AOP ID and Title:

AOP 363: Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure
Short Title: TPOi retinal layer structure

Graphical Representation

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Status

Author status	OECD status	OECD project	SAAOP status
Open for comment. Do not cite			

Abstract

Visual function is particularly important for survival, especially of developing life stages. Some chemicals, including thyroid hormone system disrupting chemicals (THSDCs), can impair eye development. The chain of events, from the molecular interaction of thyroid hormone system disruption (THSD) to the consequences at the level of vision, is not yet fully understood. The development of this AOP aims to contribute to filling these gaps and investigates how inhibition of thyroperoxidase and resulting changes in hormone levels can lead to effects on the retinal layers and subsequently at the population level.

The focus of this AOP is on fish, as the largest amount of data is available for this taxonomic group. Data obtained with a variety of different techniques to induce or mimick TH synthesis disruption have been included (addressing KE 227: "Decreased thyroid hormone synthesis"), for example, exposure to THSDCs, generation of transgenic or mutant fish, microinjection, morpholino knockdown, thyroid ablation, etc. The resulting changes in hormone levels have been studied (KE 281: "Decreased thyroxine (T4) in serum" , KE 1003: "Decreased triiodothyronine (T3) in serum"), as well as changes in the retinal layers (KE 1877: "Altered retinal layer structure"). These include e.g. cell size, cell layer structure, organisation and number of photoreceptors, pigmentation and information on morphological changes (e.g. cell shapes). At a higher level of biological organization, physiological and behavioural changes were investigated (AO 1643: "Altered visual function"), including e.g. optokinetic response, optomotor reponse, light response, etc. The present AOP is closely linked to AOPs 155-159 on THSD leading to impaired swim bladder inflation in fish, as well as AOP 297 on retinoic acid effects on eyes.

Background

This AOP is based on data derived from several extensive literature searches. First, data was collected on different biological levels: Results at the molecular level, data on hormone levels, data on the tissue level and on the behavioural/physiological level. In a next step, KEs and KERs were identified and defined and a more detailed search was initiated. While initially an AOP network including several different effects on eye developemnt was considered, in a next step AOP 363 was selected and further refined, and again an intensive and very detailed final literature search was conducted. The search for bibliographic data was conducted online in "pubmed", "sciencedirect/Scopus" and "Web of Science". The initial search terms were: "fish", "eye development", "retina", "thyroid/hormone disorders", "visual behaviour", "photoreceptors" and combinations of these terms. A very detailed manual search followed for the various KEs and KERs. Not only articles on chemical exposure of different animals were considered, but also more basic studies using other THSD induction techniques such as transgenic or mutant fish, microinjection, morpholino oligonucleotides, thyroidectomy, etc. The range of data that was assessed is wide, from gene expression and hormone levels to physiological and behavioural changes in different animals. In total, around 120 articles from these structured studies were analysed in terms of experimental design and information on different biological levels. Most of the data is on fish, especially zebrafish (85%), which is why this AOP focuses on fish, but it can probably be applied to other vertebrate species as well.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	279	Thyroperoxidase, Inhibition	Thyroperoxidase, Inhibition
	KE	277	Thyroid hormone synthesis, Decreased	TH synthesis, Decreased
	KE	281	Thyroxine (T4) in serum, Decreased	T4 in serum, Decreased
	KE	1003	Decreased, Triiodothyronine (T3) in serum	Decreased, Triiodothyronine (T3) in serum
	KE	1877	Altered, retinal layer structure	Altered, retinal layer structure
	KE	1643	Altered, Visual function	Altered, Visual function
	AO	351	Increased Mortality	Increased Mortality
	AO	360	Decrease, Population trajectory	Decrease, Population trajectory

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Thyroperoxidase, Inhibition	adjacent	Thyroid hormone synthesis, Decreased	High	Moderate
Thyroid hormone synthesis, Decreased	adjacent	Thyroxine (T4) in serum, Decreased	Moderate	Moderate
Thyroxine (T4) in serum, Decreased	adjacent	Decreased, Triiodothyronine (T3) in serum	Moderate	Moderate
Decreased, Triiodothyronine (T3) in serum	adjacent	Altered, retinal layer structure	Moderate	Low
Altered, retinal layer structure	adjacent	Altered, Visual function	High	Low
Altered, Visual function	adjacent	Increased Mortality	Moderate	Low
Increased Mortality	adjacent	Decrease, Population trajectory	Moderate	Moderate
Thyroperoxidase, Inhibition	non-adjacent	Thyroxine (T4) in serum, Decreased	High	Moderate

Stressors

Name	Evidence
Propylthiouracil	High
Methimazole	High

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Larvae	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic applicability: The weight of evidence supporting the first linkage of this AOP between the MIE, TPO inhibition, and the KE of decreased TH synthesis, is strong and supported by more than three decades of research in animals including humans. Several papers have measured alterations in TPO and subsequent effects on TH synthesis (Cooper et al., 1982; Cooper et al.,1983; Divi and Doerge, 1994).

Also for the next KER, it is widely accepted that TPO inhibition leads to declines in serum T4 levels in adult mammals. Strong qualitative and quantitative relationships exist between reduced TH synthesis and reduced serum T4 (Ekerot et al., 2013; Degon et al., 2008; Cooper et al., 1982; 1983; Leonard et al., 2016; Zoeller and Tan, 2007). Nevertheless, a majority of the empirical evidence comes from work with laboratory rodents, there is a large amount of supporting data from humans (with anti-hyperthyroidism drugs including propylthiouracil and methimazole), some amphibian species (e.g., frog), fish species (e.g., zebrafish and fathead minnow), and some avian species (e.g, chicken) (Cooper et al. (1982; 1983); Hornung et al. (2010); Van Herck et al. (2013); Paul et al. (2013); Nelson et al. (2016); Alexander et al. (2017); Stinckens et al. (2020)).

Although the following KER (T4 in serum decreased leads to Triiodothyronine (T3) in serum decreased) is plausibly applicable across vertebrates, too, variation can be expecteddue to feedback/compensatory mechanisms that can also differ across species. In zebrafish and fathead minnow, several studies reported the evidence for a relationship between circulating T4 and T3 levels (Nelson et al., 2016; Stinckens et al., 2020, Wang et al., 2020).

The linkage between the MIE, decreased T3 in serum, and the KE of altered retinal layer structure, is evidental in the different vertebrate classes. There is ample evidence that THs have an influence on the development of the retinal layer structure. Although there are some differences in eye structure between species, it is known that the retina follows the typical organisation of vertebrates. Within vertebrates, it consists of several layers of RPE, photoreceptors, neurons and choroid. It is plausible to assume that TH levels are important for healthy eye development in all vertebrates.

Thyroid hormone receptors have a general function in different cell types of the vertebrate retina, they mediate specific events in retinal and photoreceptor development. The decrease of TH levels can lead to disturbances of the retinal layers, as shown by studies in various vertebrates such as fish species, rats, mice and humans (Baumann (2016), Komoike et al. (2013), Besson et al. (2020), Gamborino (2000), Houbrechts (2016), (Li et al. 2021)). In humans, hypothyroidism is also linked to impaired color vision (Racheva et al., 2020).

Life stage applicability: This AOP considers effects of TPO inhibitors on the development of the retina during the embryolarval life stage. In order to more specifically evaluate the life stage applicability of the impact on thyroperoxidase inhibition on retinal layer structure and visual function leading to increased mortality, the timing of the ontogeny of the target organ needs to be matched to the timing of the ontogeny of the HPT-axis. Fish, amphibians and birds develop externally and rely on maternally transferred THs and TH machinery during the earliest stages of embryonic development.

In zebrafish, effects on retinal layer structure are typically observed at 96 or 120 hpf. By 60 hpf, the different layers of the retina can be distinguished (Morris and Fadool 2005; Schmitt and Dowling 1999) but differentiation and maturation continues until well beyond 84 hpf (Raymond and others 1995). The first thyroid follicle appears around 55 hpf and endogenous T4 production has been observed at 72 hpf (Walter and others 2019). Since thyroperoxidase is principally located in the thyroid follicles and responsible for the synthesis of TH which are released to circulation, important impacts on thyroidal TH synthesis due to thyroperoxidase inhibition are not expected before 72 hpf. This hypothesis is in line with the observation that inflation of the posterior chamber of the swim bladder appears to be unaffected by thyroperoxidase inhibition in zebrafish and fathead minnow (Nelson and others 2016; Stinckens and others 2016). Thyroperoxidase expression has however additionally been observed locally in the eyes of mice (Li and others 2012), suggesting a potential role of local TH synthesis in eye development before the thyroid follicles become active.

In summary, in zebrafish the formation of the retinal layers occurs before the activation of thyroidal TH synthesis but further differentiation and maturation continues until well after the onset of thyroidal TH synthesis. There is ample evidence of the impact of thyroperoxidase inhibition on retinal layer structure at the age of 5 dpf, and there is some evidence showing early effects at 48, 66 and 72 hpf (Komoike and others 2013; Reider and Connaughton 2014) suggesting the importance of local TH synthesis in the eyes. Additional mechanisms (e.g., deiodinase inhibition) could also play a role. Currently there is insufficient evidence to clearly evaluate the importance of inhibition of local TPO in the eyes versus thyroidal TPO for the development of proper retinal layer structure.

Mammals on the other hand continuously receive maternal THs via the placenta. Therefore, exposure to inhibitors of TH synthesis is expected to have an effect on the earliest phases of embryonic development by inhibiting maternal TH synthesis (REF).

Taken together, there is strong support for applicability of the current AOP to embryolarval/embryofoetal stages of vertebrates. Since the term 'eleutheroembryo' (stage starting at hatching and ending with free-feeding fish) is not available, the terms 'embryo' and 'larvae' were selected to reflect this.

Sex applicability: Fish species have different patterns of gonadal differentiation. Many species are undifferentiated gonochorists[LB1] (e.g., zebrafish, fathead minnows), in which an indifferent gonad first develops into an ovary-like gonad which then further differentiates into either a mature ovary or a testis (Maack and Segner, 2003). Other fish species such as medaka are differentiated gonochorists where the indifferent gonad develops directly into an ovary or a testis. In both cases, in the early life stages where the eyes develop, the gonads have not yet started to differentiate. For example, in zebrafish the eyes develop in the first 5 days of development and the gonads differentiate in the period around 20-50 dpf. In species such as zebrafish, even sex determination has not occurred by the time the eyes develop, since it is dependent on environmental factors. This means that in the life stages of interest for this AOP (embryo-larval), sex has not been established yet nor has gonad differentiation started. Therefore, sex is not assumed to be an important factor in determining the effect of TPO inhibitors on retinal structure development.

This does however not preclude the occurrence of sex dependent changes in eye structure during later life after gonadal differentiation. For example, Chen et al. (2018) exposed marine medaka to perfluorobutane sulfonate (PFBS) for an entire life cycle and this resulted in sex dependent changes in eye water content and neurotransmitter levels in the eyes.

Essentiality of the Key Events

Essentiality means that a stressor can activate an AOP and its various KEs, and that cessation of this stressor can prevent this activation or lead to a recovery of the adverse effects. Certain studies, such as gene knockdown, recovery or knockout experiments, have been reviewed to evaluate this. Evidence for essentiality in this AOP can be classified as **high**. Direct evidence from specifically designed experimental studies illustrating essentiality is available for several KEs in the AOP. Especially the evidence of essentiality of decreased T3 levels for effects on the eyes is very important and strongly supports this AOP.

Weight of Evidence Summary

1. Biological plausibility:

   Most of the **KERs (309, 305, 366, 2374, 2375, 2013)** were found to be **highly biologically plausible**. For example, TPO is known to be a key enzyme of the TH system and plays an important role in controlling important functions such as neuronal development, including eye development. Similarly, the thyroid hormone T4 is known to be activated to T3 by DIOs in the liver and other organs. Both T3 and T4 are present during retinal development (Roberts and others 2006), and key components such as DIOs (Heijlen and others 2013; summarized by Viets and others 2016), TH receptors (Gan and Flamarique 2010), and TPO (Li and others 2012) are also expressed in the vertebrate retina during retinal development. However, there are compensatory mechanisms that limit the impact on T3 levels, possibly through increased deiodinase activity or other feedback or compensatory mechanisms, as well as some gaps in knowledge. Therefore, the biological plausibility of **KER 2038 and 2373** was determined to be **moderate.**

2. Empirical support is **moderate** for most KERs in the AOP and **low** for the most upstream KERs.

3. Overall WoE ranges from **moderate to high**. As prescribed by the User's handbook, biological plausibility was given slightly more weight in this decision compared to empirical evidence.

Quantitative Consideration

The difficulties in generating quantitative data for this AOP may be due to the fact that both decreased and increased T3 levels affect the development of retinal structure, confirming that this process is under strict control of balanced TH levels, but also making it difficult to describe the quantitative relationship between T3 levels and altered retinal structure (Stinckens et al. (2020)). Furthermore, disrupted retinal layers are often observed using a semi-quantitative classification system rather than quantitative measurements.

However, the combinations of some studies show some correlations:
For example, the study by Rehberger et al. (2018) shows a tendency for T3 and T4 to decrease with increasing PTU concentration, Baumann et al. (2016) found both a disturbed retina and behavioural abnormalities due to impaired visual performance in larvae at these concentrations (and at much higher concentrations). Baumann et al. (2016) also shows a correlation between increased TPO expression (measured as a fold change) and decreased RPE diameter with increasing PTU exposure.

There are quantitative data on KER1 (TPO, inhibition (KE 279) results in TH synthesis, reduced (KE 277), also. For example, Hassan et al (2017) quantified TH synthesis blocked by PTU and MMI in an in vitro TPO inhibition study to predict TH concentration in rat serum. Similarly, Fisher et al. (2013) modelled the effect of TPO inhibition on serum TH concentrations during early development in rats, Haselman et al. (2020), in Xenopus laevis, demonstrated the temporal profiles of thyroid iodotyrosines (MIT/DIT) and iodothyronines (T4/T3), the products of TPO activity, after exposure to three different model TPO inhibitors (MMI, PTU, MBT) at different concentrations.

Considerations for Potential Applications of the AOP (optional)

THSDCs are increasingly recognized as a serious environmental problem for aquatic species, as well as for humans. Especially the THSD effects on (neuro-)developmental processes pose a risk to different vertebrate species. The current framework for assessment of THSD effects is separated between human and environmental health, and in the latter, it is restricted to amphibians. The implementation of thyroid-related endpoints into test guidelines using fish is urgently needed and is currently being addressed in different EU-funded research projects (Holbech et al. 2020) and in project 2.64 of the OECD TG work plan, “Inclusion of thyroid endpoints in OECD fish Test Guidelines”. Moreover, this testing gap has been recognized by OECD VMG-Eco in 2016 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.

The present AOP provides strong evidence that eye development represents a very promising endpoint that could be implemented into existing OECD test guidelines that cover developmental phases of fish, such as the Fish Embryo Acute Toxicity (FET) test (OECD TG 236), the Fish Early Life Stage Toxicity (FELS) Test (OECD TG 210) and the Fish Sexual Development Test (FSDT, OECD TG 234). Especially the FET seems to be well suited for implementation of histopathological analyses of retinal structures for the detection of cellular changes that will ultimately result in decreased visual capacities and fitness impairment of exposed larvae. Combined with mechanistic analyses, such as gene expression or TH level measurements, a modified FET for detection of THSD in fish seems very promising for future THSD testing with fish. A major advantage is that a large part of the proposed endpoints in zebrafish can be assessed in embryonic life stages, which are considered “non-protected” alternatives to animal testing.

Consequently, based on AOP 363, together with other AOPs linking THSD to visual function that are under development (AOP 364, 365), we provide evidence that fish eye development, with focus on morphological and structural alterations, can be included as apical endpoint into fish endocrine disruption test guidelines for THSD. However, the TH-specificity of eye-related endpoints should be examined, since other signaling pathways, such as the estrogenic, retinoid, IGF-1 and aryl hydrocarbon receptor, can also affect eye development (Molla et al., 2019; Chen et al., 2020). Consequently, measurement of TH levels or performance of thyroid histopathology are required to support the causal link between the THSD mechanism and the observed effects.

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Appendix 1

List of MIEs in this AOP

Event: 279: Thyroperoxidase, Inhibition

Short Name: Thyroperoxidase, Inhibition

Key Event Component

Process	Object	Action
iodide peroxidase activity	thyroid peroxidase	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent
Aop:119 - Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	MolecularInitiatingEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	MolecularInitiatingEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	MolecularInitiatingEvent
Aop:271 - Inhibition of thyroid peroxidase leading to impaired fertility in fish	MolecularInitiatingEvent
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	MolecularInitiatingEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	MolecularInitiatingEvent
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	MolecularInitiatingEvent

Stressors

Name
2(3H)-Benzothiazolethione
2-mercaptobenzothiazole

Name
Ethylene thiourea
Mercaptobenzothiazole
Methimazole
Propylthiouracil
Resorcinol
Thiouracil
Ethylenethiourea
Amitrole
131-55-5
2,2',4,4'-Tetrahydroxybenzophenone
Daidzein
Genistein
4-Nonylphenol
4-propoxyphenol
Sulfamethazine

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
thyroid follicular cell

Organ term

Organ term
thyroid follicle

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

There is a wealth of information on the inhibition of TPO by drugs such as MMI and PTU, as well as environmental xenobiotics. In the landmark paper on thyroid disruption by environmental chemicals, Brucker-Davis (1998) identified environmental chemicals that depressed TH synthesis by inhibiting TPO. Hurley (1998) listed TPO as a major target for thyroid tumor inducing pesticides. More recent work has tested over 1000 chemicals using a high-throughput screening assay (Paul-Friedman et al., 2016).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
humans	Homo sapiens	High	NCBI
pigs	Sus scrofa	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
chicken	Gallus gallus	High	NCBI

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	High	NCBI
mouse	Mus musculus		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

Taxonomic: This KE is plausibly applicable across vertebrates. TPO inhibition is a MIE conserved across taxa, with supporting data from experimental models and human clinical testing. This conservation is likely a function of the high degree of protein sequence similarity in the catalytic domain of mammalian peroxidases (Taurog, 1999). Ample data available for human, rat, and porcine TPO inhibition demonstrate qualitative concordance across these species (Schmultzer et al., 2007; Paul et al., 2013; Hornung et al., 2010). A comparison of rat TPO and pig TPO, bovine lactoperoxidase, and human TPO inhibition by genistein demonstrated good qualitative and quantitative (40–66%) inhibition across species, as indicated by quantification of MIT and DIT production (Doerge and Chang, 2002). Ealey et al. (1984) demonstrated peroxidase activity in guinea pig thyroid tissue using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate that is oxidized by the peroxidase to form a brown insoluble reaction product. Formation of this reaction product was inhibited by 3-amino-1,2,4-triazole and the TPO inhibitor, methimazole (MMI). A comparative analysis of this action of MMI between rat- and human-derived TPO indicates concordance of qualitative response. Data also suggest an increased quantitative sensitivity to MMI in rat compared to human (Vickers et al., 2012). Paul et al. (2013) tested 12 chemicals using the guaiacol assay using both porcine and rat thyroid microsomes. The authors concluded that there was an excellent qualitative concordance between rat and porcine TPO inhibition, as all chemicals that inhibited TPO in porcine thyroid microsomes also inhibited TPO in rat thyroid microsomes when tested within the same concentration range. In addition, these authors noted a qualitative concordance that ranged from 1.5 to 50-fold differences estimated by relative potency. Similary, Takayama et al. (1986) found a very large species difference in potency for sulfamonomethoxine between cynomologus monkeys and rats.

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf and not at 24 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TPO inhibition.

Sex: This KE is plausibly applicable to both sexes. The molecular components responsible for thyroid hormone synthesis, including thyroperoxidase, are identical in both sexes. Therefore inhibition of thyroperoxidase is not expected to be sex-specific.

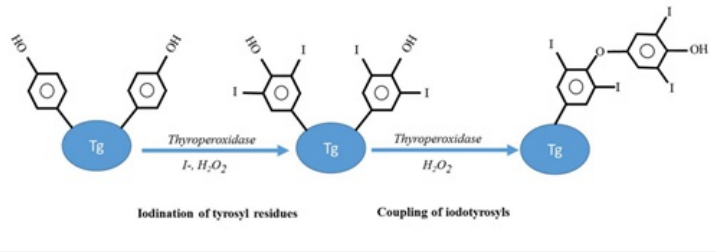
Key Event Description

Thyroperoxidase (TPO) is a heme-containing apical membrane protein within the follicular lumen of thyrocytes that acts as the enzymatic catalyst for thyroid hormone (TH) synthesis. TPO catalyzes several reactions in the thyroid gland, including: the oxidation of iodide; nonspecific iodination of tyrosyl residues of thyroglobulin (Tg); and, the coupling of iodotyrosyls to produce Tg-bound monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Divi et al., 1997; Kessler et al., 2008; Ruf et al., 2006; Taurog et al., 1996). The outcome of TPO inhibition is decreased synthesis of thyroxine (T4) and triiodothyronine (T3), a decrease in release of these hormones from the gland into circulation, and unless compensated, a consequent decrease in systemic concentrations of T4, and possibly T3. The primary product of TPO-catalyzed TH synthesis is T4 (Taurog et al., 1996; Zoeller et al., 2007) that would be peripherally or centrally deiodinated to T3.

It is important to note that TPO is a complex enzyme and that has two catalytic cycles and is capable of iodinating multiple species (Divi et al., 1997). Alterations in all of these events are not covered by some of the commonly used assays that measure “TPO inhibition” (e.g., guaiacol and AmplexUltraRed, see below). Therefore, in the context of this AOP we are using TPO inhibiton not in the classical sense, but instead to refer to the empirical data derived from the assays commonly used to investigate environmental chemicals.

Figure 1 below illustrates the enzymatic and nonenzymatic reactions mediated by TPO that result in the synthesis of thyroxine (T4) .

Figure 1. Synthesis of thyroxine (T4) by thryoperoxidase showing the iodination of tyrosyl residues and subsequent coupling of iodotyrosyls to form T4.



Inhibition of TPO can be reversible, with transient interaction between the enzyme and the chemical, or irreversible, whereby suicide substrates permanently inactivate the enzyme. Reversible and irreversible TPO inhibition may be determined by the chemical structure, may be concentration dependent, or may be influenced by other conditions, including the availability of iodine (Doerge and Chang, 2002).

The ontogeny of TPO has been determined using both direct and indirect evidence in **mammals**. Available evidence suggests the 11th to 12th fetal week as the beginning of functional TPO in humans. In rodents, TPO function begins late in the second fetal week, with the first evidence of T4 secretion on gestational day 17 (Remy et al., 1980). Thyroid-specific genes appear in the thyroid gland according to a specific temporal pattern; thyroglobulin (*Tg*), TPO (*Tpo*), and TSH receptor (*Tshr*) genes are expressed by gestational day 14 in rats, and the sodium iodide symporter, NIS (*Nis*), is expressed by gestational day 16 in rats. Maturation to adult function is thought to occur within a few weeks after parturition in rats and mice, and within the first few months in neonatal humans (Santisteban and Bernal, 2005). Tg is first detected in human fetuses starting at 5th week of gestation and rises throughout gestation (Thorpe-Beeston et al., 1992), but iodine trapping and T4 production does not occur until around 10-12 weeks. Also, the dimerization of Tg, a characteristic of adult TH storage, is not found until much later in human gestation (Pintar, 2000). In rats, Tg immunoreactivity does not appear until day 15 of gestation (Fukiishi et al., 1982; Brown et al., 2000). The vast majority of research and knowledge on Tg is from mammals, although genomic orthologs are known for a variety of other species (Holzer et al., 2016). It is important to note that prior to the onset of fetal thyroid function, TH are still required by the developing fetus which until that time relies solely on maternal sources. Chemical-induced TPO inhibition can affect synthesis in the maternal gland and in the fetal gland.

The components of the TH system responsible for TH synthesis are highly conserved across vertebrates. In fish and amphibians TPO and NIS inhibition result in an expected decrease of TH synthesis (Hornung et al., 2010; Tietge et al., 2013; Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020) like in mammals. Although the thyroid hormone system is highly conserved across vertebrates, there are some taxon-specific considerations.

Zebrafish and fathead minnows are oviparous **fish** species in which maternal thyroid hormones are transferred to the eggs and regulate early embryonic developmental processes during external (versus intra-uterine in mammals) development (Power et al., 2001; Campinho et al., 2014; Ruuskanen and Hsu, 2018) until embryonic thyroid hormone synthesis is initiated. Maternal transfer of thyroid hormones to the eggs has been demonstrated in zebrafish (Walpita et al., 2007; Chang et al., 2012) and fathead minnows (Crane et al., 2004; Nelson et al., 2016).

Inhibition of thyroperoxidase can only occur after activation of embryonic TH synthesis mediated by thyroperoxidase. Endogenous transcription profiles of thyroid-related genes in zebrafish and fathead minnow showed that mRNA coding for thyroid peroxidase is maternally transferred in relatively high amounts with subsequent mRNA degradation followed by initiation of embryonic transcription around hatching (Vergauwen et al., 2018).

How it is Measured or Detected

There are no approved OECD or EPA guideline study protocols for measurement of TPO inhibition. However, there is an OECD scoping document on identification of chemicals that modulate TH signaling that provides details on a TPO assay (OECD, 2017).

From the early 1960's, microsomal fractions prepared from porcine thyroid glands and isolated porcine follicles were used as a source of TPO for inhibition experiments (Taurog, 2005). Microsomes from human goiter samples (Vickers et al., 2012) and rat thyroid glands (Paul et al., 2013; 2014; Paul-Friedman et al., 2016) have also been used as a source of TPO.

TPO activity has been measured for decades via indirect assessment by kinetic measurement of the oxidation of guaiacol (Chang & Doerge 2000; Hornung et al., 2010; Schmutzler et al., 2007). This method is a low-throughput assay due to the very rapid kinetics of the guaiacol oxidation reaction. More recently, higher-throughput methods using commercial fluorescent and luminescent substrates with rodent, porcine, and human microsomal TPO have been developed (Vickers et al., 2012; Paul et al., 2013; 2014; Kaczur et al., 1997). This assay substitutes a pre-fluorescent substrate (Amplex UltraRed) for guaiacol, that when incubated with a source of peroxidase and excess hydrogen peroxidase, results in a stable fluorescent product proportional to TPO activity (Vickers et al., 2012). The stability of the fluorescent reaction product allows this assay to be used in a higher throughput format (Paul-Friedman et al., 2016). This approach is appropriate for high-throughput screening but does not elucidate the specific mechanism by which a chemical may inhibit TPO (Paul-Friedman et al., 2016), and as with most in vitro assays, is subject to various sources of assay interference (Thorne et al., 2010).

HPLC has been used to measure the activity of TPO via formation of the precursors monoiodotyrosine (MIT), diiodotyrosine (DIT),

and both T3 and T4, in a reaction mixture containing TPO, or a surrogate enzyme such as lactoperoxidase (Divi & Doerge 1994). The tools and reagents for this method are all available. However, HPLC or other analytical chemistry techniques make this a low throughput assay, depending on the level of automation. A primary advantage of this in vitro method is that it directly informs hypotheses regarding the specific mechanism by which a chemical may impact thyroid hormone synthesis in vitro.

In fish, increases of TPO mRNA levels are often used as indirect evidence of TPO inhibition in in vivo experiments (Baumann et al., 2016; Nelson et al., 2016; Wang et al., 2020).

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List of Key Events in the AOP

Event: 277: Thyroid hormone synthesis, Decreased

Short Name: TH synthesis, Decreased

Key Event Component

Process	Object	Action
thyroid hormone generation	thyroid hormone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:128 - Kidney dysfunction by decreased thyroid hormone	MolecularInitiatingEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:188 - Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:192 - Pendrin inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:193 - Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:271 - Inhibition of thyroid peroxidase leading to impaired fertility in fish	KeyEvent
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	KeyEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	KeyEvent
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	KeyEvent
Aop:119 - Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent
Aop:110 - Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent

Stressors

Name
Propylthiouracil

Methimazole

Name

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
thyroid follicular cell

Organ term

Organ term
thyroid gland

Evidence for Perturbation by Stressor

Propylthiouracil

6-n-proylthiouracil is a common positive control for inhibition of TPO

Methimazole

Methimazole is a very common positve control for inhibition of TPO

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
Xenopus laevis	Xenopus laevis	Moderate	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI
Sus scrofa	Sus scrofa	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxonomic: This KE is plausibly applicable across vertebrates. Decreased TH synthesis resulting from TPO or NIS inhibition is conserved across vertebrate taxa, with *in vivo* evidence from humans, rats, amphibians, some fish specis, and birds, and *in vitro* evidence from rat and porcine microsomes. Indeed, TPO and NIS mutations result in congenital hypothyroidism in humans (Bakker et al., 2000; Spitzweg and Morris, 2010), demonstrating the essentiality of TPO and NIS function toward maintaining euthyroid status. Though decreased serum T4 is used as a surrogate measure to indicate chemical-mediated decreases in TH synthesis, clinical and veterinary management of hyperthyroidism and Grave's disease using propylthiouracil and methimazole, known to decrease TH synthesis, indicates strong medical evidence for chemical inhibition of TPO (Zoeller and Crofton, 2005).

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. The earliest life stages of teleost fish (e.g., fathead minnow, zebrafish) rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). In externally developing fish species, decreases in TH synthesis can only occur after initiation of embryonic TH synthesis. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. Therefore, it is still uncertain when exactly embryonic TH synthesis is activated and thus when exactly this process becomes sensitive to disruption. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It currently remains unclear when exactly embryonic thyroid hormone production is initiated in zebrafish.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Description

The thyroid hormones (TH), triiodothyronine (T3) and thyroxine (T4) are thyrosine based hormones. Synthesis of TH is regulated by thyroid-stimulating hormone (TSH) binding to its receptor and thyroidal availability of iodine via the sodium iodide symporter (NIS). Other proteins contributing to TH production in the thyroid gland, including thyroperoxidase (TPO), dual oxidase enzymes (DUOX), and pendrin are also necessary for iodothyronine production (Zoeller et al., 2007).

The production of THs in the thyroid gland and resulting serum concentrations are controlled by a negatively regulated feedback mechanism. Decreased T4 and T3 serum concentrations activates the hypothalamus-pituitary-thyroid (HPT) axis which upregulates thyroid-stimulating hormone (TSH) that acts to increase production of additional THs (Zoeller and Tan, 2007). This regulatory system includes: 1) the hypothalamic secretion of the thyrotropin-releasing hormone (TRH); 2) the thyroid-stimulating hormone (TSH) secretion from the anterior pituitary; 3) hormonal transport by the plasma binding proteins; 4) cellular uptake mechanisms at the tissue level; 5) intracellular control of TH concentration by deiodinating mechanisms; 6) transcriptional function of the nuclear TH receptor; and 7) in the fetus, the transplacental passage of T4 and T3 (Zoeller et al., 2007).

TRH and the TSH primarily regulate the production of T4, often considered a “pro-hormone,” and to a lesser extent of T3, the transcriptionally active TH. Most of the hormone released from the thyroid gland into circulation is in the form of T4, while peripheral deiodination of T4 is responsible for the majority of circulating T3. Outer ring deiodination of T4 to T3 is catalyzed by the deiodinases 1 and 2 (DIO1 and DIO2), with DIO1 expressed mainly in liver and kidney, and DIO2 expressed in several tissues including the brain (Bianco et al., 2006). Conversion of T4 to T3 takes place mainly in liver and kidney, but also in other target organs such as in the brain, the anterior pituitary, brown adipose tissue, thyroid and skeletal muscle (Gereben et al., 2008; Larsen, 2009).

In **mammals**, most evidence for the ontogeny of TH synthesis comes from measurements of serum hormone concentrations. And, importantly, the impact of xenobiotics on fetal hormones must include the influence of the maternal compartment since a majority of fetal THs are derived from maternal blood early in fetal life, with a transition during mid-late gestation to fetal production of THs that is still supplemented by maternal THs. In humans, THs can be found in the fetus as early as gestational weeks 10-12, and concentations rise continuously until birth. At term, fetal T4 is similar to maternal levels, but T3 remains 2-3 fold lower than maternal levels. In rats, THs can be detected in the fetus as early as the second gestational week, but fetal synthesis does not start until gestational day 17 with birth at gestational day 22-23. Maternal THs continue to supplement fetal production until parturition. (see Howdeshell, 2002; Santisteban and Bernal, 2005 for review). *The ontogeny of TPO inhibition during development by environmental chemicals is a data gap.*

Decreased TH synthesis in the thyroid gland may result from several possible molecular-initiating events (MIEs) including: 1) Disruption of key catalytic enzymes or cofactors needed for TH synthesis, including TPO, NIS, or dietary iodine insufficiency. Theoretically, decreased synthesis of Tg could also affect TH production (Kessler et al., 2008; Yi et al., 1997). Mutations in genes that encode requisite proteins in the thyroid may also lead to impaired TH synthesis, including mutations in pendrin associated with Pendred Syndrome (Dossena et al., 2011), mutations in TPO and Tg (Huang and Jap 2015), and mutations in NIS (Spitzweg and Morris, 2010). 2) Decreased TH synthesis in cases of clinical hypothyroidism may be due to Hashimoto's thyroiditis or other forms of thyroiditis, or physical destruction of the thyroid gland as in radioablation or surgical treatment of thyroid lymphoma. 3) It is possible that TH synthesis may also be reduced subsequent to disruption of the negative feedback mechanism governing TH homeostasis, e.g. pituitary gland dysfunction may result in a decreased TSH signal with concomitant T3 and T4 decreases. 4) More rarely, hypothalamic dysfunction can result in decreased TH synthesis.

Increased fetal thyroid levels are also possible. Maternal Graves disease, which results in fetal thyrotoxicosis (hyperthyroidism and increased serum T4 levels), has been successfully treated by maternal administration of TPO inhibitors (c.f., Sato et al., 2014).

It should be noted that different species and different lifestages store different amounts of TH precursor and iodine within the thyroid gland. Thus, decreased TH synthesis via transient iodine insufficiency or inhibition of TPO may not affect TH release from the thyroid gland until depletion of stored iodinated Tg. Adult humans may store sufficient Tg-DIT residues to serve for several months to a year of TH demand (Greer et al., 2002; Zoeller, 2004). Neonates and infants have a much more limited supply of less than a week.

While the thyroid hormone system is highly conserved across vertebrates, there are some taxon-specific considerations.

Zebrafish and fathead minnows are oviparous **fish** species in which maternal thyroid hormones are transferred to the eggs and regulate early embryonic developmental processes during external (versus intra-uterine in mammals) development (Power et al., 2001; Campinho et al., 2014; Ruuskanen and Hsu, 2018) until embryonic thyroid hormone synthesis is initiated. Maternal transfer of thyroid hormones to the eggs has been demonstrated in zebrafish (Walpita et al., 2007; Chang et al., 2012) and fathead minnows (Crane et al., 2004; Nelson et al., 2016).

Decreases in TH synthesis can only occur after initiation of embryonic TH synthesis. The components of the TH system responsible for TH synthesis are highly conserved across vertebrates and therefore interference with the same molecular targets compared to mammals can lead to decreased TH synthesis (TPO, NIS, etc.) in fish. Endogenous transcription profiles of thyroid-related genes in zebrafish and fathead minnow showed that mRNA coding for these genes is also maternally transferred and increasing expression of most transcripts during hatching and embryo-larval transition indicates a fully functional HPT axis in larvae (Vergauwen et al., 2018). Although the HPT axis is highly conserved, there are some differences between fish and mammals (Blanton and Specker, 2007; Deal and Volkoff, 2020). For example, in fish, corticotropin releasing hormone (CRH) often plays a more important role in regulating thyrotropin (TSH) secretion by the pituitary and thus thyroid hormone synthesis compared to TSH-releasing hormone (TRH). Also, in most fish species thyroid follicles are more diffusely located in the pharyngeal region rather than encapsulated in a gland.

How it is Measured or Detected

Decreased TH synthesis is often implied by measurement of TPO and NIS inhibition measured clinically and in laboratory models as these enzymes are essential for TH synthesis. Rarely is decreased TH synthesis measured directly, but rather the impact of chemicals on the quantity of T4 produced in the thyroid gland, or the amount of T4 present in serum is used as a marker of decreased T4 release from the thyroid gland (e.g., Romaldini et al., 1988). Methods used to assess TH synthesis include, incorporation of radiolabel tracer compounds, radioimmunoassay, ELISA, and analytical detection.

Recently, amphibian thyroid explant cultures have been used to demonstrate direct effects of chemicals on TH synthesis, as this model contains all necessary synthesis enzymes including TPO and NIS (Hornung et al., 2010). For this work THs was measured by HPLC/ICP-mass spectometry. Decreased TH synthesis and release, using T4 release as the endpoint, has been shown for thiouracil antihyperthyroidism drugs including MMI, PTU, and the NIS inhibitor perchlorate (Hornung et al., 2010).

Techniques for in vivo analysis of thyroid hormone system disruption among other drug-related effects in fish were reviewed by Raldua and Piña (2014). TIQDT (Thyroxine-immunofluorescence quantitative disruption test) is a method that provides an immunofluorescent based estimate of thyroxine in the gland of zebrafish (Raldua and Babin, 2009; Thienpont et al., 2011; Jomaa et al., 2014; Rehberger et al., 2018). Thienpont used this method with ~25 xenobiotics (e.g., amitrole, perchlorate, methimazole, PTU, DDT, PCBs). The method detected changes for all chemicals known to directly impact TH synthesis in the thyroid gland (e.g., NIS and TPO inbibitors), but not those that upregulate hepatic catabolism of T4. Rehberger et al. (2018) updated the method to enable simultaneous semi-quantitative visualization of intrafollicular T3 and T4 levels. Most often, whole body thyroid hormone level measurements in fish early life stages are used as indirect evidence of decreased thyroid hormone synthesis (Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020). Analytical determination of thyroid hormone levels by LC-MS is becoming increasingly available (Hornung et al., 2015).

More recently, transgenic zebrafish with fluorescent thyroid follicles are being used to visualize the compensatory proliferation of the thyroid follicles following inhibition of thyroid hormone synthesis (Opitz et al., 2012).

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Event: 281: Thyroxine (T4) in serum, Decreased

Short Name: T4 in serum, Decreased

Key Event Component

Process	Object	Action
abnormal circulating thyroxine level	thyroxine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:42 - Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	KeyEvent
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	KeyEvent
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in	

AOP ID and Name	Event Type
Mammals	KeyEvent
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:194 - Hepatic nuclear receptor activation leading to altered amphibian metamorphosis	KeyEvent
Aop:366 - Competitive binding to thyroid hormone carrier protein transthyretin (TTR) leading to altered amphibian metamorphosis	KeyEvent
Aop:367 - Competitive binding to thyroid hormone carrier protein thyroid binding globulin (TBG) leading to altered amphibian metamorphosis	KeyEvent
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	KeyEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	KeyEvent
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	KeyEvent
Aop:119 - Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent
Aop:110 - Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	KeyEvent
Aop:162 - Enhanced hepatic clearance of thyroid hormones leading to thyroid follicular cell adenomas and carcinomas in the rat and mouse	KeyEvent
Aop:128 - Kidney dysfunction by decreased thyroid hormone	KeyEvent
Aop:188 - Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:192 - Pendrin inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:193 - Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole
Perchlorate

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
serum

Evidence for Perturbation by Stressor

Propylthiouracil

6-n-propylthouracil is a classic positive control for inhibition of TPO

Methimazole

Methimazole is a classic positive control for inhibition of TPO.

Perchlorate

Perchlorate ion (ClO_4^-) is a classic positive control for inhibition of NIS

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
chicken	Gallus gallus	Moderate	NCBI
Xenopus laevis	Xenopus laevis	Moderate	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	High	NCBI
Sus scrofa	Sus scrofa	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Female	High
Male	High

Taxonomic: This KE is plausibly applicable across vertebrates and the overall evidence supporting taxonomic applicability is strong. THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in zebrafish development, embryo-to-larval transition and larval-to-juvenile transition (Thienpont et al., 2011; Liu and Chan, 2002), and amphibian and lamprey metamorphoses is well established (Manzon and Youson, 1997; Yaoita and Brown, 1990; Furlow and Neff, 2006). Their existence and importance has also been described in many different animal and plant kingdoms (Eales, 1997; Heyland and Moroz, 2005), while their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of TH in the different species depends on the expression and function of specific proteins (e.g receptors or enzymes) under TH control and may vary across species and tissues. As such extrapolation regarding TH action across species and developmental stages should be done with caution.

With few exceptions, vertebrate species have circulating T4 (and T3) that are bound to transport proteins in blood. Clear species differences exist in serum transport proteins (Dohler et al., 1979; Yamauchi and Isihara, 2009). There are three major transport proteins in mammals; thyroid binding globulin (TBG), transthyretin (TTR), and albumin. In adult humans, the percent bound to these proteins is about 75, 15 and 10 percent, respectively (Schussler 2000). In contrast, in adult rats the majority of THs are bound to TTR. Thyroid binding proteins are developmentally regulated in rats. TBG is expressed in rats until approximately postnatal day (PND) 60, with peak expression occurring during weaning (Savu et al., 1989). However, low levels of TBG persist into adult ages in rats and can be experimentally induced by hypothyroidism, malnutrition, or caloric restriction (Rouaze-Romet et al., 1992). While these species differences impact TH half-life (Capen, 1997) and possibly regulatory feedback mechanisms, there is little information on quantitative dose-response relationships of binding proteins and serum hormones during development across different species. Serum THs are still regarded as the most robust measurable key event causally linked to downstream adverse outcomes.

Life stage: The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, T4 levels are not expected to decrease in response to exposure to inhibitors of TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH disruptors.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to

males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Description

All iodothyronines are derived from the modification of tyrosine molecules (Taurog, 2000). There are two biologically active thyroid hormones (THs) in serum, triiodothyronine (T3) and T4, and a few less active iodothyronines (rT3, 3,5-T2). T4 is the predominant TH in circulation, comprising approximately 80% of the TH excreted from the thyroid gland in mammals and is the pool from which the majority of T3 in serum is generated (Zoeller et al., 2007). As such, serum T4 changes usually precede changes in other serum THs. Decreased thyroxine (T4) in serum results from one or more MIEs upstream and is considered a key biomarker of altered TH homeostasis (DeVito et al., 1999).

Serum T4 is used as a biomarker of TH status because the circulatory system serves as the major transport and delivery system for TH delivery to tissues. The majority of THs in the blood are bound to transport proteins (Bartalena and Robbins, 1993). In serum, it is the unbound, or 'free' form of the hormone that is thought to be available for transport into tissues. Free hormones are approximately 0.03 and 0.3 percent for T4 and T3, respectively. There are major species differences in the predominant binding proteins and their affinities for THs (see below). However, there is broad agreement that changes in serum concentrations of THs is diagnostic of thyroid disease or chemical-induced disruption of thyroid homeostasis across vertebrates (DeVito et al., 1999; Miller et al., 2009; Zoeller et al., 2007; Carr and Patiño, 2011).

Normal serum T4 reference ranges can be species and lifestage specific. In **rodents**, serum THs are low in the fetal circulation, increasing as the fetal thyroid gland becomes functional on gestational day 17, just a few days prior to birth. After birth serum hormones increase steadily, peaking at two weeks, and falling slightly to adult levels by postnatal day 21 (Walker et al., 1980; Harris et al., 1978; Goldey et al., 1995; Lau et al., 2003). Similarly, in **humans**, adult reference ranges for THs do not reflect the normal ranges for children at different developmental stages, with TH concentrations highest in infants, still increased in childhood, prior to a decline to adult levels coincident with pubertal development (Corcoran et al. 1977; Kapelari et al., 2008).

In some **frog** species, there is an analogous peak in thyroid hormones in tadpoles that starts around embryonic NF stage 56, peaks at Stage 62 and the declines to lower levels by Stage 56 (Sternberg et al., 2011; Leloup and Buscaglia, 1977).

Additionally, ample evidence is available from studies investigating responses to inhibitors of thyroid hormone synthesis in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

How it is Measured or Detected

Serum T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone concentrations are clinically considered more direct indicators of T4 and T3 activities in the body, but in animal studies, total T3 and T4 are typically measured. Historically, the most widely used method in toxicology is the radioimmunoassay (RIA). The method is routinely used in rodent endocrine and toxicity studies. The ELISA method is commonly used as a human clinical test method. Analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates, through methods employing HPLC, liquid chromatography, immuno luminescence, and mass spectrometry are less common, but are becoming increasingly available (Hornung et al., 2015; DeVito et al., 1999; Baret and Fert, 1989; Spencer, 2013; Samanidou V.F et al., 2000; Rathmann D. et al., 2015). In fish early life stages most evidence for the ontogeny of thyroid hormone synthesis comes from measurements of whole body thyroid hormone levels using LC-MS techniques (Hornung et al., 2015) are increasingly used to accurately quantify whole body thyroid hormone levels as a proxy for serum thyroid hormone levels (Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020). It is important to note that thyroid hormones concentrations can be influenced by a number of intrinsic and extrinsic factors (e.g., circadian rhythms, stress, food intake, housing, noise) (see for example, Döhler et al., 1979).

Any of these measurements should be evaluated for the relationship to the actual endpoint of interest, repeatability, reproducibility, and lower limits of quantification using a fit-for-purpose approach (i.e., different regulatory needs will require different levels of confidence in the AOP). This is of particular significance when assessing the very low levels of TH present in fetal serum. Detection limits of the assay must be compatible with the levels in the biological sample. All three of the methods summarized above would be fit-for-purpose, depending on the number of samples to be evaluated and the associated costs of each method. Both RIA and ELISA measure THs by an indirect methodology, whereas analytical determination is the most direct measurement available. All these methods, particularly RIA, are repeatable and reproducible.

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Event: 1003: Decreased, Triiodothyronine (T3) in serum

Short Name: Decreased, Triiodothyronine (T3) in serum

Key Event Component

Process	Object	Action
abnormal circulating hormone level	3,3',5'-triiodothyronine	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	KeyEvent
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	KeyEvent
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:189 - Type I iodothyronine deiodinase (DIO1) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	KeyEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	KeyEvent
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

serum

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	High	NCBI
African clawed frog	Xenopus laevis	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: The overall evidence supporting taxonomic applicability is strong. With few exceptions vertebrate species have circulating T3 and T4 that are bound to transport proteins in blood. Therefore, the current key event is plausibly applicable to vertebrates in general. Clear species differences exist in transport proteins (Yamauchi and Isihara, 2009). Specifically, the majority of supporting data for TH decreases in serum come from rat studies, and the predominant iodothyronine binding protein in rat serum is transthyretin (TTR). TTR demonstrates a reduced binding affinity for T4 when compared with thyroxine binding globulin (TBG), the predominant serum binding protein for T4 in humans. This difference in serum binding protein affinity for THs is thought to modulate serum half-life for T4; the half-life of T4 in rats is 12-24 hr, wherease the half-life in humans is 5-9 days (Capen, 1997). While these species differences impact hormone half-life, possibly regulatory feedback mechanisms, and quantitative dose-response relationships, measurement of serum THs is still regarded as a measurable key event causatively linked to downstream adverse outcomes.

THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in amphibian and larbean metamorphoses (Manzon and Youson, 1997; Yaoita and Brown, 1990) as well as fish development, embryo-to-larval transition and larval-to-juvenile transition (Thienpont et al., 2011; Liu and Chan, 2002) is well established. Their existence and importance has been also described in many differrent animal and plant kingdoms (Eales, 1997; Heyland and Moroz, 2005), while their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of TH in the different species may differ depending on the expression or function of specific proteins (e.g receptors or enzymes) that are related to TH function, and therefore extrapolation between species should be done with caution.

Life stage: Thyroid hormones are essential in all life stages, but decreases of circulating levels are associated with specific developmental events. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, T4 levels are not expected to decrease in response to exposure to inhibitors of TH synthesis during these earliest stages of development. However, T3 levels are expected to decrease upon exposure to deiodinase inhibitors in any life stage, since maternal T4 needs to be activated to T3 by deiodinases similar to embryonically synthesized T4.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Description

There are two biologically active thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), and a few less active iodothyronines (rT3, 3,5-T2), which are all derived from the modification of tyrosine molecules (Hulbert, 2000). However, the plasma concentrations of the other iodothyronines are significantly lower than those of T3 and T4. The different iodothyronines are formed by the sequential outer or inner ring monodeiodination of T4 by the deiodinating enzymes, Dio1, Dio2, and Dio3 (Gereben et al., 2008). Deiodinase structure is considered to be unique, as THs are the only molecules in the body that incorporate iodide.

The circulatory system serves as the major transport and delivery system for THs from synthesis in the gland to delivery to tissues. The majority of THs in the blood are bound to transport proteins (Bartalena and Robbins, 1993). In **humans**, the major transport proteins are TBG (thyroxine binding globulin), TTR (transthyretin) and albumin. The percent bound to these proteins in adult humans is about 75, 15 and 10 percent, respectively (Schussler 2000). Unbound (free) hormones are approximately 0.03 and 0.3 percent for T4 and T3, respectively. In serum, it is the free form of the hormone that is active.

There are major species differences in the predominant binding proteins and their affinities for THs (see section below on Taxonomic applicability). However, there is broad agreement that changes in serum concentrations of THs is diagnostic of thyroid disease or chemical-induced disruption of thyroid homeostasis (Zoeller et al., 2007).

It is notable that the changes measured in the TH concentration reflect mainly the changes in the serum transport proteins rather than changes in the thyroid status. These thyroid-binding proteins serve as hormonal store which ensure their even and constant distribution in the different tissues, while they protect the most sensitive ones in the case of severe changes in thyroid availability, like in thyroidectomies (Obregon et al., 1981). Until recently, it was believed that all of the effects of TH were mediated by the binding of T3 to the thyroid nuclear receptors (TRa and TRb), a notion which is now questionable due to the increasing evidence that support the non-genomic action of TH (Davis et al., 2010, Moeller et al., 2006). Many non-nuclear TH binding sites have been identified to date and they usually lead to rapid cellular response in TH-effects (Bassett et al., 2003), but the specific pathways that are activated in this regard need to be elucidated.

The production of THs in the thyroid gland and the circulation levels in the bloodstream are self-controlled by an efficiently regulated feedback mechanism across the Hypothalamus-Pituitary-Thyroid (HPT) axis. TH levels are regulated, not only in the plasma level, but also in the individual cell level, to maintain homeostasis. This is succeeded by the efficient regulatory mechanism of the thyroid hormone axis which consists of the following: (1) the hypothalamic secretion of the thyrotropin-releasing hormone (TRH), (2) the thyroid-stimulating hormone (TSH) secretion from the anterior pituitary, (3) hormonal transport by the plasma binding proteins, (4) cellular uptake mechanisms in the cell level, (5) intracellular control of TH concentration by the deiodinating mechanism (6) transcriptional function of the nuclear thyroid hormone receptor and (7) in the fetus, the transplacental passage of T4 and T3 (Cheng et al., 2010).

In regards to the brain, the TH concentration involves also an additional level of regulation, namely the hormonal transport through the Blood Brain Barrier (BBB) (Williams, 2008). The TRH and the TSH are actually regulating the production of pro-hormone T4 and in a lesser extent of T3, which is the biologically active TH. The rest of the required amount of T3 is produced by outer ring deiodination of T4 by the deiodinating enzymes D1 and D2 (Bianco et al., 2006), a process which takes place mainly in liver and kidneys but also in other target organs such as in the brain, the anterior pituitary, brown adipose tissue, thyroid and skeletal muscle (Gereben et al., 2008; Larsen, 2009). Both hormones exert their action in almost all tissues of mammals and they are acting intracellularly, and thus the uptake of T3 and T4 by the target cells is a crucial step of the overall pathway. The trans-membrane transport of TH is performed mainly through transporters that differ depending on the cell type (Hennemann et al., 2001; Friesema et al., 2005; Visser et al., 2008). Many transporter proteins have been identified up to date but the monocarboxylate transporters (Mct8, Mct10) and the anion-transporting polypeptide (OATP1c1) show the highest degree of affinity towards TH (Jansen et al., 2005).

T3 and T4 have significant effects on normal development, neural differentiation, growth rate and metabolism (Yen, 2001; Brent, 2012; Williams, 2008), with the most prominent ones to occur during the fetal development and early childhood. The clinical features of hypothyroidism and hyperthyroidism emphasize the pleiotropic effects of these hormones on many different pathways and target organs. The thyroidal actions though are not only restricted to mammals, as their high significance has been identified also for other vertebrates, with the most well-studied to be the amphibian metamorphosis (Furlow and Neff, 2006). The importance of the thyroid-regulated pathways becomes more apparent in iodine deficient areas of the world, where a higher rate of cretinism and growth retardation has been observed and linked to decreased TH levels (Gilbert et al., 2012). Another very common cause of severe hypothyroidism in human is the congenital hypothyroidism, but the manifestation of these effects is only detectable in the lack of adequate treatment and is mainly related to neurological impairment and growth retardation (Glinoer, 2001), emphasizing the role of TH in neurodevelopment in all above cases. In adults, the thyroid-related effects are mainly linked to metabolic activities, such as deficiencies in oxygen consumption, and in the metabolism of the vitamin, proteins, lipids and carbohydrates, but these defects are subtle and reversible (Oetting and Yen, 2007). Blood tests to detect the amount of thyroid hormone (T4) and thyroid stimulating hormone (TSH) are routinely done for newborn babies for the diagnosis of congenital hypothyroidism at the earliest stage possible.

Although the components of the thyroid hormone system as well as thyroid hormone synthesis and action are highly conserved across vertebrates, there are some taxon-specific considerations.

Although the HPT axis is highly conserved, there are some differences between fish and mammals (Blanton and Specker, 2007;

Deal and Volkoff, 2020). For example, in **fish**, corticotropin releasing hormone (CRH) often plays a more important role in regulating thyrotropin (TSH) secretion by the pituitary and thus thyroid hormone synthesis compared to TSH-releasing hormone (TRH). TTRs from fish have low sequence identity with human TTR, for example seabream TTR has 54% sequence identity with human TTR but the only amino acid difference within the thyroxine-binding site is the conservative substitution of Ser117 in human TTR to Thr117 in seabream TTR (Santos and Power, 1999; Yamauchi et al., 1999; Eneqvist et al., 2004). In vitro binding experiments showed that TH disrupting chemicals bind with equal or weaker affinity to seabream TTR than to the human TTR with polar TH disrupting chemicals, in particular, showing a more than 500-fold lower affinity for seabream TTR compared to human TTR (Zhang et al., 2018).

Zebrafish and fathead minnows are oviparous fish species in which maternal thyroid hormones are transferred to the eggs and regulate early embryonic developmental processes during external (versus intra-uterine in mammals) development (Power et al., 2001; Campinho et al., 2014; Ruuskanen and Hsu, 2018) until embryonic thyroid hormone synthesis is initiated. Maternal transfer of thyroid hormones, both T4 and T3, to the eggs has been demonstrated in zebrafish (Walpita et al., 2007; Chang et al., 2012) and fathead minnows (Crane et al., 2004; Nelson et al., 2016).

Several studies have reported evidence of T3 decreases after exposure to TPO inhibitors and deiodinase inhibitors in early life stages of zebrafish (Stinckens et al., 2016; Stinckens et al., 2020; Wang et al., 2020) and fathead minnow (Nelson et al., 2016; Cavallin et al., 2017).

How it is Measured or Detected

T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone are considered more direct indicators of T4 and T3 activities in the body. The majority of T3 and T4 measurements are made using either RIA or ELISA kits. In animal studies, total T3 and T4 are typically measured as the concentrations of free hormone are very low and difficult to detect. Historically, the most widely used method in toxicology is RIA. The method is routinely used in rodent endocrine and toxicity studies. The ELISA method has become more routine in rodent studies. The ELISA method is a commonly used as a human clinical test method.

Recently, analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates through methods employing HPLC and mass spectrometry have become more common (DeVito et al., 1999; Miller et al., 2009; Hornung et al., 2015; Nelson et al., 2016; Stinckens et al., 2016).

Any of these measurements should be evaluated for fit-for-purpose, relationship to the actual endpoint of interest, repeatability, and reproducibility. All three of the methods summarized above would be fit-for-purpose, depending on the number of samples to be evaluated and the associated costs of each method. Both RIA and ELISA measure THs by a an indirect methodology, whereas analytical determination is the most direct measurement available. All of these methods, particularly RIA, are repeatable and reproducible.

In fish early life stages most evidence for the ontogeny of thyroid hormone synthesis comes from measurements of whole body thyroid hormone levels and using LC-MS techniques (Hornung et al., 2015) are increasingly used to accurately quantify whole body thyroid hormone levels as a proxy for serum thyroid hormone levels (Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020).

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Event: 1877: Altered, retinal layer structure

Short Name: Altered, retinal layer structure

Key Event Component

Process	Object	Action
retina layer formation	retina	morphological change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	KeyEvent

Stressors

Name
Propylthiouracil
Methimazole
Perchlorate

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
eye

Evidence for Perturbation by Stressor

Propylthiouracil

6-n-propylthouracil is a classic positive control for inhibition of TPO.

Methimazole

Methimazole is a classic positive control for inhibition of TPO.

Perchlorate

Perchlorate ion (ClO_4^-) is a classic positive control for inhibition of NIS.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic applicability: In general, the eye structure is very conserved among vertebrates, but some differences exist with regard to shape and expression of the different retinal layers. Fig. 1 (from Richardson 2012) demonstrates the histology of the human vs the zebrafish eye. As in humans, the mature zebrafish retina consists of three nuclear layers separated by two plexiform layers. The photoreceptor rod and cone nuclei are located in the outer nuclear layer; the amacrine, horizontal, and Müller glial cell bodies are found in the inner nuclear layer and the ganglion cell bodies are placed in the ganglion cell layer. The plexiform layers connect these layers. In contrast to zebrafish, the human retina lacks UV-sensitive cones.

Other structural differences between species are mostly related to their lifestyle (e.g. nocturnal vs diurnal) (Bibliowicz 2011) and cannot be generalized for specific vertebrate classes.

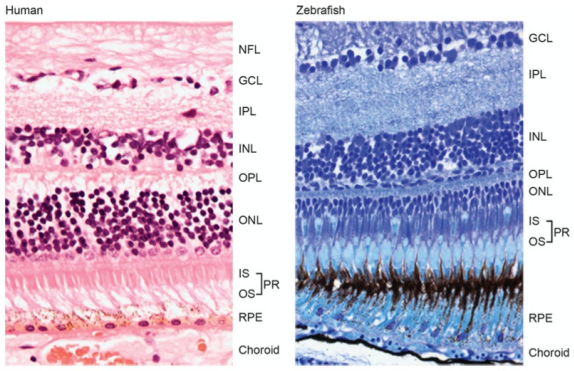

Figure 1 Cross-sectional histology of the human and zebrafish retina demonstrating similarities in the arrangement of cells and structural features that define the distinct retinal layers. RPE, pigmented epithelium; IS, inner segment; OS, outer segment; PR, photoreceptor; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; and NFL, nerve fibre layer.

Life-stage applicability: Eye structure differs between life stages, as the different retinal layers do not develop at the same time and the eye itself grows with the organism. Eye development in zebrafish closely resembles the one in humans and other vertebrates. The eye develops from three different embryological tissues that form the specific structures of the eye, starting with the optic vesicle at 16 hpf, which further develops into the two-layered optic cup composed of the retinal neuroepithelium and pigmented epithelium until 20 hpf. Lens development begins as a lens placode that forms a solid lens mass by 22 hpf. Afterwards, the neuroectodermal layers of the optic vesicle invaginate ventrally by 24 hpf. By 48 hpf, zebrafish eye morphogenesis is almost complete with only retinal neurogenesis continuing. Retinal pigment epithelium flattening and final differentiation occurs around 27 hpf (Moreno-Marmol and others 2018). By 60 hpf, the different layers of the retina can be distinguished (Morris and Fadool 2005; Schmitt and Dowling 1999). Thereafter, further differentiation and maturation of the layers and cell types continues (Raymond and others 1995). For example, rods continue to mature until around 20 dpf (Morris and Fadool 2005). Impacts on retinal layer structure have been reported at 48, 66, 72, 96 and 120 hpf during zebrafish embryo-eleutheroembryo development (Baumann and others 2016; Komoike and others 2013; Reider and Connaughton 2014). Since the term 'eleutheroembryo' (stage starting at hatching and ending with free-feeding) is not available, the terms 'embryo' and 'larvae' were selected to reflect this.

Sex applicability: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Effects on retinal layers during early development are therefore expected to be independent of sex.

At later life stages, however, sex dependency cannot be excluded. Sexual dimorphism of eye sclera surface exposure has been recently discovered (Danel et al. 2018; Danel et al., 2020). Danel et al. (2020) also found that women have rounder eye fissures and brighter irises compared to men. Maekawa et al. (2010) observed eye abnormalities such as microphthalmia and cataract in female mice but not in male mice when the fatty acid composition of the diet was changed during gestation. The authors hypothesized that this was due to differences in lipid metabolism. This suggests that effects of other factors on eye structure could also be sex dependent in vertebrates.

Evidence for perturbation by stressor: Multiple studies demonstrate that eye development and its resulting structure can be disrupted by different stressors (reviewed for example by Chen 2020).

Key Event Description

The anatomy and histology of the eye are highly conserved among vertebrates. The cornea and lens refract and focus light onto the posterior chamber of the eye, the vitreous cavity, which is covered by the retina. The retina consists of three specialised layers

of cells, the outermost of which is formed by photoreceptors that absorb light and transmit the subsequent neural signal to the innermost layers, which consist of neurons specialised in processing and transmitting this neural signal (Wässle and Riemann, 1978; Cameron and Carney, 2000 ; Rockhill et al, 2000; Fadool, 2003). The neurons of the innermost layer converge to form the optic nerve, which transmits visual information to the brain (Gestri et al., 2012). The retina has different types of photoreceptors, the cones, which are responsible for colour vision, and the rods, which enable vision in the dark or in very low light conditions. In adults, cones are distributed in the retina in a precise and very regular arrangement, forming a photoreceptor mosaic. The precise spatiotemporal pattern of maturation of cones may affect the organization of this mosaic, and THs appear to play a role in the coordination of this maturation process (Suzuki et al., 2013). In the fish retina, this arrangement is most evident in the outer nuclear layer where the position of each cone subtype is precisely arranged relative to the others (Fadool, 2003; Robinson et al., 1993) resulting in a highly ordered crystalline-like mosaic.

The retinal pigment epithelium (RPE) is important to maintain a healthy and functional retina (Strauss 2005). The strong connection between the RPE cells with the tight junction, creates a blood-retinal barrier to mediate the directional transport of ions, water and nutrients while removing waste products. Another key function of the RPE is to absorb excess light energy to protect the neural retina from phototoxicity (Plafker 2012). Phagocytosis of spilled photoreceptor outer segments (Lister 2002) is another function of the RPE to maintain balanced photoreceptor growth, which is important for function.

Studies that detect and measure altered retinal layer structure after exposure to thyroid hormones or endocrine disruptors show, for example, altered cone cell number (Allison et al., 2006; Houbrechts et al., 2016; Vancamp et al., 2019), altered retinal cell number (Dong et al., 2014), or a general alteration of retinal morphology (Gamborino et al., 2001; Houbrechts et al., 2016; Komoike et al., 2013; Li et al., 2012; Reider & Connaughton, 2014), alteration of the pigment epithelium (Baumann et al., 2016), abnormal cone differentiation (Duval & Allison, 2018; Suzuki et al., 2013; Viets et al., 2016) or prevention of the opsin switch (Gan & Flamarique, 2010; Raine & Hawryshyn, 2009).

How it is Measured or Detected

For assessment of eye structure and layers, mostly simple morphometric analyses based on histological sections are sufficient. This can either be electron microscopy for subcellular changes, or normal light microscopy for cellular changes. Specific antibody staining might help to identify the different retinal layers, but usually, they are easily distinguishable by normal histological staining (e.g. HE staining).

Measurement of cell layer diameter is the most popular and simple method to assess changes in eye structure and layers. Moreover, measurement of the pigmentation grade of the retinal pigment epithelium can be used to assess structural changes.

(reviewed in Chen 2020)

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Event: 1643: Altered, Visual function

Short Name: Altered, Visual function

Key Event Component

Process	Object	Action
vision trait	eye	functional change

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline	KeyEvent
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	KeyEvent
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	AdverseOutcome
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	AdverseOutcome
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	KeyEvent

Stressors

Name
Propylthiouracil

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
eye

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Life Stage	Evidence
Juvenile	Moderate
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic applicability: Visual function decrease can be evaluated in wide range of species including mammals, amphibians, fish and humans. Evaluation of these visual function modification change according to the species and its environment.

Life-stage applicability: Vision plays a crucial role in the early life stages of most species, as timing of eye development and establishment of functional vision is essential for perception of food or avoidance of predators for example (Carvalho et al., 2002).The first visual responses based on retinal functionality appear around 70 hpf in zebrafish (Schmitt and Dowling 1999). It is plausible to assume that alterations of the eye structure would result in altered visual function across all life stages, but such alterations are most likely to occur during the development of the normal eye structure, which occurs in the embryo-eleutheroembryo phase. Some studies have also shown a decrease in vision related to age (Brastrom et al., 2019; Martínez-Roda et al., 2016; Segura et al., 2018) including on visual acuity, visual fields, colour vision and dark adaptation, are well documented (Hennelly et al, 1998).

Sex applicability: Sex does not seem relevant for most of the visual function decreases observed in different studies. Differences according to the sex of the individuals have however been reported concerning the basic visual capacities (e.g. color perception, contrast sensitivity, visual acuity, motion perception,...) but also concerning the frequency of certain diseases influencing these diminished visual functions, notably in humans (Vanston and Strother, 2017).

Key Event Description

The decrease in visual function can have different aspects, such as loss of chromatic vision, changes in eye movements, differences in sensitivity to light, but also changes in the retinal pigment epithelium (RPE) that may be related to a decrease in visual function (Strauss, 2005). The visual system is highly variable from one species to another, and this variability is a key factor influencing animal behaviour (Corral-López et al., 2017).

Decreases in these visual functions can have a strong impact on behaviour, leading to changes in individual response and abilities in the environment, including, for example, perception of food or avoidance of predators. Variation in the visual system can also influence learning tasks when visual stimuli are used (Corral-López et al., 2017).

Studies have detected visual impairments in fish at different temperatures (Babkiewicz et al., 2020) after treatment with the endocrine disruptor propylthiouracil (Baumann et al 2016), after chronic dietary selenomethionine exposure (Raine et al 2016), exposure to PCBs (Zhang et al, 2015) or deiodinase knockdown (Houbrechts et al 2016, Vancamp et al 2018).

How it is Measured or Detected

Measurements of visual function can be performed at the level of neuronal activity:

- Electroretinography (Chrispell et al., 2015)
- Analysis of neural activity in the optic tectum can be quantified as the ratio of phosphorylated ERK () to total ERK in the optic tectum using immunofluorescent antibodies (Randlett et al., 2015, Dehnert et al., 2019).
- Babkiewicz et al. (2020) used an advanced technique to display an artificial prey on a miniature OLED screen and use functional calcium imaging with light sheet microscopy to visualize a neural response in the optic tectum.

Other measurements are performed at the level of the eyes:

- Opto Kinetic response, OKR (similar protocol for Rat/mice (Segura et al., 2018), fish (Zou et al., 2010) and humans (Kang and Wildsoet, 2016)). The OKR is a visually-mediated assay in which an individual will respond to alternating black and white stripes by exhibiting eye saccades, eye movements without coordinated body movements, in the same direction as rotating stripes. An eye saccade relies on the ability to rapidly move the eye from focusing on one external target to the next in a repeated manner (Magnuson et al., 2020). Optokinetic tracking has a robust performance and does not require training the animal, allowing for the quick assessment (and at earlier ages) of visual features such as visual acuity (VA) and contrast sensitivity (CS)11–14. However, the main disadvantage of optokinetic tracking is that it is a subjective method in which the decision about whether the animal is performing the optokinetic tracking or not is made by an experimenter (Segura et al., 2018).

Yet other studies use assessment of vision-related behaviours:

- Opto Motor Reponses, OMR. OMR tracks the ability of fish to swim in the direction of a perceived motion when presented with a whole-field stimulus (Neuhauss, 2003), (Gould et al., 2017)).
- Light-dark transition or vision startle response: reaction to change in light intensity (light sensitivity) (Brastrom et al., 2019)
- Black-white preference test (Baumann et al., 2016)
- Diverse Mobility assay including Tracking, touch-evoked escape-response assays, Swirl assays, locomotion assay, swimming activity, phototactic swimming activity assay, induced locomotor response (LLR) (Baumann et al., 2016; Gao et al., 2015; Zhao et al., 2014, Dehnert et al., 2019).

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List of Adverse Outcomes in this AOP

Event: 351: Increased Mortality

Short Name: Increased Mortality

Key Event Component

Process	Object	Action

mortality increased

Process	Object	Action

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	AdverseOutcome
Aop:96 - Axonal sodium channel modulation leading to acute mortality	AdverseOutcome
Aop:104 - Altered ion channel activity leading impaired heart function	AdverseOutcome
Aop:113 - Glutamate-gated chloride channel activation leading to acute mortality	AdverseOutcome
Aop:160 - Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality	AdverseOutcome
Aop:161 - Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality	AdverseOutcome
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:186 - unknown MIE leading to renal failure and mortality	AdverseOutcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	AdverseOutcome
Aop:320 - Binding of viral S-glycoprotein to ACE2 receptor leading to acute respiratory distress associated mortality	AdverseOutcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	AdverseOutcome
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	AdverseOutcome
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	AdverseOutcome
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	AdverseOutcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	AdverseOutcome
Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	AdverseOutcome
Aop:410 - GSK3beta inactivation leading to increased mortality via defects in developing inner ear	AdverseOutcome

Biological Context

Level of Biological Organization

Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

All living things are susceptible to mortality.

Key Event Description

Increased mortality refers to an increase in the number of individuals dying in an experimental replicate group or in a population over a specific period of time.

How it is Measured or Detected

Mortality of animals is generally observed as cessation of the heart beat, breathing (gill or lung movement) and locomotory movements. Mortality is typically measured by observation. Depending on the size of the organism, instruments such as microscopes may be used. The reported metric is mostly the mortality rate: the number of deaths in a given area or period, or from a particular cause.

Depending on the species and the study setup, mortality can be measured:

- in the lab by recording mortality during exposure experiments
- in dedicated setups simulating a realistic situation such as mesocosms or drainable ponds for aquatic species
- in the field, for example by determining age structure after one capture, or by capture-mark-recapture efforts. The latter is a method commonly used in ecology to estimate an animal population's size where it is impractical to count every individual.

Regulatory Significance of the AO

Increased mortality is one of the most common regulatory assessment endpoints, along with reduced growth and reduced reproduction.

Event: 360: Decrease, Population trajectory

Short Name: Decrease, Population trajectory

Key Event Component

Process	Object	Action
population growth rate	population of organisms	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)	AdverseOutcome
Aop:25 - Aromatase inhibition leading to reproductive dysfunction	AdverseOutcome
Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction	AdverseOutcome
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	AdverseOutcome
Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior	AdverseOutcome
Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	AdverseOutcome
Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	AdverseOutcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome

AOP ID and Name	Event Type
Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release	AdverseOutcome
Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I /metaphase I transition	AdverseOutcome
Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction	AdverseOutcome
Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint	AdverseOutcome
Aop:292 - Inhibition of tyrosinase leads to decreased population in fish	AdverseOutcome
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	AdverseOutcome
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	AdverseOutcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	AdverseOutcome
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	AdverseOutcome
Aop:336 - DNA methyltransferase inhibition leading to population decline (1)	AdverseOutcome
Aop:337 - DNA methyltransferase inhibition leading to population decline (2)	AdverseOutcome
Aop:338 - DNA methyltransferase inhibition leading to population decline (3)	AdverseOutcome
Aop:339 - DNA methyltransferase inhibition leading to population decline (4)	AdverseOutcome
Aop:340 - DNA methyltransferase inhibition leading to transgenerational effects (1)	AdverseOutcome
Aop:341 - DNA methyltransferase inhibition leading to transgenerational effects (2)	AdverseOutcome
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	AdverseOutcome
Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline	AdverseOutcome
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	AdverseOutcome
Aop:299 - Excessive reactive oxygen species production leading to population decline via reduced fatty acid beta-oxidation	AdverseOutcome
Aop:311 - Excessive reactive oxygen species production leading to population decline via mitochondrial dysfunction	AdverseOutcome
Aop:216 - Excessive reactive oxygen species production leading to population decline via follicular atresia	AdverseOutcome
Aop:238 - Excessive reactive oxygen species production leading to population decline via lipid peroxidation	AdverseOutcome
Aop:326 - Thermal stress leading to population decline (3)	AdverseOutcome
Aop:325 - Thermal stress leading to population decline (2)	AdverseOutcome
Aop:324 - Thermal stress leading to population decline (1)	AdverseOutcome
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	AdverseOutcome
Aop:349 - Inhibition of 11β-hydroxylase leading to decresed population trajectory	AdverseOutcome
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory	AdverseOutcome
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	AdverseOutcome
Aop:386 - Increased reactive oxygen species production leading to population decline via inhibition of photosynthesis	AdverseOutcome
Aop:387 - Increased reactive oxygen species production leading to population decline via mitochondrial dysfunction	AdverseOutcome
Aop:388 - DNA damage leading to population decline via programmed cell death	AdverseOutcome
Aop:389 - Oxygen-evolving complex damage leading to population decline via inhibition of photosynthesis	AdverseOutcome
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	AdverseOutcome
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	AdverseOutcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	AdverseOutcome
Aop:410 - GSK3beta inactivation leading to increased mortality via defects in developing inner ear	AdverseOutcome

Biological Context

Level of Biological Organization

Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Consideration of population size and changes in population size over time is potentially relevant to all living organisms.

Key Event Description

Population ecology is the study of the sizes (and to some extent also the distribution) of plant and animal populations and of the processes, mainly biological in nature, that determine these sizes. As such, it provides an integrated measure of events occurring at lower levels of biological organization (biochemical, organismal, etc.). The population size in turn determines community and ecosystem structure. For fish, maintenance of sustainable fish and wildlife populations (i.e., adequate to ensure long-term delivery of valued ecosystem services) is an accepted regulatory goal upon which risk assessments and risk management decisions are based.

How it is Measured or Detected

Population trajectories, either hypothetical or site specific, can be estimated via population modeling based on measurements of vital rates or reasonable surrogates measured in laboratory studies. As an example, Miller and Ankley 2004 used measures of cumulative fecundity from laboratory studies with repeat spawning fish species to predict population-level consequences of continuous exposure.

Regulatory Significance of the AO

Maintenance of sustainable fish and wildlife populations (i.e., adequate to ensure long-term delivery of valued ecosystem services) is a widely accepted regulatory goal upon which risk assessments and risk management decisions are based.

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- Miller DH, Ankley GT. 2004. Modeling impacts on populations: fathead minnow (Pimephales promelas) exposure to the endocrine disruptor 17ß-trenbolone as a case study. Ecotoxicology and Environmental Safety 59: 1-9.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 309: Thyroperoxidase, Inhibition leads to TH synthesis, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Low

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	High	Low
Inhibition of thyroid peroxidase leading to impaired fertility in fish	adjacent	High	High
Thyroperoxidase inhibition leading to altered amphibian metamorphosis	adjacent	High	Moderate
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	High	Moderate
Thyroperoxidase inhibition leading to altered visual function via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	adjacent		
Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxonomic: This KER is plausibly applicable across vertebrates. Inhibition of TPO activity is widely accepted to directly impact TH synthesis. This is true for both rats and humans, as well as some fishes, frogs and birds. Most of the data supporting a causative relationship between TPO inhibition and altered TH synthesis is derived from animal studies, in vitro thyroid microsomes from rats or pigs, and a limited number of human ex vivo (Nagasaka and Hidaka, 1976; Vickers et al., 2012) and clinical studies. There are data to support that gene mutations in TPO result in congenital hypothyroidism, underscoring the essential role of TPO in human thyroid hormone synthesis.

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH disruptors.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Relationship Description

Thyroperoxidase (TPO) is a heme-containing apical membrane protein within the follicular lumen of thyrocytes that acts as the enzymatic catalyst for thyroid hormone (TH) synthesis (Taurog, 2005) across vertebrates. Two commonly used reference chemicals, propylthiouracil (PTU) and methimazole (MMI), are drugs that inhibit the ability of TPO to: a) activate iodine and transfer it to thyroglobulin (Tg) (Davidson et al., 1978); and, b) couple thyroglobulin (Tg)-bound iodotyrosyls to produce Tg-bound thyroxine (T4) and triiodothyronine (T3) (Taurog, 2005).

Evidence Supporting this KER

The weight of evidence supporting a direct linkage between the MIE, TPO inhibition, and the KE of decreased TH synthesis, is strong and supported by more than three decades of research in animals, including humans (Cooper et al., 1982; Cooper et al.,1983; Divi and Doerge, 1994).

Biological Plausibility

The biological plausibility for this KER is rated Strong. TPO is the only enzyme capable of de novo systhesis of TH. TPO catalyzes several reactions, including the oxidation of iodide, nonspecific iodination of tyrosyl residues of thyroglobulin (Tg) to form monoiodotyrosyl (MIT) or diiodotyrosyl (DIT) residues, and the coupling of these Tg-bound iodotyrosyls to produce Tg-bound T3 and T4 (Divi and Doerge, 1994; Kessler et al., 2008; Ruf et al., 2006; Taurog et al., 1996, 2005). Therefore, inhibition of TPO activity is widely accepted to directly impact TH synthesis.

Empirical Evidence

Empirical support for this KER is strong. There are several papers that have measured alterations in TPO and subsequent effects on TH synthesis across vertebrates. Taurog et al. (1996) showed decreased guicaol activity, decreased bound I^{125}, and subsequent decreases in newly formed T3 and T4 per molecule of Tg, following exposure to PTU, MMI and some antibiotics. There is important evidence in **mammals**. Following in vivo exposure to PTU in rats (Cooper et al., 1982; 1983), there are concentration and time-dependent decreases in thyroid protein bound iodine and serum T4 and T3 that recovered one month after cessation of PTU exposure. In addition, measures of thyroidal iodine content were highly correlated with intra-thyroidal PTU concentration. Vickers et al. (2012) demonstrated dose- and time- dependent inhibition of TPO activity in both human and rat thyroid homogenates exposed to MMI. Hassan et al. (2017, 2020) and Handa et al. (2021) predicted the level of thyroid hormones in serum after treatment with PTU and MMI in rats. They developed a quantitative model by comparing dose- response data.

Tietge et al (2010) showed decreases in thyroidal T4 following MMI exposure in **Xenopus**. Also in Xenopus, Haselman et al (2020) showed decreases in thyroidal iodotyrosines (MIT/DIT) and iodothyronines (T4/T3) following exposure to MMI. Doerge et al (1998) showed that a tryphenylmethane dye, malachite green, inhibited TPO and lowered thyroxine production. A recent paper used a series of benzothiazoles and showed TPO inhibition (guicaol assay) and inhibition of TSH stimulated thyroxine release from Xenopus thyroid gland explant cultures (Hornung et al., 2015).

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. Several other studies have also shown that chemically induced Inhibition of TPO results in reduced TH synthesis in zebrafish (Van der Ven et al., 2006; Raldua and Babin, 2009; Liu et al., 2011; Thienpont et al., 2011; Rehberger et al., 2018). A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Temporal Evidence: In **mammals**, the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). The impact of decreased TPO activity on thyroidal hormone synthesis is similar across all ages in mammals. Good evidence for the temporal relationship of the KER comes from thyroid system modeling (e.g., Degon et al., 2008; Fisher et al., 2013) using data from studies of iodine deficiency and chemicals that inhibit NIS. In addition, there is ample evidence of the temporal impacts of TPO inhibition on TH synthesis, using ex vivo and in vitro measures that demonstrate the time course of inhibition following chemical exposures, including some data from human thyroid microsomes and ex vivo thyroid slices (Vickers et al., 2012). Future work is needed that measures both TPO inhibition and TH production during development.

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal thyroid hormones transferred to the eggs. Embryonic thyroid hormone synthesis is activated later during embryo-larval development. (See Domain of applicability)

Dose-Response Evidence: Dose-response data is available from a number of studies in **mammals** that correlate TPO inhibition with decreased TH production measured using a variety of endpoints including iodine organification (e.g., Taurog et al., 1996), inhibition of guicaol oxidation in thyroid microsomes (e.g., Doerge and Chang, 2002), and direct measure of thyroid gland T4 concentrations (e.g., Hornung et al., 2015). However, there is a lack of dose-response data from developmental studies showing direct linkages

from TPO inhibition to thyroidal TH synthesis.

Uncertainties and Inconsistencies

While it is clear that TPO inhibition will lead to altered hormone synthesis, there is a need for data that will inform quantitative modeling of the relationship between TPO inhibition and the magnitude of effects on thyroid hormone synthesis.

Data from studies on genistein highlight this uncertainty. Doerge and colleagues have demonstrated that for this compound up to 80% TPO inhibition did not result in decreased serum T4 in rats (Doerge and Chang, 2002). This is not consistent with other prototypical TPO inhibitors (e.g., PTU, MMI). Genistein is however a well-known phytoestrogen and the observed inconsistency may be the result of feedback mechanisms resulting from its estrogenic effect.

Quantitative Understanding of the Linkage

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. This study established that, in *Xenopus*, measurable decreases in the products of TPO activity can occur as early as 2 days of exposure during pro-metamorphosis. However, despite consistent profiles of some iodo-species across chemicals, other iodo-species showed inconsistent profiles across chemicals. This highlights the multiple mechanisms of TPO (iodination and coupling) and differential susceptiblity to inhibition of those mechanisms depending on the chemical's type of interaction with TPO. The most consistent concentration-response relationship across chemicals and over time was demonstrated by thyroidal T4, which is the most relevant product to subsequent key events. At the highest concentrations tested for each chemcial, thyroidal T4 was below detection by 7 days of exposure across all three TPO inhibitors. Keeping in mind that the thyroid gland has follicular lumen space where thyroglobulin/T4 is stored until proteolysis and release to the blood, full inhibition of TPO would result in a delayed measureable response due to the time it takes to deplete stored hormone. Regardless of the delay, the results from this study imply full inhibition of TPO by each of these three chemicals at the highest test concentrations, but would require chemical residue analysis and/or toxicokinetic modeling to relate cellular/tissue concentrations at the site of TPO catalysis to levels of inhibition via Michaelis-Menten kinetic descriptions.

Profiles of thyroidal iodinated species demonstrated by Haselman et al. (2020) across three different TPO inhibitors suggests that a high level of TPO inhibition must occur in order to elicit responses in subsequent key events. Although the level of TPO inhibition is not directly quantifiable from this study, these data suggest that at least 90-100% inhibition was occurring since circulating T4 was not detectable at 10 days of exposure to the highest concentrations of MMI and MBT. However, additional efforts would be necessary to determine the minimum level of TPO inhibition that leads to a measurable decrease in thyroidal T4 and subsequently circulating T4. Furthermore, Hassan et al. (2017, 2020) and Handa et al. (2021) predicted the level of thyroid hormones in serum after treatment with PTU and MMI in rats. They developed a quantitative model by comparing dose- response data.

Response-response relationship

There are only a limited number of studies where both TPO inhibition and iodine organification have been measured in vivo, and there are not enough data available to make any definitive quantitative correlations. One in vivo study in rats exposed to the TPO inhibitor genistein found no in vivo impact on serum thyroid hormone concentrations, even when TPO was inhibited up to 80% (Chang and Doerge, 2000). Genistein is however a well-known phytoestrogen and the observed inconsistency may be the result of feedback mechanisms resulting from its estrogenic effect.

Given that this is an MIE to KE relationship, there is only one response to evaluate in the relationship. Decreased TH synthesis, as measured by responses of iodinated species in the thyroid gland, is the result of TPO inhibition, which cannot be measured directly in vivo.

Time-scale

In vivo, evaluations of TPO inhibition are limited to evaluation of the iodinated species, or products of TPO activity, present in the thyroid gland at a particular time. However, as stated previously, any measurable response in these iodinated species is not a discreet assessment of TPO activity given that the gland maintains storage of hormone in the follicular lumen space and any alteration of TPO activity would be detected once the stores begin to be depleted. In *Xenopus laevis*, Haselman et al. (2020) showed a decrease in thyroidal iodinated species after only 2 days of exposure to potent TPO inhibitor MMI during thyroid-mediated metamorphosis and within 4 days for PTU and MBT, both model TPO inhibitors. In zebrafish, Walter et al. (2019) reported a similar time frame, namely a decrease in T4 levels at 72 hpf after starting the exposure to PTU at 0-2 hpf. It should be noted that the time-scale is probably depending on the developmental stage and whether the embryo is capable of thyroid hormone synthesis, rather than on the exposure duration.

Known modulating factors

Iodine availability will impact the ability of TPO to iodinate tyrosine residues on thyroglobulin. Iodine availability to TPO can be impacted a number of ways. First, environmental availability of iodine can vary greatly depending on whether and how much iodine exists in surface waters for aquatic organisms (gill respirators) and in the diets of both terrestrial and aquatic organisms. Second, somewhat regardless of iodine availability through environmental uptake (i.e., barring extremely high iodine exposure), iodine is actively transported into the thyroid follicular cell from the blood via sodium-iodide symporter (NIS), which has been shown to be susceptible to inhibition by, for example, perchlorate. As such, iodine availability to TPO is mediated by functional NIS. Finally, iodine is not fully available to TPO on the apical surface of the thyroid follicular cell until it is transported through the apical

membrane by pendrin, an anion exchange protein - mutations or inhibition of pendrin could affect iodine availability to TPO.

Hydrogen peroxide is also needed by TPO to mediate the oxidation of iodide, which is produced locally by dual oxidase (DUOX). A mutation or inhibition of DUOX will impact local production of H_2O_2 leading to lower oxidizing potential of TPO and less organification of iodide.

Known Feedforward/Feedback loops influencing this KER

Thyroid stimulating hormone (TSH) released from the pituitary positively regulates the synthesis and release of thyroid hormones from the thyroid gland. As such, when TPO is inhibited and thyroid hormone synthesis is decreased, lower systemic levels of hormone cause feedback from the pituitary via TSH to upregulate a number of processes in the thyroid gland as a means of compensation, including (but not limited to) enhanced gene expression of NIS and thyrocyte cell proliferation (Tietge et al., 2010; Haselman et al., 2020).

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Relationship: 305: TH synthesis, Decreased leads to T4 in serum, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Moderate
XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	Moderate
Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	adjacent	High	High
Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	adjacent	High	Moderate
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low
Thyroperoxidase inhibition leading to altered amphibian metamorphosis	adjacent	High	Moderate
Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	adjacent	High	High
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to altered visual function via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	adjacent		
Inhibition of thyroid peroxidase leading to follicular cell adenomas and carcinomas (in rat and mouse)	adjacent		
Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	adjacent		
Kidney dysfunction by decreased thyroid hormone	adjacent	High	
Iodotyrosine deiodinase (IYD) inhibition leading to altered amphibian metamorphosis	adjacent	High	High
Pendrin inhibition leading to altered amphibian metamorphosis	adjacent	High	High
Dual oxidase (DUOX) inhibition leading to altered amphibian metamorphosis	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
zebrafish	Danio rerio	Low	NCBI
fathead minnow	Pimephales promelas	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxonomic: This KER is plausibly applicable across vertebrates. While a majority of the empirical evidence comes from work with laboratory rodents, there is a large amount of supporting data from humans (with anti-hyperthyroidism drugs including propylthiouracil and methimazole), some amphibian species (e.g., frog), fish species (e.g., zebrafish and fathead minnow), and some avian species (e.g, chicken). The following are samples from a large literature that supports this concept: Cooper et al. (1982; 1983); Hornung et al. (2010); Van Herck et al. (2013); Paul et al. (2013); Nelson et al. (2016); Alexander et al. (2017);

Stinckens et al. (2020).

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH disruptors.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Relationship Description

Thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3) are synthesized by NIS and TPO in the thyroid gland as iodinated thyroglobulin (Tg) and stored in the colloid of thyroid follicles across vertebrates. Secretion from the follicle into serum is a multi-step process. The first involves thyroid stimulating hormone (TSH) stimulation of the separation of the peptide linkage between Tg and TH. The next steps involve endocytosis of colloid, fusion of the endosome with the basolateral membrane of the thyrocyte, and finally release of TH into blood. More detailed descriptions of this process can be found in reviews by Braverman and Utiger (2012) and Zoeller et al. (2007).

Evidence Supporting this KER

The weight of evidence linking these two KEs of decreased TH synthesis and decreased T4 in serum is strong. It is commonly accepted dogma that decreased synthesis in the thyroid gland will result in decreased circulating TH (serum T4).

Biological Plausibility

The biological relationship between two KEs in this KER is well understood and documented fact within the scientific community.

Empirical Evidence

It is widely accepted that TPO inhibition leads to declines in serum T4 levels in adult **mammals**. This is due to the fact that the sole source for circulating T4 derives from hormone synthesis in the thyroid gland. Indeed, it has been known for decades that insufficient dietary iodine will lead to decreased serum TH concentrations due to inadequate synthesis. Strong qualitative and quantitative relationships exist between reduced TH synthesis and reduced serum T4 (Ekerot et al., 2013; Degon et al., 2008; Cooper et al., 1982; 1983; Leonard et al., 2016; Zoeller and Tan, 2007). There is more limited evidence supporting the relationship between decreased TH synthesis and lowered circulating hormone levels during development. Lu and Anderson (1994) followed the time course of TH synthesis, measured as thyroxine secretion rate, in non-treated pregnant rats and correlated it with serum T4 levels. Modeling of TH in the rat fetus demonstrates the quantitative relationship between TH synthesis and serum T4 concentrations (Hassan et al., 2017, 2020; Handa et al., 2021). Furthermore, a wide variety of drugs and chemicals that inhibit TPO are known to result in decreased release of TH from the thyroid gland, as well as decreased circulating TH concentrations. This is evidenced by a very large number of studies that employed a wide variety of techniques, including thyroid gland explant cultures, tracing organification of 131-I and in vivo treatment of a variety of animal species with known TPO inhibitors (King and May,1984; Atterwill et al., 1990; Brown et al., 1986; Brucker-Davis, 1998; Haselman et al., 2020; Hornung et al., 2010; Hurley et al., 1998; Kohrle, 2008; Tietge et al., 2010).

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. Several other studies have also shown that chemically induced Inhibition of TPO results in reduced TH synthesis in zebrafish (Van der Ven et al., 2006; Raldua and Babin, 2009; Liu et al., 2011; Thienpont et al., 2011; Rehberger et al., 2018). A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Temporal Evidence: In **mammals**, the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). There are currently no studies that measured both TPO synthesis and TH production during development. However, the impact of decreased TH synthesis on serum hormones is similar across all ages in mammals. Good evidence for the temporal relationship comes from thyroid system modeling of the impacts of iodine deficiency and NIS inhibition (e.g., Degon et al., 2008; Fisher et al., 2013). In addition, recovery experiments have demonstrated that serum thyroid hormones recovered in athyroid mice following grafting of in-vitro derived follicles (Antonica et al., 2012). In *Xenopus*, it has been shown that depression of TH synthesis in the thyroid gland precedes depression of circulating TH within 7 days of exposure during pro-metamorphosis (Haselman et al., 2020).

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal thyroid hormones transferred to the eggs. Embryonic thyroid hormone synthesis is activated later during embryo-larval development. (See Domain of applicability)

Dose-response Evidence: Dose-response data is lacking from studies that include concurrent measures of both TH synthesis and serum TH concentrations. However, data is available demonstrating correlations between thyroidal TH and serum TH concentrations during gestation and lactation during development (Gilbert et al., 2013). This data was used to develop a rat quantitative biologically-based dose-response model for iodine deficiency (Fisher et al., 2013). In *Xenopus*, dose-responses were demonstrated in both thyroidal T4 and circulating T4 following exposure to three TPO inhibitors (Haselman et al., 2020).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. The first uncertainty stems from the paucity of data for quantitative modeling of the relationship between the degree of synthesis decrease and resulting changes in circulating T4 concentrations. In addition, most of the data supporting this KER comes from inhibition of TPO, and there are a number of other processes (e.g., endocytosis, lysosomal fusion, basolateral fusion and release) that are not as well studied.

For example, Kim et al. (2015) investigated the adverse effects of Triphenyl phosphate (TPP), a substance that disrupts the thyroid system.
Therefore, **Rat pituitary** (GH3) and **thyroid follicular cell lines** (FRTL-5) were studied. In the GH3 cells, TPP led to an upregulation of the expression of important thyroid genes (tshˇ, tr˛ and trˇ) while T3, a positive control, downregulated the expression of these genes. In FRTL-5 cells, the expression of nis and tpo genes was significantly upregulated, suggesting that TPP stimulates thyroid hormone synthesis in the thyroid gland.

In **zebrafish larvae** at the age of 7 days post-fertilisation (dpf), TPP exposure resulted in a significant **increase in T3 and T4** concentrations and the expression of genes involved in thyroid hormone synthesis. Exposure to TPP also significantly regulated the expression of genes involved in the metabolism (dio1), transport (ttr) and excretion (ugt1ab) of thyroid hormones. The down-regulation of the crh and tshˇ genes in the zebrafish larvae suggests the activation of a central regulatory feedback mechanism that is triggered by the increased T3 levels in vivo. Taken together, these observations indicate that TPP increases thyroid hormone concentrations in early life stages of zebrafish by disrupting central regulatory and hormone synthesis pathways.

Quantitative Understanding of the Linkage

In rats, Hassan et al. (2020) demonstrated in vitro: ex vivo correlations of TPO inhibition using PTU and MMI and constructed a quantitative model relating level of TPO inhibition with changes in circulating T4 levels. They determined that 30% inhibition of TPO was sufficient to decrease circulating T4 levels by 20%. This is further supported by studies of Hassan et al. (2017) and Handa et al. (2021)

In *Xenopus*, Haselman et al. (2020) collected temporal and dose-response data for both thyroidal and circulating T4 which showed strong qualitative concordance of the response-response relationship. A quantitative relationship exists therein, but is yet to be demonstrated mathematically in this species.

Response-response relationship

Fisher et al. (2013) published a quantitative biologically-based dose-response model for iodine deficiency in the rat. This model provides quantitative relationships for thyroidal T4 synthesis (iodine organification) and predictions of serum T4 concentrations in developing rats. There are other computational models that include thyroid hormone synthesis. Ekerot et al. (2012) modeled TPO, T3, T4 and TSH in dogs and humans based on exposure to myeloperoxidase inhibitors that also inhibit TPO. This model was recently adapted for rat (Leonard et al., 2016) and Hassan et al (2017) have extended it to include the pregnant rat dam in response to TPO inhibition induced by PTU. While the original model predicted serum TH and TSH levels as a function of oral dose, it was not used to explicitly predict the relationship between serum hormones and TPO inhibition, or thyroidal hormone synthesis. Leonard et al. (2016) recently incorporated TPO inhibition into the model. Degon et al (2008) developed a human thyroid model that includes TPO, but does not make quantitative prediction of organification changes due to inhibition of the TPO enzyme. Further empirical support for the response-response relationship has been demonstrated in the amphibian model, *Xenopus laevis*, exposed to TPO inhibitors during pro-metamorphosis (Haselman et al., 2020) wherein temporal profiles were measured for both thyroidal and circulating T4.

Time-scale

Given that the thyroid gland contains follicular lumen space filled with stored thyroglobulin/T4, complete inhibition of thyroid hormone synthesis at a given point in time will not result in an instantaneous decrease in circulating T4. The system will be capable of maintaining sufficient circulating T4 levels until the gland stores are depleted. The time it takes to deplete stored hormone will greatly depend on species, developmental status and numerous other factors.

In *Xenopus*, Haselman et al. (2020) demonstrated an approximately 5 day difference between a significant decrease in thyroidal T4 preceding a significant decrease in circulating T4 while exposed to a potent TPO inhibitor (MMI) continuously during pro-metamorphosis.

Known modulating factors

During *Xenopus* metamorphosis, circulating T4 steadily increases to peak levels at metamorphic climax. Therefore, during *Xenopus* metamorphosis, this KER is operable at an increased rate as compared to a system that is maintaining steady circulating T4 levels through homeostatic control. In this case, developmental status is a modulating factor for the rates and trajectories of these KEs.

Known Feedforward/Feedback loops influencing this KER

This KER is entirely influenced by the feedback loop between circulating T4 originating from the thyroid gland and circulating TSH originating from the pituitary. Intermediate biochemical processes exist within the hypothalamus to affirm feedback and coordinately release TSH from the pituitary. However, quantitative representations of these feedback processes are limited to models discussed previously.

In *Xenopus*, circulating levels of T4 increase through pro-metamorphosis indicating a "release" of feedback to allow circulating levels of T4 to increase and drive metamorphic changes (Sternberg et al., 2011). This provides evidence that homeostatic control of feedback can be developmentally dependent, and likely species dependent.

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Relationship: 2038: T4 in serum, Decreased leads to Decreased, Triiodothyronine (T3) in serum

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to altered visual function via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Juvenile	Moderate
Larvae	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Thyroid follicles mainly produce T4 and to a lesser extent T3 across vertebrates. When serum T4 levels are decreased, less T4 is available for conversion to the more biologically active T3. This key event relationship is not always evident. This could be due to feedback/compensatory mechanisms that in some cases seem to be able to maintain T3 levels even though T4 levels are reduced, for example through increased conversion of T4 to T3 by deiodinases. These feedback mechanisms can also differ across species. Therefore, although this KER is plausibly applicable across vertebrates, variation can be expected. In zebrafish and fathead minnow, several studies reportedThe evidence for a relationship between circulating T4 and T3 levels (Nelson et al., 2016; Stinckens et al., 2020, Wang et al., 2020).

Life stage: This key event relationship is applicable to late larvae and juveniles rather than to embryos, because of the presence of maternal TH in embryos.

Uncertainties during embryonic lifestage:

- A decrease in T4 was observed in fathead minnows exposed to 1 mg/L 2-mercaptobenzothiazole (MBT), a thyroperoxidase inhibitor, through 6 dpf (Nelson et al., 2016). In contrast, there was no observed effect on T3 in fathead minnows exposed to MBT through 6 dpf. Comparably, zebrafish exposed to 0.4 or 0.7 mg/L MBT through 120 hpf showed decreased T4 but not T3 (Stinckens et al., 2016). During this early larval life stage, T3 may have been derived from maternal T4. In addition, it could be produced from further depletion of any T4 still produced by the thyroid gland (as thyroperoxidase may not have been fully inhibited at the tested exposure concentrations).
- Since exposure to PFAS did result in decreased whole-body T4 and T3 in 5 day old zebrafish, the life-stage specificity possibly depends on the mechanism that lies at the basis of the TH changes (Wang et al., 2020). The exact mechanisms by which PFAS disrupt the thyroid hormone system remain uncertain. Compounds that directly reduce T3 levels (e.g., deiodinase inhibitors) in addition to reducing T4 levels via another mechanism can be expected to result in decreased T4 and T3 levels.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Relationship Description

When serum thyroxine (T4) levels are decreased, less T4 is available for conversion to the more biologically active triiodothyronine (T3). While some thyroid hormone (TH) disrupting mechanisms can immediately affect T3 levels, including deiodinase inhibition, other mechanisms reduce T4 levels, for example through inhibition of TH synthesis, leading to decreased T3 levels.

Since in fish early life stages TH are typically measured on a whole body level, it is currently uncertain whether TH levels changes occur at the serum and/or tissue level. Pending more dedicated studies, whole body TH levels are considered a proxy for serum TH levels.

This key event relationship is not always evident. This could be due to feedback/compensatory mechanisms that in some cases seem to be able to maintain T3 levels even though T4 levels are reduced, for example through increased conversion of T4 to T3 by deiodinases.

Evidence Supporting this KER

Biological Plausibility

When serum thyroxine (T4) levels are decreased, less T4 is available for conversion to the more biologically active triiodothyronine (T3). It is plausible to assume that while some thyroid hormone (TH) disrupting mechanisms can immediately affect T3 levels, including deiodinase inhibition, other mechanisms reduce T4 levels, for example through inhibition of TH synthesis, leading to decreased T3 levels.

Empirical Evidence

- A decrease in whole-body T4 and T3 was observed in zebrafish exposed to methimazole from fertilization until the age of 21 and 32 days and to propylthiouracil until the age of 14, 21 and 32 days (Stinckens et al., 2020). Additionally, a strong correlation was observed between T4 and T3 levels. Both compounds are thyroperoxidase inhibitors expected to inhibit thyroid hormone synthesis.
- A dose-dependent decrease in whole-body T4 and T3 was observed in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids from fertilization until the age of 5 days (Wang et al., 2020). The exact mechanisms by which PFAS disrupt the thyroid hormone system remain uncertain.
- While T4 measurements could not be acquired in fathead minnows exposed to 1 mg/L 2-mercaptobenzothiazole, a thyroperoxidase inhibitor, for 14 days, a significant decrease in T3 was observed (Nelson et al., 2016). The decreased T3 levels were likely the result of reduced T4 synthesis.
- Besson et al. (2020) showed both decreased T4 levels and decrased T3 levels in metamorphosing convict surgeonfish exposed to chlorpyrifos.

Uncertainties and Inconsistencies

- Since in fish early life stages THs are typically measured on a whole body level, it is currently uncertain whether TH level changes occur at the serum and/or tissue level. Pending more dedicated studies, whole body TH levels are considered a proxy for serum TH levels.
- This key event relationship is not always evident. This could be due to feedback/compensatory mechanisms that in some cases seem to be able to maintain T3 levels even though T4 levels are reduced, for example through increased conversion of T4 to T3 by deiodinases. Examples of studies showing reduced T4 levels in the absence of reduced T3 levels:
    - Zebrafish exposed to 0.35 mg/L 2-mercaptobenzothiazole, a thyroperoxidase inhibitor, through 32 dpf showed decreased whole-body T4, but T3 levels showed particularly large variation and overall were not significantly decreased (Stinckens et al., 2016).
    - Although T4 content of 28 dpf larval fathead minnows exposed to 32 or 100 µg/l methimazole, a thyroperoxidase inhibitor, was reduced, these fish showed no change in whole body T3 content (Crane et al., 2006). Significantly higher T3/T4 ratios in fish held in 100 µg/l methimazole suggest an increased conversion of T4 to T3 or reduced degradation and conjugation during continued exposure to methimazole

Quantitative Understanding of the Linkage

Stinckens et al. (2020, supplementary information) showed a significant linear relationship between whole body T3 and T4 concentrations at 21 and 32 days post fertilization after continuous exposure of zebrafish to methimazole and propylthiouracil, two inhibitors of TH synthesis.

Known Feedforward/Feedback loops influencing this KER

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This relationship depends on the MIE that is causing the decrease in T3. For example, deiodinase inhibition results in reduced activation of T4 to T3 and thus in reduced T3 levels; increased T4 levels have been observed, probably as a compensatory mechanism in response to the lower T3 levels. For example, Cavallin et al. (2017) exposed fathead minnows to iopanoic acid, a deiodinase inhibitor, and observed T4 increases together with T3 decreases.

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Relationship: 2373: Decreased, Triiodothyronine (T3) in serum leads to Altered, retinal layer structure

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Life-stage applicability: Most studies on TH-regulated retinal structure are performed during vertebrate development. There is evidence of the impact of reduced T3 (caused by inhibition of thyroperoxidase) on retinal layer structure at 48, 66, 72, 96 and 120 hpf during zebrafish embryo-eleutheroembryo development (Baumann and others 2016; Komoike and others 2013; Reider and Connaughton 2014).

Taxonomic applicability: The visual system of the zebrafish follows the typical organisation of vertebrates and is often used as a model to study human eye diseases. Although there are some differences in eye structure between fish and mammals, it is plausible to assume that TH levels are important for healthy eye development across all vertebrates.

Sex applicability: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Effects on retinal layers resulting from TH level changes during early development are therefore expected to be independent of sex.

Key Event Relationship Description

Although the exact mechanisms need further investigation, studies show that thyroid hormones (THs) are required for healthy eye development in vertebrates (Wester et al. 1990, Suliman & Novales Flamarique 2013, Deveau et al., 2019) and it has been described that retinal development, photoreceptor differentiation and colour vision are directly regulated by THs. Not only in zebrafish (Bertrand et al. 2007), but also in mice (Ng et al. 2010) and chickens (Trimarchi et al. 2008), THs are directly linked to the transcription of essential visual opsins and the differentiation of retinal cells, as well as the overall structure of the retina, which is essential for proper functioning. Therefore, decreased triidothyronine (T3) levels in serum during eye development are likely to lead to structural and morphological alterations of the retina.

Evidence Supporting this KER

Biological Plausibility

THs, TH receptors, and deiodinase (DIO) enzymes are important for eye and retinal development in vertebrates. Dio enzymes activate and inactivate THs, consequently playing a central role in regulating TH levels in target tissues. In zebrafish, TH receptors and dio enzymes have been localized in the retina from 24 hpf onwards, probably regulating the differentiation of retinal structures and photoreceptors (Gan et al. (2010),Duval, M. G., & Allison, W. T. (2018)). It is known from amphibians that when TH levels start to rise at the beginning of metamorphosis, the morphology of the eyes starts changing. In chicken, the developing eye shows a dynamic expression pattern of Deiodinase 2 (DIO2) and Deiodinase 3 (DIO3), probably regulating photoreceptor differentiation and cornea development (reviewed by Darras 2015).

Empirical Evidence

There is ample evidence that reduced THs have an influence on development of the retinal layer structure in fish and other vertebrates.

Evidence from exposure to PTU, 6-n-propylthouracil, a classic positive control for inhibition of thyroperoxidase responsible for TH synthesis:

- Reduced T4 and T3 levels at 14, 21 and 32 days post fertilization (dpf) were observed after exposure of zebrafish to 111 mg/L PTU (Stinckens et al., 2020). Exposure to 37 mg/L PTU reduced T4 levels at 14, 21 and 32 dpf and significantly reduced T3 levels at 32 dpf, while the more limited decrease of T3 levels at 14 and 21 dpf was not statistically significant (Stinckens et al., 2020). Schmidt and Braunbeck (2011) also showed reduced T4 levels in juvenile zebrafish exposed to PTU for 5 weeks. PTU was also shown to reduce T4 levels already at 72 and 120 hours post fertilization (Walter et al., 2019). T3 levels tended to decrease at 72 and 120 hpf but these changes were not significant. Exposures were always continuous and started immediately after fertilization.
- Baumann et al. (2016) described alterations in retinal structure, pigmentation and eye size in 5 day old zebrafish embryos after exposure to PTU. Exposures to 100 and 250 mg/L PTU reduced retinal pigment epithelial diameter and exposure to 250 mg/L increased the grey value of the pigment layer which is a measure of decreased pigmentation.
- Gan et al. (2010) showed that thyroid hormones accelerate opsin expression in differentiating cones and induce the opsin switch, a shift from expression of UV opsin to blue opsin, in differentiated single cones in salmonids. Using in situ hybridization, they characterized the spatiotemporal dynamics of opsin expression and switching in embryos treated with exogenous thyroid hormone or propylthiouracil. The results show that PTU repressed the opsin switch. Thyroid hormone is required for opsin switching in the retina of salmonid fishes.

Evidence from exposure to methimazole, a model thyroperoxidase inhibitor:

- Methimazole was shown to reduce T4 and T3 levels at 14, 21 and 32 days post fertilization after exposure of zebrafish to 50 and 100 mg/L (Stinckens et al., 2020). Exposures were always continuous and started immediately after fertilization. (any data on TH for MMI at 5 dpf or earlier?)
- Komoike et al. (2013) exposed zebrafish embryos to 10 mM methimazole and observed moderately disrupted retinal structure with apoptosis of renital cells already at 48 hpf and more severely disrupted retinal structure at 72 hpf. Major gaps and malformations of the retinal structure occured at 72 hpf. The observed retinal anomalous morphologies have a direct analogy to the congenital anomalies observed in children exposed to methimazole in utero.
- Reider and Connaughton (2014) exposed zebrafish embryos to methimazole until 66, 70 or 72 hpf and analysed the retina at 72 hpf. The thickness of the ganglion cell layer (GCL) was decreased in embryos exposed to MMI until 66 hpf compared to controls. An increase in GCL thickness was observed in embryos exposed until 70 hpf, and normal thickness was observed in embryos exposed until 72 hpf. Although the impact of the exposure windows cannot be entirely explained, this confirms the relation between reduced T3 and altered retinal structure.

Evidence from other chemical exposures:

- Baumann et al. (2016): After exposure to 200 and 300 mg/L TBBPA, a compound with several mechanisms including a direct interaction with the thyroid hormone receptor and binding to the thyroid hormone binding protein transthyretin, grey values were increased at 5 dpf indicative of reduced pigmentation in the eyes. There were no significant effects on the retinal pigment epithelium diameter. Experiments from Zhu et al. (2018) and Yu et al. (2021) confirm a reduction in T3 levels in both the larvae and embryos after exposure to 300 µg/L TBBPA and the locomotor activity of larval offspring was significantly reduced.
- Besson et al. (2020) used treatment with NH3 to highlight the role that thyroid hormones (TH) play in retinal development in metamorphosing convict surgeonfish. They analysed different cell segments, types, and layers of the retina, such as (i) the densities of photoreceptor external segments (perceiving light signals), (ii) photoreceptor nuclei, (iii) bipolar cells (which integrate the synaptic signals originating from the photoreceptors), and (iv) ganglion cells (which integrate signals from bipolar cells and create action potential toward the optic nerve). They investigated the role of TH in the development of these sensory structures by injecting fish daily from d0 to d5 with NH3 (10−6 M), a TH antagonist, to achieve TH signal disruption. NH3 prevents the binding of TH such as T3 to TR, therefore impairing the binding of transcriptional coactivators to TR, which therefore remain in an inactive and repressive conformation.The NH3 treatment was thus applied to repress TH signaling

by disrupting the TH pathway leading to an adverse outcome on retinal layer level. Repressed retinal development at both d2 and d5 with a 10- 25 % decrease of bipolar cell density was detected.

- Besson et al. (2020) further showed that treatment with chlorpyrifos reduced T3 levels and reduced bipolar cell density by 10%.

- Bhumika et al. (2014) found that lowering T3 signaling through exposure to different chemicals accelerates optic tectum reinnervation following optiv nerve crush in zebrafish and that this is accompanied by a more rapid resolution of the inflammatory response. Unlike in mammals, full recovery of the damaged CNS is possible in adult fish and amphibians and, for instance, the optic nerve of fish can regenerate completely after injury. Adult zebrafish were exposed to 10 µM of iopanoic acid (IOP), which lowered intracellular 3,5,3′-triiodothyronine (T3) availability, or to 7 µM of the thyroid hormone receptor β antagonist methylsulfonylnitrobenzoate (C1). Both treatments accelerated optic tectum (OT) reinnervation. At 7 days post injury (7 dpi) there was a clear increase in the biocytin labeled area in the OT following anterograde tracing as well as an increased immunostaining of Gap43, a protein expressed in outgrowing axons. This effect was attenuated by T3 supplementation to IOP-treated fish. ON crush induced limited cell death and proliferation at the level of the retina in control, IOP- and C1-treated fish.

Evidence from genetic knockdown and knockout studies:

- Houbrechts (2016) performed deiodinase (DIO) knockdown in zebrafish embryos and observed reduced eye size, disturbed retinal lamination and strong reduction in rods and all four cone types. DIO 1 and 2 are both responsible for converting T4 to the more active T3. Combined knockdown of DIO 1 and 2, leading to reduced T3 levels, altered the structure of the ganglion cell layer (GCL), making it wider and less dense. DIO3 deactivates T3 and defects were more prominent and persistent in D3-deficient fish with observations of marked disorganization across all retinal layers.

- Using genetic zebrafish experiments Duval and Allison (2018) investigated the role of the thyroid hormone receptor thrb in cone differentiation at different time points. Disrupting thrb activity via expression of a dominant negative thrb (dnthrb) at either early or late retinal development had differential outcomes on red cones (reduced abundance), versus UV and blue cones (increased abundance).The effects of thrb change through photoreceptor development, first promoting red cones and restricting UV cones, and later restricting UV and blue cones. Knockdown of thrb causes near-complete absence of red cones and an increase in UV cone abundance (by approximately 35%), whereas expression of dnthrb via heat shock at 52 hpf leads to increased UV (by 27%) and blue cone abundance (by 36%) relative to heat shocked nontransgenic siblings. Inducing dnthrb expression at other time points, including 24 hpf, 30 hpf, and 36 hpf, did not alter cone abundances as dramatically relative to controls (<20% change). This revealed an effect of thrb that is limited to later photoreceptor development: the endogenous receptor negatively regulates blue cone determination. In contrast, disrupting Thrb activity either early (with morpholino knock down) or late leads to more UV cones.

- Ng et al. (2010) showed in mice that knockout of the thyroid receptor, THRb2, results in important changes in the numbers of specific cone types in the retina and M opsins do not even appear at all. Knockout of a thyroid hormone receptor conceptually corresponds to decreased activation of the thyroid hormone receptor due to decreased T3 levels.

Other models of hypothyroidism:

- Gamborino (2000) analysed eye development in a rat model of congenital-neonatal hypothyroidism (HG), induced by combined chemical-surgical thyroidectomy. Histopathological analyses of the eyes of TH-deficient animals revealed decrease in photoreceptor and ganglion cell layer thickness, a delay in photoreceptor outer segment morphogenesis and significantly lower values for ganglion cell nuclear volumes and nuclear pore density.

indirect evidence:

- Trimarchi et al. (2008) observed three waves of expression of components of the HPT-axis in specific locations in the retina in progenitor cells and photoreceptor cells during development of the chicken, indicating that thyroid hormones are required for normal retinal development and photoreceptor differentiation.

Uncertainties and Inconsistencies

Several studies have shown molecular responses to hypothyroidism that are related to eye development (Bagci et al., 2015; Houbrechts et al., 2016; Baumann et al., 2019) but the exact molecular processes linking lower TH level to disturbances of the layers in the retina is not yet fully understood.

Both decreased as well as increased TH action has been shown to impact retinal development.

- For example, Ng et al. (2010) showed altered cone appearance in the retina following both DIO3 knockout (leading to hyperthyroidism) and THRb2 knockout (corresponding to hypothyroidism).
- Besson et al. (2020) used pharmacological treatments (T3 + iopanoic acid (IOP), NH3) to not only disrupt but also activate the TH signaling pathway. They used 10−6M T3 + (iopanoic acid) (T3 treatment) to achieve TH signal activation. Here, IOP was used as an inhibitor of deiodinase enzymes, following comparable work in mammals and amphibians, and as routinely used in fish to prevent the immediate degradation of injected T3. The combined treatment thus causes elevated T3 levels. Detected effects on retinal layer were elevated densities of bipolar cells at day 2 in surgeonfish.
- Suppressing TH signaling in retina dystrophy mouse models (a mouse model of retinal degeneration) seems to protect cone viability (Ma et al., 2014; 2016). The authors suggested that the impact of TH on cone survival is independent of its impact on

cone opsin expression. The mechanism underlying the effect on cone viability has not been elucidated.
- Bhumika et al. (2014) showed accelerated reinnervation of the optic tectum after optic nerve crush in zebrafish that had been treated with IOP or a TR antagonist. both treatments cause hypothyroidism. Supplementation of T3 reduced the rate of reinnervation.

Most knowledge comes from effects in developing organisms. There are some gaps in our knowledge about how TH levels affect the eyes of already fully developed organisms and/or whether they have similarly serious effects on the retinal layers. It can be assumed that the effects, if any, are weaker. Studies (Reider et al. 2014) found that layer thickness varied across ages suggesting that these retinal layers are differentially sensitive to for example MMI and/or that there are different critical periods of sensitivity of the retinal tissue.

Quantitative Understanding of the Linkage

There is no direct quantitative relation available at this point.

Known Feedforward/Feedback loops influencing this KER

- One feedback loop mechanism could be triggered by iodine deficiency or inhibition of iodine uptake. It appears probably that the inhibition increases the secretion of Thyroid stimulating hormone, which could stimulate the expression of the NIS-transporter. This increase in TSH could shift the ratio in favour of T3.

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Relationship: 2374: Altered, retinal layer structure leads to Altered, Visual function

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Adult	Moderate
Juvenile	Moderate
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic applicability: The visual system of the zebrafish follows the typical organisation of vertebrates and is often used as a model to study human eye diseases. Although there are some differences in eye structure between zebrafish and humans, it is plausible to assume that a functioning eye structure is important for visual function across all vertebrates and invertebrates that

have eyes.

Life stage applicability: The first visual responses based on retinal functionality appear around 70 hpf in zebrafish (Schmitt and Dowling 1999). It is plausible to assume that alterations of the eye structure would result in altered visual function across all life stages, but such alterations are most likely to occur during the development of the normal eye structure, which occurs in the embryo-eleutheroembryo phase.

Sex applicability: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Effects on visual function resulting from altered eye structure during early development are therefore expected to be independent of sex.

Key Event Relationship Description

The structure of the vertebrate retina is well conserved and consists of the following layers: The retinal pigment epithelium (RPE), the photoreceptor layer (PRL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL) and the ganglion cell layer (GCL). Each of these layers has a specific function for the physiology of the visual system. The RPE serves to protect and maintain the photoreceptors and absorbs excess light. The photoreceptors in the PRL consist of a light-receiving outer segment (OS) and the inner segment (IS), which contains the cell bodies. They send their signals to the bipolar cells in the INL, which transmit the signal to the ganglion cells. These form the optic nerve and are responsible for transmitting signals to the optic nerves. In both plexiform layers, the retinal neurons form their synaptic connections (Bibliowicz et al. 2011).

To study the eye, the zebrafish (Danio rerio) is at the forefront of many studies as a model organism. In zebrafish, eye development begins around 12 hpf (Houbrechts et al., 2016b) and by 72 hpf the layers of the retina are well developed (Malicki et al., 2016). Functional vision is established by 4-5 dpf (Brockerhoff, 2006; Chhetri et al., 2014).

Evidence Supporting this KER

Biological Plausibility

It should be emphasised that all layers of the retina are interdependent. The RPE plays an essential role in the retinoid cycle for the photoreceptors (PRL), which perceive the light stimulus and transmit it via the bipolar cells to the ganglia (IPL), which form the optic nerve and transmit the signal to the optic nerve (Connaughton 2005). If these key sites of the phototransduction pathway are disrupted by, for example, endocrine disruptors, it stands to reason that there would be a significant impact on the optical sense and it is plausible that disorders of the eye structure can lead to visual disorders.

Empirical Evidence

- Baumann et al., 2016 used propylthiouracil (PTU) and tetrabromobisphenol A (TBBPA) to disrupt the thyroid hormone system in zebrafish larvae. This exposure induced different molecular response patterns leading to impaired eye development (reduction of RPE cell diameter, pigmentation and eye size). Behavioural analyses showed that these larvae were also disrupted in their visual capacities, such as decrease in optokinetic response and increase in light preference of PTU-treated larvae.
- Avallone et al. (2015) studied the effects of cadmium exposure on the vision of adult zebrafish. The morpho-cytological changes of the retina (Nerve fiber layer clearly thickened and vacuolated, presence of compact pycnotic nuclei, empty area, change in the thickness of pigmented retinal epithelium and at the level of cones inner segments, extended folds of treated retinas, presence of cell debris and/of blood cells in vitreal chamber) were investigated by light and electron microscopy, while the functionality of the cadmium-exposed retinas was assessed by re-illumination behavioural tests with white or coloured light. Cadmium toxicity was shown to cause significant cell degeneration and loss of organisation at both macroscopic and microscopic levels. These changes were directly related to functional responses, particularly by increasing light sensitivity of exposed fish. Avoidance of bright light had increased in exposed fish.
- Houbrechts et al. (2016) used a knockdown of deiodinase 1 and 2 genes in zebrafish embryos to induce transient hypothyroidism and observed a wider and less dense ganglion cell layer at 3 dpf together with a reduced response (increase of swimming activity) to light at 4 dpf. By 7 dpf both the change in the ganglion cell layer as well as the altered response to light had recovered and resembled those of the untreated larvae.
- Flamarique et al. (2013) used thyroid hormone treatment to transform the UV cones of young rainbow trout into blue cones and showed that this reduced the distances and angles at which prey were located (variables that are known indicators of

foraging performance). Using optical measurements and photon-catch calculations, the study showed that control rainbow trouts perceived prey (Daphnia) with greater contrast compared to thyroid-hormone-treated fish, demonstrating that the presence of UV cones enhances foraging performance of young rainbow trout.

- Walter et al. (2019) found out that developmental exposure to either T4 or T3 in zebrafish embyros altered photomotor behavior. The response to a sudden transition from light to dark differed from that in untreated fish.
- Heijlen et al. (2014) showed that knockdown of Type 3 Iodothyronine Deiodinase, known to disrupt retinal layer structure (Houbrechts et al. (2016), causes embryos to spend significantly less time moving, and perturbs the escape response after a tactile stimulus. It is unclear to what extent this relationship is determined by alterations in muscle development or other factors contributing to these types of behaviour.
- Van Camp et al. (2018) showed that permanent deiodinase 2 deficiency in zebrafish resulted in a reduction of the number of R/G cones and rods that persisted through 7 dpf together with a reduced response to light (observed at 6 dpf).
- Chawla et al. (2018) investigated the role of Retinoic Acid (RA) in embryonic development of craniofacial structures in zebrafish. An increase in RA caused morphological changes of the eyes: a decrease of both cellular density of the corneal epithelium and cellularity of the inner segment. Inhibition of RA synthesis with 4-diethylamino- benzaldehyde (DEAB) resulted in structural changes of the retina, including the obliteration of photoreceptors and ganglion cell layer, and decreased cellularity of the outer and inner nuclear layers. Treated fish showed strong impairment of the optokinetic reflex.

Uncertainties and Inconsistencies

Often, high variances occur in the results of behavioural studies because an organism has some compensatory mechanisms that allow it to survive.
It is more difficult to compare data from different laboratories in such experiments.
Similarly, extrapolating data from fish to mammalian data is particularly difficult for behavioural studies.

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage is currently limited.

Time-scale

Temporal evidence is supported by the studies of Houbrechts et al. (2016) and Van Camp et al. (2018) in genetic knockdown and knockout zebrafish respectively. Houbrechts et al. (2016) used a DIO 1 and 2 knockdown, which causes transient hypothyroidism. At 3 dpf they showed altered retinal layer structure and at 4 dpf they showed an altered response to light. By 7 dpf both the retinal layer structure and the response to light had returned to normal. Van Camp et al. (2018) used a DIO2 knockout model causing permanent hypothyroidism. They did shown both altered numbers of rods and cones in the retina and an altered response to light at 7 dpf.

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Relationship: 2375: Altered, Visual function leads to Increased Mortality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	adjacent		
Thyroperoxidase inhibition leading to altered visual function via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic applicability: The visual system of the fish (e.g., zebrafish) follows the typical organisation of vertebrates and is often used as a model to study human eye diseases. Although there are some differences, it is plausible to assume that visual function is important for survival across all vertebrates and invertebrates that have eyes.

Sex applicability: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Effects on mortality resulting from altered visual function are therefore expected to be independent of sex.

Life stage applicability: It is plausible to assume that altered visual function of the eye would result in a higher mortality across all life stages. This could be especially true for the embryonic stages, the most sensitive stage of life. Vision plays a crucial role (in the early life stages) of most species, as eye development and establishment of functional vision is essential for perception of food or avoidance of predators for example (Carvalho et al., 2002).

Key Event Relationship Description

In animals, whatever the taxa, visual abilities are strongly linked to their lifestyle (feeding, avoidance of predators, movement, protection....). When these capacities are impaired, they lead to reduced fitness and are therefore strongly linked to a decrease in survival, particularly in the early stages of life.

Evidence Supporting this KER

Biological Plausibility

Decreases in visual functions can have a strong impact on behavior, leading to changes in individual response and abilities in the environment, including, for example, perception of food or avoidance of predators. Variation in the visual system can also influence learning tasks when visual stimuli are used (Corral-López et al., 2017).

Sensory drive has been implicated in speciation in various taxa, largely based on phenotype-environment correlations and signatures of selection in sensory genes, including view (Maan et al, 2017).

It can be assumed that an animal which has difficulties in finding food and avoiding predators will have lower survival chances in wildlife.

Empirical Evidence

Only very few studies are available in which it was demonstrated that decreased visual capacities lead to reduced survival of the organism. In general, mortality is rarely assessed but survival-reducing factors (feeding, predation) are mainly investigated. Here we consider the work about different toxicants that disrupt complex fish behaviors, such as predator avoidance, reproductive, and social behaviors. Toxicant exposure often completely eliminates the performance of behaviors that are essential to fitness and survival in natural ecosystems, frequently after exposures of lesser magnitude than those causing significant mortality (Brown et al., 2004).

- Fuiman et al. (2006) specifically investigated the importance of several putative survival skills for escaping a predator. They first analysed routine swimming, acoustic startle stimulus and visual startle stimulus of red drum larvae and subsequently performed a predation experiment using the same larvae in the presence of a live predator. The authors found that the effectiveness of escape responses was almost 100% and thus responsiveness determined survival under predation. Of the different putative survival skills, only visual responsiveness was significantly correlated to escape potential, while others such as acoustic responsiveness were not significantly contributing to escape potential. Further investigation showed that only visual responsiveness differed significantly between poorly responding larvae and better responders.

- Dehnert et al., 2019 In zebrafish, 2, 4-Dichlorophenoxyacetic acid exposure during eye development impaired visual behavior, i.e. reduced prey capture. Additonally, exposed fish showed reduced neural activity within the optic tectum following prey exposure. (more details needed, this is very convincing with the details)
- Besson et al., 2020 exposed metamorphosing convict surgeonfish to pharmacological treatments.They performed a 10^{-6} M NH3 treatment (a TH antagonist) to achieve TH signal disruption and they observed an adverse outcome on retinal layer level. Repressed retinal development at both day 2 and day 5 with a 10-25 % decrease of bipolar cell density was detected. They followed up with a behavior test at day 2 with blacktail snapper as a predator and got the following results:

  1. In the test using chemical cues of the predator the NH3-treated fish did not discriminate between water sources, while control fish clearly avoided predator cues.

  2. In the viusual cues test the NH3-treated fish showed no preference and spent 25 % more time in visual stimulus compared to controls.

  3. In a survival predation test in an in situ arena they observed that day 2 NH3 treated fish exhibited 30% lower survival than d2 control fish.
- Furthermore Besson et al., 2020 conducted a Chlorpyrifos (CPF) treatment 30 µg L–1 and observed a significant reduction (25%) in T4 levels at day 2 in CPF30 fish, as well as significantly reduced T3 levels in CPF30 fish (28%) compared with control fish. CPF30 fish also exhibited reduced densities of bipolar cell (10%) of retinal layer and CPF30 fish experienced lower survival.
- Flamarique et al. (2013) showed that thyroid hormone treatment impacted the development of the visual system in rainbow trout and reduced the distances and angles at which prey were located (variables that are known indicators of foraging performance). Using optical measurements and photon-catch calculations, the study showed that control rainbow trouts perceived prey (Daphnia) with greater contrast compared to thyroid-hormone-treated fish. Reduced foraging performance is likely to reduce survival in the wild.
- Heijlen et al. (2014) showed that knockdown of Type 3 Iodothyronine Deiodinase, known to disrupt eye development (Houbrechts et al. (2016), causes embryos to spend significantly less time moving, and perturbs the escape response after a tactile stimulus. It is unclear to what extent this relationship is determined by alterations in muscle development or other factors contributing to these types of behaviour. An inability to escape predators likely reduces survival in the wild.

Uncertainties and Inconsistencies

It is obvious that impaired vision leads to higher mortality, as the sense of sight is important for survival, and if it is impaired, feeding or escape becomes more difficult. However, the number of studies investigating this connection is limited. Also, the natural conditions, which depend on many variables, are difficult to reproduce in the laboratory or to compare between different laboratories.

Quantitative Understanding of the Linkage

Known modulating factors

Increase according to global health of the population (e.g on trout (Post and Parkinson, 2001)

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Relationship: 2013: Increased Mortality leads to Decrease, Population trajectory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	adjacent		
Acetylcholinesterase inhibition leading to acute mortality	adjacent	Moderate	Moderate
Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	adjacent	Moderate	Moderate
Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Moderate
Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	adjacent	Moderate	Moderate
Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to altered visual function via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	adjacent		
Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	adjacent	High	High
GSK3beta inactivation leading to increased mortality via defects in developing inner ear	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: All organisms must survive to reproductive age in order to reproduce and sustain populations. The additional considerations related to survival made above are applicable to other fish species in addition to zebrafish and fathead minnows with the same reproductive strategy (r-strategist as described in the theory of MaxArthur and Wilson (1967). The impact of reduced survival on population size is even greater for k-strategists that invest more energy in a lower number of offspring.

Life stage: Density dependent effects start to play a role in the larval stage of fish when free-feeding starts (Hazlerigg et al., 2014).

Sex: This linkage is independent of sex.

Key Event Relationship Description

Increased mortality in the reproductive population may lead to a declining population. This depends on the excess mortality due to the applied stressor and the environmental parameters such as food availability and predation rate. Most fish species are r-strategist, meaning they produce a lot of offspring instead of investing in parental care. This results in natural high larval mortality causing only a small percentage of the larvae to survive to maturity. If the excess larval mortality due to a stressor is small, the population dynamics might result in constant population size. Should the larval excess be more significant, or last on the long-term, this will affect the population. To calculate the long-term persistence of the population, population dynamic models should be used.

Evidence Supporting this KER

Survival rate is an obvious determinant of population size and is therefore included in population modeling (e.g., Miller et al., 2020).

Biological Plausibility

- Survival to reproductive maturity is a parameter of demographic significance. Assuming resource availability (i.e., food, habitat, etc.) is not limiting to the extant population, sufficient mortality in the reproductive population may ultimately lead to declining population trajectories.
- Under some conditions, reduced larval survival may be compensated by reduced predation and increased food availability, and therefore not result in population decline (Stige et al., 2019).

Empirical Evidence

- According to empirical data, combined with population dynamic models, feeding larvae are the crucial life stage in zebrafish (and other r-strategists) for the regulation of the population. (Schäfers et al., 1993)
- In fathead minnow, natural survival of early life stages has been found to be highly variable and influential on population growth (Miller and Ankley, 2004)
- Rearick et al. (2018) used data from behavioural assays linked to survival trials and applied a modelling approach to quantify changes in antipredator escape performance of larval fathead minnows in order to predict changes in population abundance. This work was done in the context of exposure to an environmental oestrogen. Exposed fish had delayed response times and slower escape speeds, and were more susceptible to predation. Population modelling showed that his can result in population decline.
- In the context of fishing and fisheries, ample evidence of a link between increased mortality and a decrease of population size has been given. Important insights can result from the investigation of optimum modes of fishing that allow for maintaining a population (Alekseeva and Rudenko, 2018). Jacobsen and Essington (2018) showed the impact of varying predation mortality on forage fish populations.
- Boreman (1997) reviewed methods for comparing the population-level effects of mortality in fish populations induced by pollution or fishing.

Uncertainties and Inconsistencies

- The extent to which larval mortality affects population size could depend on the fraction of surplus mortality compared to a natural situation.
- There are scenarios in which individual mortality may not lead to declining population size. These include instances where populations are limited by the availability of habitat and food resources, which can be replenished through immigration. Effects of mortality in the larvae can be compensated by reduced competition for resources (Stige et al., 2019).
- The direct impact of pesticides on migration behavior can be difficult to track in the field, and documentation of mortality during migration is likely underestimated (Eng 2017).

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List of Non Adjacent Key Event Relationships

Relationship: 366: Thyroperoxidase, Inhibition leads to T4 in serum, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	non-adjacent	High	Moderate
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	non-adjacent	High	Low
Thyroperoxidase inhibition leading to altered amphibian metamorphosis	non-adjacent	High	High
Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	non-adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Xenopus laevis	Xenopus laevis	High	NCBI
rat	Rattus norvegicus	High	NCBI
chicken	Gallus gallus	Moderate	NCBI
human	Homo sapiens	High	NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Taxonomic: Use of TPO inhibitors as anti-hyperthyroidism drugs in humans and pets (Emiliano et al., 2010; Trepanier, 2006) and effects of these drugs on serum TH concentrations in rats (US EPA, 2005), amphibian, fish and avian species (Coady et al., 2010; Grommen et al., 2011; Nelson et al., 2016; Rosebrough et al., 2006; Stinckens et al.; 2020; Tietge et al., 2012), strongly supports a causative linkage between inhibition of TPO and decreased serum T4 across species. Therefore, this KER is plausibly applicable across vertebrate species. Therefore, this KER is plausibly applicable across vertebrates.

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). Therefore, iIt is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH disruptors.

Sex: The KE is plausibly applicable to both sexes. Thyroid hormones are essential in both sexes and the components of the HPT-axis are identical in both sexes. There can however be sex-dependent differences in the sensitivity to the disruption of thyroid hormone levels and the magnitude of the response. In humans, females appear more susceptible to hypothyroidism compared to males when exposed to certain halogenated chemicals (Hernandez-Mariano et al., 2017; Webster et al., 2014). In adult zebrafish, Liu et al. (2019) showed sex-dependent changes in thyroid hormone levels and mRNA expression of regulatory genes including corticotropin releasing hormone (crh), thyroid stimulating hormone (tsh) and deiodinase 2 after exposure to organophosphate flame retardants. The underlying mechanism of any sex-related differences remains unclear.

Key Event Relationship Description

Thyroperoxidase (TPO) is the enzyme that catalyzes iodine organification of thyroglobulin to produce thyroglobulin (Tg)-bound T3 and T4 in the lumen of thyroid follicles. Tg-bound THs are endocytosed across the apical lumen-follicular cell membrane, undergo thyroglobulin proteolysis, followed by hormone section into the blood stream (see Taurog, 2005 for review). This indirect KER describes the relationship of TPO inhibition to reduced circulating levels of thyroid hormone (TH) in the serum.

Evidence Supporting this KER

The weight of evidence linking thyroperoxidase inhibition to reductions in circulating serum TH is strong. Many studies support this basic linkage. There is no inconsistent data.

Biological Plausibility

It is a well-accepted fact that inhibition of the only enzyme capable of synthesizing THs, TPO, results in subsequent decrease in serum TH concentrations. A large amount of evidence from clinical and animal studies clearly support the commonly accepted dogma that inhibition of TPO leads to decreased serum THs.

Empirical Evidence

The majority of research in support of this KER involve exposure to known TPO inhibitors and measurement of serum hormones. There are many in vivo studies that link decreases in serum TH concentrations with exposure to xenobiotics that inhibit

thyroperoxidase (TPO) in **mammals** (Brucker-Davis, 1998; Hurley, 1998; Boas et al., 2006; Crofton, 2008; Kohrle, 2008; Pearce and Braverman, 2009; Murk et al., 2013).

While these studies support the connection between exposure to a known TPO inhibitor and decreased TH, many of these studies do not empirically measure TPO inhibition or decreased TH synthesis. Thus, many studies support the indirect linkage between TPO inhibition (for chemicals identified as TPO inhibitors in in vivo or ex vivo studies) and decreased TH, with the well accepted theory that these proceed via decreased TH synthesis. That exposure to TPO inhibitors leads to decreased serum TH concentrations, via decreased TH synthesis is strongly supported by decades of mechanistic research in a variety of species.

This indirect relationship is also evidenced by the use of clinically-relevant anti-hyperthyroidism drugs, MMI and PTU (Laurberg & Anderson, 2014; Sundaresh et al., 2013). These drugs are both recognized TPO inhibitors and are part of a standard drug-based regimen of care for clinically hyperthyroid patients including those with Grave's disease. Serum THs are measured as the bioindicator of successful treatment with anti-hyperthyroidism drugs; the actual decrease in TH synthesis in the thyroid gland is implied in the efficacious use of these drugs (Trepanier, 2006).

In **rats**, MMI and PTU are often used as control chemicals to decrease serum THs to study biological phenomena related to disruption of TH homeostasis (many examples, including Zoeller and Crofton, 2005; Morreale de Escobar et al, 2004; Schwartz et al., 1997; Herwig et al., 2014; Wu et al., 2013; Pathak et al., 2011). Further, MMI is recommended as a positive control for use in the **Amphibian** Metamorphosis (Frog) Assay within Tier 1 of the U.S. EPA Endocrine Disruptor Screening Program (US EPA, 2009; Coady et al., 2010), an assay used to evaluate the potential for chemicals to disrupt TH homeostasis. PTU has been suggested as positive control chemical in the guidance for the Comparative Developmental Thyroid Assay (US EPA, 2005), a non-guideline assay used to evaluate the potential for chemicals to disrupt TH homeostasis during gestation and early neonatal development.

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Thus, an indirect key event relationship between TPO inhibition and decreased serum THs is strongly supported by a large database of clinical medicine and investigative research with whole animals (with a great deal of supporting evidence in rats and frogs).

Temporal Evidence: In **mammals**, the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). The qualitative impact of thyroperoxidase inhibition on serum hormones is similar across all ages in mammals. The temporal nature of the impact on serum THs by TPO inhibitors in developmental exposure studies is evidenced by the duration of exposure and developmental age (Goldey et al., 1995; Ahmed et al., 2010; Tietge et al., 2010), as well as recovery after cessation of exposure (Cooke et al., 1993; Goldey et al., 1995; Sawin et al., 1998; Axelstad et al., 2008; Shibutani et al., 2009; Lasley and Gilbert, 2011). The temporal relationship between TPO inhibitor exposure duration and serum hormone decreases in adult organisms has been widely demonstrated (e.g., Hood et al., 1999; Mannisto et al., 1979). In addition, MMI and PTU induced decreases in serum T4 are alleviated by thyroid hormone replacement in both fetal and postnatal age rats (Calvo et al., 1990; Sack et al., 1995; Goldey and Crofton, 1998). Computational modeling of the thyroid also provides evidence for the indirect temporal relationship between these two KEs (e.g., Degon et al., 2008; Fisher et a., 2013).

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal thyroid hormones transferred to the eggs. Embryonic thyroid hormone synthesis is activated later during embryo-larval development. (See Domain of applicability)

Dose-Response Evidence: Empirical data is available from enough studies in animals treated with TPO inhibitors during development to make it readily accepted dogma that a dose-response relationship exists between TPO inhibition and serum TH concentrations. Again, these studies do not empirically measure TPO inhibition or decreased TH synthesis, but rely on the strong support of decades of mechanistic research in a variety of species of the causative relationship between these KEs. Examples of dose-responsive changes in TH concentrations following developmental exposure to TPO inhibitors include studies a variety of species, including: rodents (Blake and Henning, 1985; Goldey et al., 1995; Sawin et al., 1998); frogs (Tietge et al., 2013); fish tissue levels (Elsalini and Rohr, 2003.); and, chickens (Wishe et al., 1979). Computational modeling of the thyroid also provides evidence for the indirect dose-response relationship between these two KEs (e.g., Leonard et al., 2016; Fisher et a., 2013).

Uncertainties and Inconsistencies

There are no inconsistencies in this KER, but there are some uncertainties. The predominant uncertainty regarding the indirect key event relationship between inhibition of TPO activity and decreased serum T4 is the quantitative nature of this relationship, i.e., to what degree must TPO be inhibited in order to decrease serum T4 by a certain magnitude. Many animal (rat) studies typically employ relatively high exposures of TPO-inhibiting chemicals that result in hypothyroidism (severe decrements in T4 and T3). Thus, a dose-response relationship between TPO inhibition and decreased serum T4 is not typically defined. However, there are

numerous publications demonstrating clear dose- and duration- dependent relationships between TPO inhibitors dose and reduced serum T3 and T4 in rodent models (see for example: Cooper et al., 1983; Hood et al., 1999; Goldey et al., 2005; Gilbert, 2011). The relationship between maternal and fetal levels of hormone following chemically-induced TPO inhibiton has not been well characterized and may differ based on kinetics. Reductions in serum TH in the fetus, in rat and human is derived a chemical's effect on the maternal thyroid gland as well as the fetal thyroid gland.

Quantitative Understanding of the Linkage

Hassan et al. (2017, 2020) and Handa et al. (2021) worked out a quantitative model to predict serum T4 levels based on TPO inhibition in rats.

Response-response relationship

The indirect linkage between exposure to known TPO inhibitors and decreased serum TH has not been defined quantitatively. The two key event relationships that mediate this relationship (TPO inhibition leading to decreased TH synthesis, and decreased TH synthesis leading to decreased serum TH) have been incorporated into some quantitative models. A quantitative biologically-based dose-response model for iodine deficiency in the rat includes relationships between thyroidal T4 synthesis and serum T4 concentrations in developing rats Fisher et al. (2013). Ekerot et al. (2012) modeled TPO, T3, T4 and TSH in dogs and humans based on exposure to myeloperoxidase inhibitors that also inhibit TPO and was has recently adapted for rat (Leonard et al., 2016). While the original model predicted serum TH and TSH levels as a function of oral dose, it was not used to explicitly predict the relationship between serum hormones and TPO inhibition, or thyroidal hormone synthesis. Leonard et al. (2016) recently incorporated TPO inhibition into the model. Degon et al (2008) developed a human thyroid model that includes TPO but does not make quantitative prediction of organification changes due to inhibition of the TPO enzyme.

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