, in which the light signal is received and communicated to the brain. With a variety of embryological, genetic and imaging tools, the zebrafish (*Danio rerio*) is an important model organism for studying vertebrate eye development. In zebrafish, eye development begins around 12 hours post-fertilization (hpf) (Houbrechts et al., 2016). By 72 hpf, the retinal layers can be distinguished (Malicki et al., 2016), but differentiation and maturation continue beyond 72 hpf. Functional vision is established around 4-5 days post-fertilization (dpf; Brockerhoff, 2006; Chhetri et al., 2014). In adult zebrafish, the cone photoreceptors, together with a type of rod photoreceptor expressing rhodopsin (Ebrey & Koutalos, 2001), are arranged in a mosaic pattern with alternating rows of UV/blue-sensitive single cones and red/green-sensitive double cones (Fadool, 2003; H. Li et al., 2009; Viets et al., 2016).

THs are required for neuronal and neurosensory development, including retinal development in fish (Cohen et al., 2022), mice (Ng et al., 2001) chicken (Trimarchi et al., 2008) and humans (Eldred et al., 2018). Although the exact mechanisms need further investigation, THs have been shown to regulate retinal neurogenesis and are required for neuronal maturation and cell fate of cones (Harpavat and Cepko, 2003; Roberts et al., 2006). Among others, Harpavat and Cepko (2003) concluded that binding of THs to TRβ determines the correct expression of cone opsins in both zebrafish (Suzuki et al., 2013; Volkov et al., 2020) and rodents (Roberts et al., 2006). The role of THs in eye development is further supported by the fact that TH receptors (TRs), iodothyronine deiodinase (DIO) enzymes and thyroperoxidase (TPO) are expressed in vertebrate eyes (Suzuki et al., 2013; Heijlen et al., 2014; Bagci et al., 2015; Houbrechts et al., 2016; Marelli et al., 2016; Deveau et al., 2020). In particular, 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) and are needed, for example, for the expression of cone opsins (Suzuki et al., 2013). It should be noted that TRs can also mediate downstream effects in the absence of a ligand, and, thus, the sole presence of TRs does not prove that THs are required. Since retinal development, photoreceptor differentiation and color vision are directly regulated by THs, they can be disrupted by TH system disrupting compounds (THSDCs) in vertebrates (Raine & Hawryshyn, 2009); Suzuki et al., 2013; Wester et al., 1990; Suliman & Novales Flamarique, 2014; Deveau et al., 2019). Apart from THs, estrogen signaling is also crucial for proper eye development and function (Cohen et al., 2022). Consequently, developmental exposure to EDCs that interfere with aromatase activity or estrogen receptors can also impair eye development (Hamad et al., 2007; Cascio et al., 2015; Gould et al., 2017; Cohen et al., 2022). The present AOP is focused on the role of THs in eye development, but a link with estrogen-related AOPs could be envisioned in the future.

The structure and organization of the retina in fish has been shown to be affected after exposure to various THSDCs. Potential effects on retinal layer structure include alterations of cell size and shape, cell layer structure, organization and number of photoreceptors or pigmentation. In this respect, it is important to note that the studies evaluated in this review used model substances only at exposure concentrations far below LC values to avoid non-specific toxic effects, which is required for the unequivocal identification of endocrine-specific effects (Wheeler et al., 2018). LC50 values of typical THSDCs, including the TPO inhibitors discussed here, are given in Stinckens et al. (2018), and most studies analyzed for this review provide a statement that chosen concentration ranges were based on preliminary tests or previous studies

to ensure sub-lethal ranges as well as the absence of obvious systemic toxicity. For example, treatment with propylthiouracil (PTU, LC50 556 mg/L) reduced pigmentation in zebrafish eyes at 30 hpf (170 mg/L PTU; Macaulay et al., 2015) and retinal pigment epithelial (RPE) layer diameter in the eye of 5 dpf zebrafish larvae (250 mg/L PTU; Baumann et al., 2016). PTU is a model TPO inhibitor. The additional DIO inhibitory potential of PTU in fish is uncertain, but exposure of zebrafish embryos to 37 or 111 mg/L PTU resulted in decreased T4 and T3 levels and decreased T4/T3 ratio at 32 dpf suggesting that PTU may act primarily as a TPO inhibitor in zebrafish (Stinckens et al., 2020). In our own ongoing work (Goelz et al. 2022, in preparation), we found that various THSDCs such as perchlorate (PCL) and iopanoic acid (IOP) altered the photoreceptor layer and inner plexiform layer (Fig. 2) in zebrafish at sub-lethal concentrations. PCL is an inhibitor of the Na^+/I^- -symporter (NIS), which is responsible for the uptake of iodine into the thyrocytes, and thus leads to reduced TH synthesis similar to TPO inhibition (Schmidt et al., 2012). IOP inhibits all three DIO isoforms and additionally is a relatively weak TPO inhibitor (Renko et al., 2012; Stinckens et al., 2018; Paul et al., 2014). Consistent with the general thought that the inhibition of the conversion of T4 to T3 by DIO1 and DIO2 is the most important mechanism, IOP exposure resulted in reduced T3 levels, while T4 levels remained unaltered in 32 day old zebrafish (0.35 or 1 mg/L IOP) (Stinckens et al., 2020). Chen et al. (2013) as well as Xu et al. (2015) also confirmed that different THSDCs altered the size of the inner plexiform and nuclear layer, reduced the density of ganglion cells, and induced a disrupted photoreceptor structure in the zebrafish retina. Different studies have demonstrated that such morphological changes translate into altered vision-related physiology and behavior in fish after exposure to different THSDCs (Li et al., 2021; Huang et al., 2013; Reider & Connaughton, 2014, 2015; Heijlen et al., 2014; Bagci et al., 2015). For example, PTU-exposed 5 dpf zebrafish showed a concentration-dependent impairment of their optokinetic response (i.e. a compensatory ocular motor reflex triggered by moving black stripes on a white background surrounding the embryos; Baumann et al., 2016). Moreover, DIO deficiency induced by knocking down the DIO genes resulted in impaired eye development and function, as well as impaired light sensitivity at physiological level (Houbrechts et al., 2016). Together, this shows that reductions in TH levels caused by TPO inhibition as well as by other THSD modes of action lead to altered retinal layer structure and subsequent higher level adverse effects.

2. Brief AOP description

The present AOP (Fig. 3, BOX 1) links THSD to altered visual function in fish. Specifically, the AOP is initiated by inhibition of TPO (MIE, Event #279), an enzyme that is located in the thyroid follicles and is essential for TH synthesis across vertebrates. Inhibition of TPO therefore results in decreased TH synthesis (Key Event (KE) 1, Event #277). This results in decreased release of TH, mainly thyroxine (T4), from the thyroid follicles to the serum, leading to decreased T4 levels in the serum (KE2, Event #281) and reduced availability of T4 for conversion to the more biologically active triiodothyronine (T3). Although reduced TH synthesis can also directly decrease T3 levels, we re-use this upstream path from previously reviewed AOP 159. This does not exclude the potential contribution by other upstream paths. Since eye development is under TH regulation, decreased T3 levels (KE3, Event #1003) during early development interfere with the normal formation of the retina. Alterations in retinal layer structure (KE4, Event #1877) often include changes in the diameter, density and morphology of the retinal cell layers as well as altered pigmentation level of the retinal pigment epithelium. Alterations in the retinal layer structure subsequently affect normal functioning of the eye causing alterations in visual function (KE5, Event #1643). Vision is essential to support behavioral patterns such as foraging and predator avoidance, both of which are vital to survival. Therefore, altered visual function is linked to increased mortality (AO1, Event #351) and eventually to decreased population growth rate (AO2, Event #360).

We chose to specify the central KE as ‘retinal layer structure altered’, separating it from other effects on the eyes such as reduced eye size and altered photoreceptor patterning, since distinct methods are available to measure these different events. In the future, we envision the development of a network of AOPs initiated by multiple THSD-related MIEs, linking to different effects on the eyes and converging in altered visual function. Here, we describe the first AOP in this network focusing on altered retinal layer structure as the intermediate key event linking reduced TH levels to altered visual function in fish. The present AOP builds further on and re-uses parts of a previously reviewed AOP network linking reduced TH levels to impaired swim bladder inflation in fish (AOPs 155-159; Knapen et al., 2020) as well as previously reviewed AOPs linking reduced TH synthesis to developmental neurotoxicity in mammals (AOPs 42, 54; Crofton, 2008; Rolaki et al., 2019). Specifically, Events #279, #277,

#281, #1003, #351 and #360 are re-used, as well as the key event relationships (KERs) connecting them. The new information in the current AOP includes the link between reduced T3 levels and altered retinal layer structure and the subsequent linkages to altered visual function and increased mortality (Figure 3).

The development of the current AOP was primarily based on experimental data with fish and draws first parallels to other vertebrate taxa. This AOP is the first pathway describing the relationship between THSD and eye development and may form the basis for applicability to other vertebrate classes in the future. In the context of the research project "ERGO" (Holbech et al., 2020), one aim is to improve the risk assessment of EDCs for the protection of human health and the environment by breaking down the currently existing wall between those two areas. In other words, evidence of endocrine disruption observed in a fish or amphibian model may raise concern for an adverse effect in mammals including humans, and *vice versa*. The present AOP represents an important step forward by describing a toxicological mechanism that is likely applicable across vertebrates.

3. Overview of AOP development approach

The present AOP was developed based on data obtained from several comprehensive literature searches. Overall, the data search was based on a "middle-out" approach that focused on finding evidence for MIEs and AOs linked to changes in eye development that we found in the literature and in our own experiments with THSDCs. A first structured literature search was performed online on Pubmed, Sciencedirect/Scopus and Web of Science. The search terms used were "fish," "eye development," "retina," "thyroid/endocrine disruptors," "visual behavio(u)r," "photoreceptors," and combinations thereof. The resulting literature was analyzed in terms of data collected at different levels of biological organization, including gene expression, hormone levels, cellular and organ level effects as well as physiology and behavior. In total, this search yielded over 100 articles from the years 1994 to 2021. Apart from research articles on chemical exposure of different animals, more fundamental studies were included that used other methods for induction of THSD, such as transgenic or mutant fish, microinjection, morpholino oligonucleotides, chemical thyroid ablation, etc. Based on this literature analysis, a set of KEs describing the reported effects on eye development due to THSD was defined. An AOP network linking reduced T3 levels to altered visual function *via* altered retinal layer structure, reduced eye size and altered photoreceptor patterning was developed and more specific literature was

collected to develop preliminary KE and KER descriptions. AOP 363, focused on altered retinal layer structure, was selected for detailed development based on the level of support available from literature and own datasets. TPO inhibition was selected as the first MIE to work on, because most evidence of the impact of THSD on visual function is coming from studies using TPO inhibitors. An intensive, specific final literature search was done to collect evidence for this AOP. More specific search terms related to the KERs were used to find evidence for those specific KERs. Additionally, highly relevant reviews were used for identifying additional important publications. The AOP is further supported by data from own ongoing research that is currently being prepared for publication (referred to as "in preparation"). The majority of literature used fish, mainly zebrafish (85%) as research model, and the prime focus of this AOP is on fish, but the AOP is plausibly applicable to other vertebrate species.

4. Summary of scientific evidence assessment

The criteria for assessing the weight of evidence (WoE) were taken from the User's Handbook Supplement to the Guidance Document for Developing and Assessing AOPs (OECD 2018) according to the further developed Bradford-Hill considerations (Becker et al., 2015). The biological plausibility was rated high for the upstream KERs #309, #305, #366 reflecting the widely accepted importance of TPO for *de novo* synthesis of TH. The biological plausibility was also rated high for the downstream KERs #2374, #2375 and #2013, since a healthy retina is key for normal visual function and survival (Table 1). For the intermediate KERs #2038 and #2373, the biological plausibility was described as moderate, because there are still some knowledge gaps concerning the mechanisms. The empirical evidence for most KERs was classified as moderate, because ample studies support the linkages, but a combination of studies is often needed to support dose- and time-concordance. The overall WoE assessment of the KERs was performed by taking into account the biological plausibility and the empirical evidence, where biological plausibility is most influential (OECD 2018; Figure 1). The overall essentiality of KEs was rated high, with the most evidence found for essentiality of reduced T3 levels for downstream effects, which is highly supportive of this AOP.

Biological plausibility of KERs

TPO is the crucial enzyme for TH synthesis in the thyroid follicles, and inhibition of the enzyme is known to result in decreased TH synthesis across vertebrates. Thyroid follicles mainly secrete

T4 and, to a lesser extent, also T3 to the blood. T4 is then activated to T3 by DIOs in the liver as well as multiple target tissues. When less T4 is available for activation to T3, this is known to result in decreased T3 levels. The latter relationship depends on the extent of the inhibition, because (1) in some cases feedback/compensatory mechanisms limit the impact on T3 levels, possibly through increased DIO activity, and (2) thyroid hormone distributor proteins such as transthyretin, thyroxin-binding globulin and albumin determine the fraction of T4 that is available for conversion to T3 and can therefore to some extent buffer the consequences of T4 decreases.

The importance of THs for the development of the eye, and more specifically the retina, across vertebrates is widely accepted (Viets et al., 2016). Both T3 and T4 are present in the developing retina (Roberts et al., 2006), and key components of the TH system such as DIOs (Heijlen et al., 2013; summarized by Viets et al., 2016), TH receptors (Gan & Flamarique, 2010) and even TPO (Z. Li et al., 2012) are expressed in the vertebrate retina during retinal development. Multiple animal models with deficiencies in the TH system support the importance of THs for retinal structure development (Gamborino et al., 2001; Houbrechts et al., 2016; Ng et al., 2010; Roberts et al., 2006).

The photoreceptors in the retina are responsible for detecting light of various wavelengths and convert these visual stimuli to neural signals that are transferred to the brain *via* the optic nerve. The retina consists of different layers, which are interdependent and have specific functions in supporting this phototransduction process. If the retinal layer structure is altered, for example through disruption of the TH system, this is linked to altered visual function (Baumann et al., 2016; Houbrechts et al., 2016).

Proper visual function is essential to support physiological and behavioral patterns that are vital to survival. The most evident examples are foraging, where accurate vision is needed to locate and catch prey, as well as predator avoidance, where timely perception and localization of an approaching predator is key. Altered visual function interferes with these types of behaviors and results in increased mortality (Besson et al., 2020). Finally, if individual mortality in a population is increased, in the long term this will lead to a decreasing population growth. However, the relation between mortality and population size is the subject of population modeling and is complicated by various factors in the environment. For example, reduced larval

survival due to toxicant exposure may increase the chances of the remaining larvae to find food (Stige et al., 2019).

Empirical evidence of KERs

The upstream KERs linking TPO inhibition to decreased T3 levels are re-used from previously reviewed and endorsed AOPs leading to developmental neurotoxicity in mammals (AOPs 42, 54; Crofton et al., 2019; Rolaki et al., 2019) and to increased mortality *via* reduced swim bladder inflation in fish (AOPs 155-159; Knapen et al., 2020). The evidence for these KERs is briefly summarized below for completeness, and more focus is on the new KERs in the present AOP linking decreased T3 levels to increased mortality *via* altered retinal layer structure and altered visual function.

Empirical evidence for KER #309 'TPO, inhibition leads to TH synthesis, decreased' (previously reviewed) is considered low in fish, because evidence of this explicit relationship is limited. TPO inhibition is usually measured in *in vitro* assays using mammalian source materials (Paul Friedman et al., 2016). Nevertheless, a number of studies have directly quantified T4 present in the thyroid follicles to show that model TPO inhibitors such as the pharmaceuticals PTU and methimazole (MMI, a model TPO inhibitor) reduce TH synthesis in zebrafish (Raldúa & Babin, 2009; Rehberger et al., 2018; Thienpont et al., 2011). Studies in rats (Cooper et al., 1983, 1984) and African clawed frog (Tietge et al., 2010) further support the presence of this relationship across vertebrates. Nelson et al. (2016) reported increased TPO mRNA levels as indirect measurements of TPO inhibition as well as altered thyroid histopathology probably reflecting a compensatory response to the decrease in TH synthesis upon exposure of fathead minnows to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor (Paul Friedman et al., 2016). Although indirect, this provides evidence of time- and dose-concordance (Supporting Information, Table S1).

Evidence for KER #305 'TH synthesis, decreased leads to T4 in serum, decreased' (previously reviewed) is considered low, because studies comparing follicular and serum TH levels are not available in fish, while they are available in mammals (Cooper et al., 1983, 1984). In fish early life stages, whole-body TH level measurements are often used as a proxy for serum TH levels, since obtaining serum is practically not feasible. Time-, dose- and incidence-concordance can be derived from the combined studies of Rehberger et al. (2018) and Thienpont et al. (2011) showing reduced TH synthesis after treatment with PTU and MMI, as well as Stinckens et al.

(2020) confirming reduced T4 as well as T3 levels in whole-body samples at similar exposure concentration with the same agents, both in zebrafish (Table S1).

In fish, several studies provide evidence for the non-adjacent KER #366 'TPO, inhibition leads to T4 in serum, decreased' skipping KE #277 (TH synthesis decreased), resulting in moderate empirical evidence for this KER. Stinckens et al. (2020) showed reduced T4 levels in 14, 21 and 32 days old zebrafish exposed to PTU and MMI starting immediately after fertilization (Stinckens et al., 2020). Schmidt and Braunbeck (2011) also reported reduced T4 levels in 5 weeks old zebrafish exposed to PTU. These sampling points correspond to the larval-juvenile transition period. Additionally, Walter et al. (2019b) reported reduced T4 levels after exposure of 3 and 5 days old zebrafish embryos to PTU. Zebrafish exposed to MBT from immediately after fertilization until the age of 5 and 32 days had reduced T4 levels (Stinckens et al., 2020). Zebrafish were continuously exposed in each of these studies and exposures were started shortly after fertilization. Exposure to MMI also resulted in reduced T4 levels in fathead minnows (Crane et al., 2006). Nelson et al. (2016) showed reduced T4 levels in 6 days old fathead minnows exposed to 1 mg/L MBT, while increased T4 levels were observed after exposure to 0.25, 0.5 and 1 mg/L MBT until 21 days. This may be due to compensatory feedback mechanisms that regulate TH synthesis. Evidence of time- and dose-concordance is provided by Baumann et al. (2016) showing increased mRNA levels coding for TPO, reflecting TPO inhibition in zebrafish exposed to PTU on the one hand, and decreased T4 levels in the same exposure concentration range on the other hand (Stinckens et al., 2020; Table S1). Nelson et al. (2016) similarly showed dose-concordance in fathead minnows exposed to MBT.

The empirical evidence for KER 2038 'T4 in serum, decreased leads to decreased, T3' (previously reviewed) is considered moderate in fish. Decreases of T4 and T3 as well as a strong correlation between T4 and T3 levels were shown in zebrafish larvae and juveniles exposed to MMI or PTU (Stinckens et al., 2020). Decreased T3 levels were also shown in 21 dpf fathead minnows exposed to MBT (Nelson et al., 2016). Wang et al. (2020) showed decreased T4 and T3 levels in zebrafish embryos exposed to perfluorinated chemicals. There are some uncertainties associated with this KER. Thyroid hormone level data after exposure to TPO inhibitors and at the earlier ages that are most relevant to this AOP are scarce. Additionally, reduced T4 levels do not always result in reduced T3 levels, and this is probably the result of feedback mechanisms that in some cases are able to maintain T3 levels, when T4 levels are reduced. One potential mechanism is the induction of deiodinases responsible for converting

T4 to T3. In zebrafish continuously exposed to MBT up to 32 days of age, T3 levels were not significantly altered while T4 levels were reduced (Stinckens et al., 2016). Similarly, fathead minnows exposed to MMI until the age of 28 days had reduced T4 levels, but T3 levels remained unaltered (Crane et al., 2006). Even in the absence of whole-body T3 changes, there may be more subtle local T3 level changes. In fish early life stages, TH levels are mostly measured on a whole-body level, and the exact nature of the T3 decrease, systemic or local in specific tissues, is currently an area of uncertainty. Time-, dose- and incidence-concordance is supported by studies in fish early life stages exposed to PTU, MMI (Stinckens et al., 2020), MBT (Nelson et al., 2016) and perfluorinated chemicals (Wang et al., 2020) with minor inconsistencies for incidence-concordance (Table S1).

Evidence for KER #2373 'Decreased, T3 leads to Altered, retinal layer structure' is considered moderate based on convincing evidence from studies applying a variety of techniques to reduce TH levels. There is some evidence directly linking reduced T3 levels to altered retinal layer structure. Tetrabromobisphenol A (TBBPA) reduced T3 levels (Yu et al., 2021; Zhu et al., 2018) and reduced retinal pigmentation in zebrafish embryos (Baumann et al., 2016). Additionally, genetic knockdown and knockout studies provide highly specific evidence of this relationship. Houbrechts et al. (2016) applied morpholino knockdowns of specific DIO isoforms in zebrafish embryos. Combined knockdown of DIO 1 and 2 required for activation of T4 to T3, thus expected to result in reduced levels of T3, resulted in a wider ganglion cell layer with reduced density. Marelli et al. (2016) showed that high doses of T3 can partially reverse the dominant negative effect of mutant TRs on eye development in zebrafish.

Although T3 level data are scarce at these early ages, T3 is the most biologically active TH, and it is plausible to assume that reduced T4 levels resulting from TPO inhibition lead to reduced T3 levels at the target site and that the latter is predominantly causing the biological effects. Exposure of zebrafish embryos to PTU from fertilization until the age of 5 days, covering the most important processes in retinal development, reduced the diameter and pigmentation of the retinal pigment epithelium (Baumann et al., 2016). Results of own recent studies show that zebrafish offspring with exposed parents have altered retinal layers at both larval and juvenile stages after 28 and 60 days of exposure to PTU (Pannetier et al. 2022, in preparation; Goelz et al. 2022, in preparation). TH levels in the eyes of these juvenile zebrafish were found to be altered after exposure to PTU (Pannetier et al. 2022, in preparation). In salmonids, PTU also repressed the opsin switch from UV to blue opsin expression during the early larval stage (Gan & Flamarique, 2010). Treatment of metamorphosing convict

surgeonfish with NH_3, a THR antagonist, decreased bipolar cell density (Besson et al., 2020). Since T3 is the main ligand of the THR this supports the causal link in this KER. In zebrafish, knockout of TH receptor THRβ altered cone differentiation (Duval & Allison, 2018), and in mice disabling THRb2 altered the number of specific cone types in the retina to the extent that M opsins did not appear at all (Ng et al., 2010). Gamborino et al. (2001) applied chemical-surgical thyroid ablation as a rat model for hypothyroidism and observed reduced thickness of the photoreceptor and ganglion cell layer, delayed morphogenesis of the photoreceptor outer segment and reduced ganglion cell nuclear volumes and nuclear pore density. Although it is widely accepted that THs regulate normal eye development and several studies have shown altered expression of many genes related to eye development due to hypothyroidism (Bagci et al., 2015; Baumann et al., 2019; Houbrechts et al., 2016), several effects on the eyes are typically observed simultaneously (altered retinal layer structure, altered photoreceptor patterning and reduced eye size among others) complicating the identification of the pathways responsible for the observed changes in retinal structure. Therefore, uncertainties and inconsistencies in this KER are mainly related to the exact underlying mechanisms and timing. For example, Reider and Connaughton (2014) exposed zebrafish embryos to MMI until 66 hpf and raised them further in clean water until 72 hpf. In these embryos, ganglion cell layer thickness was reduced. In contrast, embryos exposed until 70 or 72 hpf had increased and normal ganglion cell layer thickness respectively, which cannot be explained at this point. Secondly, although the assumed site of decreased T3 is the retina, the available studies only report whole body T3 levels. Another uncertainty lies in the observation that increases of T3 levels also have an impact on retinal structure (Ng et al., 2010; Besson et al., 2020; Bhumika & Darras, 2014). The TH system is tightly regulated and both hypothyroidism as well as hyperthyroidism typically have an impact on TH-regulated processes, but the margins of normal physiology have not been determined yet. There is evidence of dose-concordance from studies exposing zebrafish to PTU or MMI and showing reduced T3 levels (Stinckens et al., 2020) as well as altered retinal structure (Baumann et al., 2016; Reider and Connaughton, 2014; Table S1). Evidence of time-concordance is missing in these studies, since effects on retinal structure were observed at 5 dpf, while TH levels were measured at 14-32 dpf. Besson et al. (2020) provided evidence of dose-, time- and incidence-concordance in surgeonfish exposed to chlorpyrifos or increased temperature (Table S1).

Evidence for KER #2374 ‘Altered, retinal layer structure leads to Altered, Visual function’ is considered moderate, because convincing evidence comes from multiple studies using different

stressors and various endpoints informing on visual function. Zebrafish embryos exposed to PTU or TBBPA showed both alterations in retinal layer structure and altered visual function as well as vision-related behavior (Baumann et al., 2016). Specifically, the optokinetic response (OKR) was reduced. In the treated embryos, there was a dose-dependent decrease in the velocity of eye movements across OKR tests with different contrasts, angular velocities and spatial frequencies of the moving stripes. Embryos treated with PTU additionally had a higher preference for light over dark, potentially due to decreased light sensitivity. Transiently, hypothyroid DIO 1 and 2 knockdown zebrafish embryos had altered retinal layer structure at 3 days together with altered response to light at 4 days (Houbrechts et al., 2016). While control embryos showed increased swim speed upon a light stimulus after a period in the dark, the DIO1 and 2 knockdown embryos showed no significant increase in swim speed. By the age of 7 days, both the retinal structure and the light response had recovered, supporting temporal concordance. Flamarique (2013) showed that TH treatment reduced the numbers of UV cones in the retina, and this decreased the ability of young rainbow trout to locate prey. Foraging on *Daphnia magna*, a natural zooplankton prey of rainbow trout, was assessed with the method of silhouette video photography. This revealed that, under the full light spectrum, control fish (UV, M/L trichromats) located prey at greater distances and angles than TH-treated fish (S, M/L trichromats; Flamarique et al., 2013). Correspondingly, Walter et al. (2019) applied TH treatment in zebrafish embryos and observed altered photomotor behavior. Both treatment with T3 and T4 altered the swimming response upon a transition from light to dark at 3, 4 and 5 dpf. Avallone et al. (2015) reported altered structure of the retina as well as increased light sensitivity (increased avoidance of bright light) after exposure of adult zebrafish to cadmium. Evidence of time- and dose-concordance comes from a study by Besson et al. (2020), who investigated the effects of T3 and NH3 on surgeonfish, and a study by Baumann et al. (2016), who exposed zebrafish to PTU (Table S1).

Evidence for KER #2375 ‘Altered, Visual function leads to Increased Mortality’ is considered moderate. Besson et al. (2020) provided convincing evidence in metamorphosing convict surgeonfish exposed to NH3 resulting in altered retinal structure, altered visual function and reduced survival. Specifically, NH3-treated fish did not avoid chemical or visual predator cues and survival in a predation test was decreased by 30%. Treatment with chlorpyrifos reduced T4 and T3 levels, reduced the density of bipolar cells and reduced survival in the predation test (Besson et al., 2020). It should be noted that chlorpyrifos is also an acetylcholinesterase inhibitor, which may influence performance in the predation test *via* neurotoxicity. Fuiman et

al. (2006) showed the relation between visual function and predator escape using an experimental setup without toxicant exposure. They specifically investigated the importance of several putative survival skills for escaping a predator on an individual basis in *Sciaenops ocellatus* larvae. Firstly, the authors found that, if larvae responded to a predator attack, they were almost 100% effective at escaping. Secondly, visual responsiveness was the only survival skill that was significantly correlated to escape potential, while parameters such as acoustic responsiveness did not significantly contribute. Moreover, when comparing poorly responding larvae and better responders, the only parameter that significantly differed was visual responsiveness, again clearly showing the importance of visual function for survival. Dehnert et al. (2019) performed a detailed study to investigate the relation between impaired vision and reduced survival after exposure to 2,4-dichlorophenoxyacetic acid (2,4-D), the active ingredient in a commercial herbicide formulation DMA4®IVM (DMA4). The ability to capture paramecia was reduced after exposure of zebrafish and yellow perch to 8 ppm 2,4-D in DMA4. More detailed studies in zebrafish revealed that this treatment reduced prey capture maneuvers in the presence of live paramecia. Furthermore, exposure concentrations leading to reduced prey capture had no effect on general locomotion in the absence of prey, while they did affect the ability of larvae to navigate to a light source (i.e., phototaxis) as well as the activity of neurons in the optic tectum, indicating that the reduced ability to catch prey was likely due to reduced visual function rather than generally reduced motility of the larvae. Exposing zebrafish larvae during development of the visual system and not during the prey capture trial showed that the underlying cause of the effects was impaired visual system development. The authors also used adult zebrafish as predators to determine predator avoidance ability of zebrafish larvae and showed 20% reduction in predator avoidance in the same treatment group. More detailed studies revealed that the larvae were in fact capable of responding to acoustic stimuli with the required escape responses, again indicating that the effects are due to impaired vision and not to locomotor capacity. While most studies observe survival under ideal lab conditions (i.e. with ample food available and in the absence of predators or competitors), both Besson et al. (2020) and Dehnert et al. (2019) convincingly show that behaviors vital to survival in a realistic scenario are affected after impaired development of the visual system and showed dose concordance (Table S1). Knockdown of DIO3 in zebrafish embryos as well as TH treatment also impacts eye development, and these treatments were shown to perturb the escape response (Heijlen et al., 2014) and the ability to catch prey (Flamarique, 2013), both likely to reduce survival in the wild. Besson et al. (2020) confirmed dose-, time- and incidence-concordance by showing predator cues and survival rates after T3 and NH3 exposure, and Dehnert et al. (2019)

showed clear dose- and incidence-concordance by reporting that with increasing concentration of pure 2,4-D as well as 2,4-D in formulation (DMA4), phototaxis is progressively reduced and prey capture also becomes impaired with increasing exposure concentration (Table S1).

The final KER #2013 'Increased Mortality leads to Decrease, Population growth rate' (previously reviewed). Empirical evidence for this KER is considered moderate. The survival of early life stages has been shown to be a crucial determinant of population size in fish (McKim, 1977; Miller & Ankley, 2004; Rearick et al., 2018). Important evidence also comes from fisheries (Alekseeva & Rudenko, 2018; Jacobsen & Essington, 2018). While survival data are the obvious basis for population modeling, the exact relation in a real-life toxicant exposure scenario is complicated by the specific context including food availability and predation pressure (Stige et al., 2019).

Essentiality of KEs

Evidence of essentiality shows that a stressor can activate an AOP and its various KEs, and that cessation of that stressor can prevent that activation or lead to recovery of the adverse effects. Evidence of the essentiality of KEs for the present AOP is provided by chemical exposure and gene knockdown or knockout experiments with subsequent recovery. There is direct evidence of essentiality of the MIE (Event #279 TPO inhibition) based on studies showing that T4 levels recover after cessation of exposure to a TPO inhibitor in mammals (Vickers et al., 2012; Taurog, 1999; Paul et al., 2013; Cooper et al., 1983, 1984; AOP 42). There is direct evidence of essentiality of KE1 (Event #277 TH synthesis, Decreased) and KE2 (Event #281 Serum T4, Decreased) from stop/recovery experiments showing recovery of serum thyroxine concentrations following cessation of developmental exposure to chemical stressors that inhibit TH synthesis (Vickers et al., 2012; Taurog, 1999; Paul et al., 2013). Similar evidence in fish is missing. There is direct evidence of essentiality of KE3 (Event #1003 Decreased, T3) from several rescue and knock-out experiments showing that retinal layers recover after KE3 is blocked (Houbrechts et al., 2016, Bhumika & Darras, 2014, Marelli et al. 2016, Duval & Allison 2018, Roberts et al. 2006). The study by Houbrechts et al. (2016) provided direct evidence for the essentiality of KE4 (Event #1877 Altered, retinal layer structure). The authors observed a corresponding recovery of the response to light after blocking this KE. We did not find studies that block KE5 (Event #1643 Altered, Visual function) and show that this leads to reduced mortality. In summary, the essentiality of this AOP can be classified as high, since

there is convincing direct evidence from multiple specifically designed experimental studies, especially for the core of the AOP, namely the essentiality of decreased T3 levels for downstream effects on the retina.

Quantitative understanding

Quantitative understanding describes whether the magnitude or probability of a key downstream event can be predicted mathematically by the magnitude or probability of a key upstream event with known uncertainties (OECD, 2018). The levels of confidence for quantitative understanding are indicated in Table 1. Quantitative Data on KERs #309, #305 and #366 linking TPO Inhibition to decreased T4 levels *via* decreased TH synthesis was reported by Hassan et al. (2020, 2017) and Handa et al. (2021). They quantified the inhibition of TH synthesis by PTU and MMI in an *in vitro* TPO inhibition study to predict the level of THs in rat serum. A quantitative model was constructed by comparing *in vitro* and *ex vivo* (in tissues from exposed rats) TPO inhibition and *in vivo* time-course and dose-response analyses. Furthermore, Fisher et al. (2013) modeled the link between TPO inhibition and serum TH concentrations during early development in rats. Likewise, in *Xenopus laevis*, Haselman et al., (2020) demonstrated temporal profiles of thyroidal iodotyrosines (MIT/DIT) and iodothyronines (T4/T3), the products of TPO activity, following exposure to three different model TPO inhibitors (MMI, PTU, MBT) at multiple concentrations.

A quantitative relation between T4 and T3 levels in fish has been shown by Stinckens et al. (2020). Further analysis of the data from Baumann et al. (2016) provides a quantitative relationship between the MIE and altered retinal layer structure in zebrafish embryos (Fig. 4). The relative gene expression of TPO and the effect on the thickness of the RPE are strongly correlated. Exposure to the TPO inhibitor PTU resulted in an increase in TPO mRNA expression that was more than seven times higher compared to the negative control and increased with increasing PTU concentrations. This could be interpreted as a compensatory response and thus an indirect measurement of TPO inhibition. The measured thickness of the RPE shows a concentration-dependent decrease by up to 30% in the highest exposure concentration of 250 mg/L PTU.

The fact that both decreased and increased levels of T3 influence the development of the eye structure complicates the collection of quantitative data for KER 2373 linking reduced T3 to

altered retinal structure. In D3bMO zebrafish larvae (knockdown of DIO 3b, resulting in increased T3 levels), there was a marked disorganization across all layers of the retina with reductions in the numbers of cones (Houbrechts et al., 2016), and Ng et al. (2010) found severely reduced numbers of cones in 2-month-old DIO3 knockout mice and showed that cones were generated and then lost by cell death. This confirms that eye development is under tight control of balanced TH levels. Another complicating factor is the fact that altered retinal structure is often observed using a semi-quantitative grading system rather than quantitative measurements.

Nevertheless, a semi-quantitative relationship between thyroid follicle TH content and visual behavior can be derived by combining two studies: Rehberger et al. (2018) performed a quantitative visualization of intrafollicular T3 and T4 in PTU-treated zebrafish embryos using fluorescence antibody staining. At 2.5 mg/L PTU, 93 % of the T4 content was still present, while at 50 mg/L only 50 % of the T4 content remained compared to the control. A similar trend could be observed for T3 contents, which decreased with increasing PTU concentrations. At 2.5 mg/L PTU, the fluorescence signal (integrated density) had decreased to 70% compared to the negative control, while at 50 mg/L only 0.4% remained. These data can be compared with the data from Baumann et al. (2016), as the protocols for rearing and handling the embryos (of the same strain) were very similar. The concentration range used in the Baumann et al. (2016) study was 50 to 250 mg/L PTU, at which strong effects on eye development were observed. The morphological changes resulted in impaired visual performance of the larvae: The optokinetic response was significantly and concentration-dependently decreased in both treatments, together with a significant increase in light preference of PTU-treated larvae. A decreased OKR response of more than 30% could already be observed at 50 mg/L PTU, which further decreased with increasing exposure concentration. The trend of both studies suggests a correlation, but further studies in the same concentration range would be needed to draw clear conclusions.

Domain of applicability

For the development of this AOP, the focus was on fish as the taxonomic domain of applicability. Within fish, zebrafish are the most studied model organism, and therefore most evidence supporting this AOP comes from zebrafish (Baumann et al., 2016; Dehnert et al., 2019; Houbrechts et al., 2016; Komoike et al., 2013; Reider & Connaughton, 2014) with some

evidence from other fish species such as surgeonfish (Besson et al., 2020) and salmonids (Gan & Flamarique, 2010). However, caution is needed when extrapolating effects to "fish" in general, as current data may not account for the many fish species that are adapted to different ecological niches. Nevertheless, the term "fish" is used, because it is plausible to assume that the sequence of events is applicable to most fish species. This facilitates the extension of the AOP to other fish species in the future as more research is conducted. Lalone et al., (2018) used the US EPA Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) tool to show that TPO is conserved across vertebrates both in terms of the nucleic acid sequence and the protein sequence of the functional domain. Likewise, the HPT axis as a whole as well as retinal structure are highly conserved in vertebrates, and it is well known that THs are essential for retinal development across vertebrate classes (Ng et al., 2010; Trimarchi et al., 2008; Viets et al., 2016). For example, impaired retinal development and altered retinal electrophysiology has also been observed in mice (Gamborino et al., 2001) and TH insufficiency in humans has been linked to visual deficits (Klein and Mitchell, 1999; Klein et al., 2001, Eldred et al. 2018). Therefore, while the current WoE evaluation is based on fish, the AOP is plausibly applicable to other vertebrate classes, and we envision that the taxonomic domain of applicability can be expanded in the future.

In terms of life-stage applicability, most of the data comes from developing fish and, subsequently, in this AOP the focus is on effects of THSDCs on the development of the retina during the embryonic period. Although the retina is known to remain sensitive to THSD in juveniles as well (Mackin et al., 2019), there are knowledge gaps relative to how THSDCs affect the eyes of developed organisms and whether they have similarly strong effects on the retinal layers. Regardless of the timing of retinal structure disruption, it is plausible to assume that such effects impose consequences at a population-relevant level, as visual perception is essential for foraging success, survival and reproduction throughout all life stages (Cohen et al., 2022).

Most evidence of the impact of TPO inhibition on fish retinal development comes from exposures of zebrafish embryos from immediately after fertilization until day 5, when eleutheroembryos start to freely feed and enter the larval life stage. To evaluate the life stage specificity of the present AOP, the ontogeny of the downstream event (i.e., altered retinal layer structure) should be investigated as a function of the ontogeny of the HPT-axis and the activation of TH synthesis by TPO in particular (the MIE). In zebrafish, the first thyroid follicles appear around 55 hpf (Alt et al., 2006), and active endogenous TH synthesis indicating active

thyroidal TPO has been detected around 72 hpf (Walter et al., 2019). Before active embryonic TH synthesis, the embryo relies on maternally transferred THs to regulate the earliest TH-dependent developmental processes (Power et al., 2001). This leads to the hypothesis that TPO inhibition has limited impact on processes occurring before 72 hpf and is thought to be the reason for the absence of effects on the inflation of the posterior chamber of the swim bladder after TPO inhibition (Stinckens et al., 2016). We therefore hypothesize that the effects on the retina are triggered between activation of TH synthesis (around 72 hpf) and 5 dpf. While the retinal layers can be distinguished at 72 hpf, the process of differentiation and maturation of the retinal layers leading to a functional retina continues until well after the onset of thyroidal TH synthesis. This is illustrated by the maturation of UV cones and the first proper optokinetic response occurring around 4 dpf (Cohen et al., 2022). In summary, we distinguish between early (< 72 hpf) and late (> 72 hpf) embryonic processes, where early processes may not be (highly) sensitive to TPO inhibition specifically, while they can be sensitive to other mechanisms of THSD such as deiodinase inhibition, since deiodinases are required to activate maternal T4 (Stinckens et al., 2016). Most evidence of effects on retinal layer structure detailed in section 4 is in line with this hypothesis, while some studies lead to uncertainties. For example, Reider and Connaughton (2014) exposed zebrafish embryos to MMI until 66 hpf and raised them further in clean water until 72 hpf. In these embryos, ganglion cell layer thickness was reduced. There is still uncertainty about the exact onset of TH synthesis. Potential TH level increases between 48 and 72 hpf have not been studied. Since in zebrafish mRNA coding for TPO and NIS is maternally transferred, expression of thyroglobulin increases before the appearance of the thyroid anlage at 32–35 hpf (Vergauwen et al., 2018) and the first follicle appears around 55 hpf, this leaves a 17-hour window for potential TH synthesis and thus potential sensitivity to TPO inhibition between 55 and 72 hpf.

There are other potential mechanisms that may contribute to altered retinal layer structure and that are not the subject of the present AOP report. For example, local expression of TPO mRNA has been reported in the mouse retina (Li et al., 2012) suggesting an additional target for TPO inhibition locally in the eyes, possibly even before the onset of TH synthesis in the thyroid follicles. Further studies are needed to establish whether local TH synthesis in the eye is biologically plausible. It is not clear to what extent essential components of the TH synthesizing machinery that are present in the thyroid gland are also present in the eyes. This includes presence of the TPO protein as well as thyroglobulin, iodide uptake, a source of hydrogen peroxide, etc. Moreover, it is not clear whether (perhaps less efficient) TH synthesis is actually possible without the presence of a follicular epithelial structure. Furthermore, there are TH-

independent mechanisms that could contribute to altered retinal layer structure. Komoike et al. (2013) already observed effects of TPO inhibition (induced by MMI) on the retinal structure at 48 and 72 hpf and suggested that this was due to TH-independent apoptosis. Li et al. (2012) hypothesized that the development of the extracellular matrix was disrupted by general peroxidase inhibition after exposure to phenylthiourea, a bleaching agent that is a TPO inhibitor. Additionally, THSDCs often act *via* multiple mechanisms, and these mechanisms could simultaneously act on retinal layer development (e.g., combined TPO and DIO inhibition by PTU).

With respect to sex applicability, this AOP considers the impact of reduced TH synthesis on the development of the retina, which starts during embryonic development and continues during the larval life stage. At this point, this AOP does not consider potential effects of THSD on the already developed retina during later life stages. At the time when zebrafish early life stages are typically sampled for observation of retinal structure (at or before 5 dpf), sex has not been determined yet and gonad differentiation has not started (Maack & Segner, 2003). Effects of THSD on the development of the retinal structure are therefore generally assumed to be independent of sex.

Known stressors triggering the AOP

For the development of the AOP, data on pharmaceuticals that are generally accepted as model TPO inhibitors were mainly found and used: PTU (also a deiodinase inhibitor) and MMI. Both pharmaceuticals are solely applied to treat hyperthyroidism, which is a disease that affects only around 1 % of the population in western countries (Bahn et al., 2011). Consequently, environmental concentrations are very low, if present at all. TPO inhibition has received a lot of attention as one mechanism of THSD that may cause adverse effects to humans and the environment, and important efforts have been made to develop a high-throughput screening approach to identify TPO inhibitors (Paul Friedman et al., 2016; Paul et al., 2013; Paul et al., 2014; Stinckens et al., 2020). Several chemicals of the ToxCast libraries were found to inhibit TPO (Paul Friedman et al., 2016). Examples include MBT and ethylene thiourea (both used in rubber industry), resorcinol (used to treat skin disorders) and benzophenone (UV-filter) (Lynch et al., 2002; Tietge et al., 2013; Doerge & Takazawa, 1990; Schmutzler et al., 2007). For the crucial KER in the AOP linking reduced T3 levels to altered retinal layer structure (KER 2373), data from exposures to additional model compounds with mechanisms other than TPO

inhibition were included: iopanoic acid (DIO inhibitor resulting in decreased T3 levels), and perchlorate (inhibitor of sodium-iodide symporter leading to reduced uptake of iodide and reduced TH synthesis), both of which have an impact on the eye development of fish (e.g. Bhumika et al., 2015).

5. Potential applications

Risk assessment of EDCs specifically targeting the TH system has recently been receiving a lot of attention from both a regulatory and scientific perspective, as there is an urgent need to establish meaningful endpoints for standard test systems. Based on the present AOP, we suggest fish eye development, with focus on morphological and structural alterations, as an apical endpoint for inclusion into fish ED test guidelines for THSD. THs contribute to the regulation of proper eye development in all vertebrates, and, consequently, environmental pollutants that target the TH system can disrupt this crucial developmental process. This, in turn, has severe consequences for survival of wildlife, as visual detection of prey and predators is vital.

Different criteria have been set to identify endocrine disrupting chemicals (EDCs) as part of chemical safety evaluation. Specifically, evidence of an endocrine mechanism, an adverse health effect and a causal link between these two elements is required (EC 2017; 2018). The definition of an AOP aligns perfectly with these criteria, and therefore AOPs can aid in assembling the necessary evidence to identify EDCs such as THSDCs. The lack of thyroid-related endpoints has recently been recognized as a major gap in existing fish test guidelines for EDC assessment 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 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. The present AOP will significantly contribute to these activities by providing the scientific evidence to establish fish eye development as thyroid-sensitive endpoint. This will not only broaden the set of ED-sensitive endpoints in fish in general, but will also support ethical considerations in the context of EDC testing. First, a major part of the proposed endpoints in zebrafish can already be assessed in embryonic “non-protected” life stages, which are usually considered alternatives to animal testing. This contributes to the development of new approach methodologies (NAMs). Second, endpoints assessing eye development can easily be added to existing fish test guidelines, reducing the need for additional testing. Third,

the establishment of thyroid-related endpoints in fish could reduce or even replace amphibian testing, which is mainly applied due to the TH-regulated metamorphosis of amphibians. This would significantly contribute to the application of the 3R principle for testing of EDCs. An overview of assays associated with this AOP is given in Table 2.

Currently, THSD-specific test systems with fish are not yet established at the OECD level. However, over the last years, the zebrafish has been the predominant non-mammalian model employed in studies assessing thyroid system function and disruption (Couderq et al., 2020). In the last 5 years, 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. Almost all OECD test guidelines using zebrafish for EDC testing appear suitable for inclusion of THSD-related endpoints, i.e. TG 210 (Fish Early-life Stage Toxicity Test), TG 229 (Fish Short Term Reproduction Assay), TG 234 (Fish Sexual Development Test), TG 250 (EASZY assay – Detection of Endocrine Active Substances, acting through estrogen receptors, using transgenic tg(cyp19a1b:GFP) Zebrafish embryos) and TG 236 (Fish Embryo Acute Toxicity (FET) test) (OECD, 2018). Additionally, TG 240 (Medaka Extended One Generation Reproduction Test, MEOGRT) using *Oryzias latipes* also covers sensitive life stages responsive to THSD. A similar test with zebrafish (ZEOGRT) is currently under validation. Moreover, there are current attempts to merge TGs 229 and 234 and to add thyroid-related endpoints, as well as to use transgenic zebrafish for analyses of thyroid follicles in a modified FET test. Our literature review and resulting AOP show that eye development endpoints, particularly retinal structure and pigmentation, are affected by THSDs and could thus be promising thyroid-related endpoints for inclusion in OECD TGs with fish. Furthermore, the TH-specificity of the eye development endpoints should be examined, since other signaling pathways, such as the retinoid, IGF-1 and aryl hydrocarbon receptor pathways, may also affect eye development (Chen et al., 2020; Molla et al., 2019). Consequently, the assessment of TH levels or thyroid histopathology are required to confirm the TH-specificity of the observed effects.

Against this background, the EU is currently funding different research projects focusing on THSD in fish, such as the Horizon 2020 project "ERGO" (EndocRine Guideline Optimization), which was launched in 2019 and aims to improve existing test guidelines for EDCs. The specific objective of ERGO is "to break down the walls between different research fields (non-mammalian vertebrates and mammalian vertebrates) and to improve the hazard assessment of

EDCs for human health and the environment". The integration of information from different animal models across vertebrate classes (amphibians, fish, mammals) should provide a conclusive assessment of adverse effects of EDCs and result not only in a standardization of tests, but also a reduction of animals used for testing. A special focus is set on THSD effects, with the purpose of integrating suitable endpoints into already existing OECD test guidelines (Holbech et al., 2020).

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Table 1: Summary of the weight-of-evidence (WoE) evaluation of all KERs in the AOP

KER ID	Upstream KE	relationship	Downstream KE	Biologial plausibility	Empirical evidence	Overall WoE	Quantitative understanding	Previously reviewed[a]
309	Thyroperoxidase, inhibition	Adjacent	TH synthesis, decreased	High	Low	High	Moderate*	Y
305	TH synthesis, decreased	Adjacent	T4 in serum, decreased	High	Low	Moderate	Moderate*	Y
366	Thyroperoxidase, inhibition	Non-adjacent	T4 in serum, decreased	High	Moderate	High	Moderate*	Y
2038	T4 in serum, decreased	Adjacent	Decreased, triiodothyronine (T3)	Moderate	Moderate	Moderate	Moderate	Y
2373	Decreased, triiodothyronine (T3)	Adjacent	Altered, retinal layer structure	Moderate	Moderate	Moderate	Low	N
2374	Altered, retinal layer structure	Adjacent	Altered, visual function	High	Moderate	High	Low	N
2375	Altered, visual function	Adjacent	Increased, mortality	High	Moderate	Moderate	Low	N
2013	Increased, mortality	Adjacent	Decrease, population growth rate	High	Moderate	Moderate	Moderate	Y

[a] N: No, Y: Yes. If Yes, levels of confidence have been taken from the previously reviewed AOP 159. *Because of recent advancements, the level of confidence for quantitative understanding was changed from low to moderate.

Table 2: Overview of assays associated with the AOP

Event	Applicable assays to assess effects	Test guidelines in which assays could be included
TPO inhibition (Event #279)	*In chemico* enzyme activity assay (e.g. Paul et al., 2014)	-
TH synthesis decreased (Event #277)	Thyroxine-immunofluorescence quantitative disruption test (Thienpont et al. 2011)	210, 229, 230, 234, 236, 240, 250
T4 in serum and T3 decreased (Events #281 and #1003)	ELISA, LC/MS (reviewed by Martin et al. 2020)	210, 229, 230, 234, 236, 240, 250
Retinal layer structure altered (Event #1877)	(Immuno-)Histology (e.g. Baumann et al., 2016; Flamarique et al. 2019; Heijlen et al. 2014))	210, 234, 236, 240, 250
Visual function altered (Event #1643)	OKR, electroretinogram, photomotor response (e.g. Baumann et al., 2016; Houbrechts et al., 2016).	210, 234, 236, 240, 250

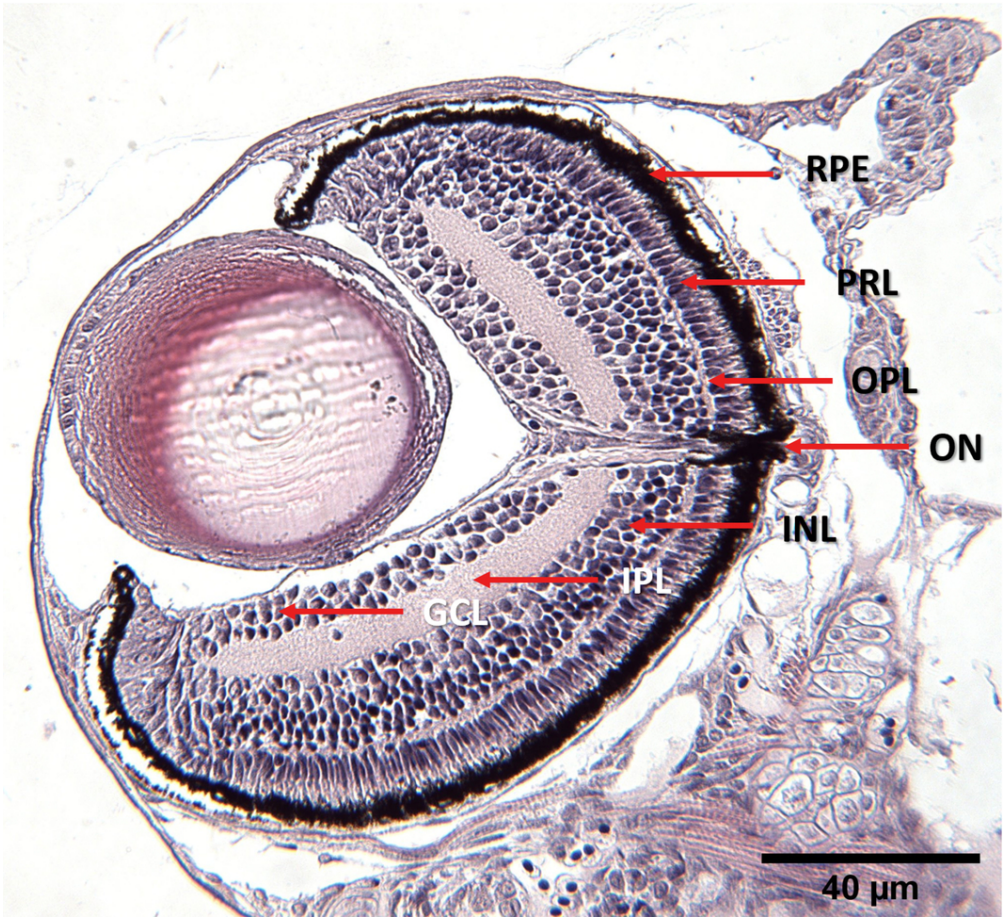

Fig. 1: Histological section of a healthy embryonic zebrafish (Danio rerio) retina at 5 dpf. RPE: retinal pigment epithelium; PRL: photoreceptor layer; OPL: outer plexiform layer; ON: optic nerve; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.

90x82mm (300 x 300 DPI)

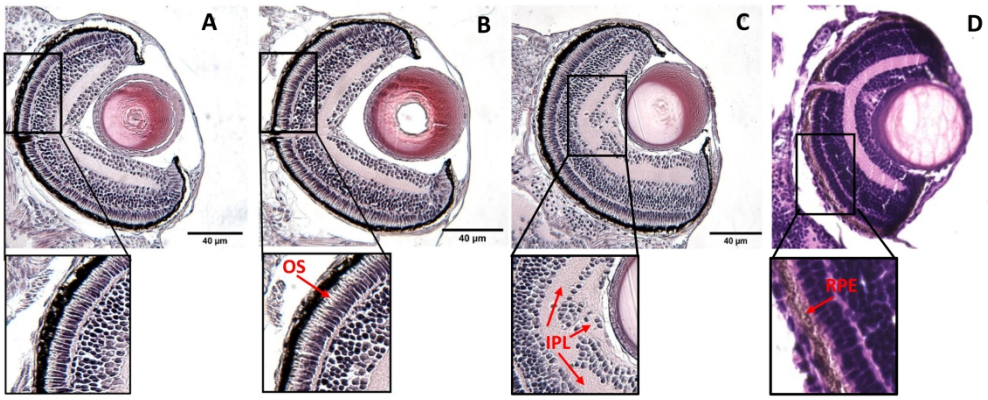

Fig. 2: Histological sections of the zebrafish (Danio rerio) retina after exposure to different THSDCs until 5 dpf; HE-stained paraffin sections. A: Control; B: Exposure to 1.5 mg/L PCL, altered photoreceptor (PR) layer: increased ratio of size of outer segment (OS) of PRs (i.e., bright area) compared to size of darker inner segment; C: Exposure to 2 mg/L IOP, malformations of the inner plexiform layer (IPL); D: Exposure to 250 mg/L PTU, reduced eye size and pigmentation of the retinal pigment epithelium (RPE). PTU: modified from Baumann et al. (2016); PCL & IOP: unpublished data from Goelz et al. 2022, in preparation.

179x73mm (600 x 600 DPI)

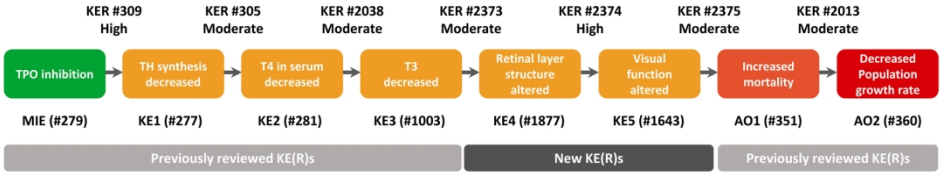

Fig. 3: Graphical representation of the AOP leading from TPO inhibition to altered visual function via altered retinal layer structure (AOP 363). Numbers preceded by # refer to AOP-Wiki IDs. Overall confidence levels resulting from the weight-of-evidence evaluation considering both the biological plausibility and the empirical evidence of the key event relationships have been included (see also Table 1).

179x34mm (300 x 300 DPI)

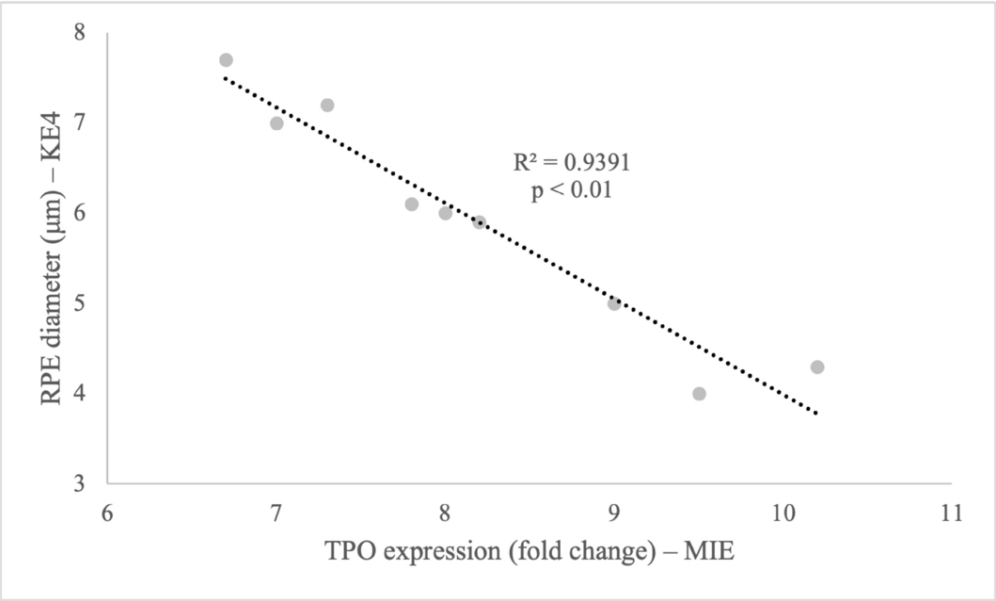

Fig. 4: Meta-analysis of MIE-KE4 response-response relationship. Correlation analysis of data from Baumann et al. (2016). Zebrafish embryos were treated with 50, 100 or 250 mg/L PTU. TPO gene expression (measured as fold change compared to control, which was set to 1) was plotted against RPE thickness. The coefficient of determination (R2=0.9391) shows a clear correlation.

90x54mm (300 x 300 DPI)

BOX 1: AOP ID Box

- Formal AOP title: Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure
- AOP authors: Lisa Gölz, Lisa Baumann, Pauline Pannetier, Lucia Vergauwen
- AOP contributors: Lisa Gölz, Lucia Vergauwen
- AOP number: 363
- Development status: *Open for comment. Do not cite.*
- OECD work plan project number: 1.35
- List of KEs:
    - MIE: Thyroperoxidase, Inhibition (Event #279)
    - KE1: Thyroid hormone synthesis, Decreased (Event #277)
    - KE2: Thyroxine (T4) in serum, Decreased (Event #281)
    - KE3: Decreased, Triiodothyronine (T3) (Event #1003)
    - KE4: Altered, retinal layer structure (Event #1877)
    - KE5: Altered, Visual function (Event #1643)
    - AO1: Increased mortality (Event #351)
    - AO2: Decrease, Population growth rate (Event #360)