OECD Series on Adverse Outcome Pathways No. 26

Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation

Lucia Vergauwen,
Evelyn Stinckens,
Daniel L. Villeneuve,
Dries Knapen

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OECD

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AOP No. 159 in the AOP-Wiki platform

Foreword

This Adverse Outcome Pathway (AOP) on Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Party of the National Coordinators for the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA).

The AOP has been reviewed for compliance with the AOP development principles following the EAGMST coaching approach. The scientific review was subsequently conducted by the UK National Centre for the 3Rs, following the OECD AOP review principles outlined in the Guidance Document on the scientific review of AOPs. This AOP was endorsed by the WNT and the WPHA on 3 August 2022.

Through endorsement of this AOP, the WNT and the WPHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The OECD's Chemicals and Biotechnology Committee agreed to declassification of this AOP on 4 November 2022.

This document is being published under the responsibility of the OECD's Chemicals and Biotechnology Committee.

The outcome of the compliance check and of the scientific review are publicly available respectively in the AOP Wiki and the eAOP Portal of the AOP Knowledge Base at the following links: [internal review] [scientific review report].

Authors:

Lucia Vergauwen [1], [lucia.vergauwen@uantwerpen.be]
Evelyn Stinckens [1], [evelyn.stinckens@uantwerpen.be]
Dan Villeneuve [2], [villeneuve.dan@epa.gov]
Dries Knapen [1], [dries.knapen@uantwerpen.be] [1]

[1] Zebrafishlab, Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
[2] United States Environmental Protection Agency, Mid-Continent Ecology Division, 6201 Congdon Blvd, Duluth, MN, USA

Abstract

This AOP describes the sequence of events leading from thyroperoxidase inhibition to increased mortality via reduced anterior swim bladder inflation. The enzyme thyroperoxidase (TPO) is essential for the synthesis of thyroxine (T4) and triiodothyronine (T3) in the thyroid follicles. Inhibition of TPO reduces thyroid hormone (TH) levels. Thyroid hormones are critical in regulating developmental processes and thyroid hormone disruption can interfere with normal development. Swim bladder inflation is known to be under TH control (Brown et al., 1988; Liu and Chan, 2002). Many fish species have a swim bladder which is a gas-filled organ that typically consists of two chambers (Robertson et al., 2007). The posterior chamber inflates during early development in the embryonic phase, while the anterior chamber inflates during late development in the larval phase. Both the posterior and the anterior chamber have an important role in regulating buoyancy, and the anterior chamber has an additional role in hearing (Robertson et al., 2007).

This AOP describes how inhibition of TPO results in decreased synthesis of THs in the thyroid follicles. This reduces the availability of T4 for conversion to the more biologically active T3. Reduced T3 levels prohibit normal inflation of the anterior swim bladder chamber. Due to its role in regulating buoyancy, this results in reduced swimming performance. Since reduced swimming performance results in a decreased ability to forage and avoid predators, this reduces chances of survival. The final adverse outcome is a decrease of the population growth rate. Since many AOPs eventually lead to this more general adverse outcome at the population level, the more specific and informative adverse outcome at the organismal level, increased mortality, is used in the AOP title. Support for this AOP is mainly based on chemical exposures in zebrafish and fathead minnows (Nelson et al., 2016; Godfrey et al., 2017; Stinckens et al., 2016, 2020). Additional evidence of a link between reduced TH synthesis and reduced anterior chamber inflation is available from a study where a mutation was inserted in the gene coding for dual oxidase, another enzyme that is important for TH synthesis since it provides hydrogen peroxide for iodide oxidation (Chopra et al., 2019).

This AOP is part of a larger AOP network describing how decreased synthesis and/or decreased biological activation of THs leads to incomplete or improper inflation of the swim bladder, leading to reduced swimming performance, increased mortality and decreased population trajectory (Knapen et al., 2018; Knapen et al., 2020; Villeneuve et al., 2018). Apart from the upstream part, the current AOP is identical to the corresponding AOPs leading from DIO1 and DIO2 inhibition to increased mortality via anterior swim bladder inflation (https://aopwiki.org/aops/156, https://aopwiki.org/aops/158).

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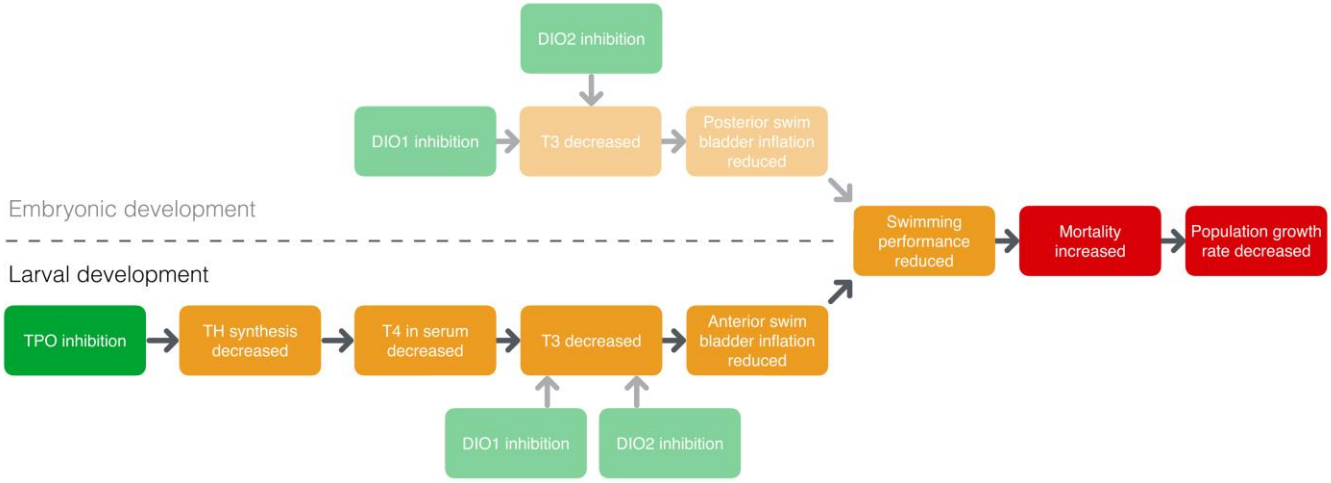
Background

The larger AOP network describing the effect of deiodinase and thyroperoxidase inhibition on swim bladder inflation consists of 5 AOPs:

- Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation: https://aopwiki.org/aops/155
- Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation: https://aopwiki.org/aops/156
- Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation : https://aopwiki.org/aops/157
- Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation : https://aopwiki.org/aops/158
- Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation: https://aopwiki.org/aops/159

The development of these AOPs was mainly based on a series of dedicated experiments (using a set of reference chemicals as prototypical stressors) in zebrafish and fathead minnow that form the core of the empirical evidence. Specific literature searches were used to add evidence from other studies, mainly in zebrafish and fathead minnow. No systematic review approach was applied.

Graphical Representation



Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	279	Thyroperoxidase, Inhibition	Thyroperoxidase, Inhibition
2	KE	277	Thyroid hormone synthesis, Decreased	TH synthesis, Decreased
3	KE	281	Thyroxine (T4) in serum, Decreased	T4 in serum, Decreased
4	KE	1003	Decreased, Triiodothyronine (T3)	Decreased, Triiodothyronine (T3)
5	KE	1007	Reduced, Anterior swim bladder inflation	Reduced, Anterior swim bladder inflation
6	KE	1005	Reduced, Swimming performance	Reduced, Swimming performance
7	AO	351	Increased Mortality	Increased Mortality
8	AO	360	Decrease, Population growth rate	Decrease, Population growth rate

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Thyroperoxidase, Inhibition	adjacent	Thyroid hormone synthesis, Decreased	High	Low
Thyroid hormone synthesis, Decreased	adjacent	Thyroxine (T4) in serum, Decreased	Moderate	Low
Thyroxine (T4) in serum, Decreased	adjacent	Decreased, Triiodothyronine (T3)	Moderate	Moderate
Decreased, Triiodothyronine (T3)	adjacent	Reduced, Anterior swim bladder inflation	Moderate	Moderate
Reduced, Anterior swim bladder inflation	adjacent	Reduced, Swimming performance	Moderate	Low
Reduced, Swimming performance	adjacent	Increased Mortality	Moderate	Low
Increased Mortality	adjacent	Decrease, Population growth rate	Moderate	Moderate
Thyroperoxidase,	Non-adjacent	Thyroxine (T4) in	High	Low

Inhibition		serum, Decreased		
Thyroxine (T4) in serum, Decreased	Non-adjacent	Reduced, Anterior swim bladder inflation	Moderate	Moderate

Stressors

Name	Evidence
Methimazole	High
Mercaptobenzothiazole	High
Propylthiouracil	High

Overall Assessment of the AOP

The document in Annex 1 includes:

- Support for biological plausibility of KERs
- Support for essentiality of KEs
- Empirical support for KERs
- Dose and temporal concordance table covering the larger AOP network

Overall, the weight of evidence for the sequence of key events laid out in the AOP is moderate to high. Nonetheless, the exact underlying mechanism of TH disruption leading to impaired swim bladder inflation is not understood.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Life stage: The current AOP is applicable to the larval life stage, the period in which the anterior chamber of the swim bladder inflates (21 days post fertilization in zebrafish).

Taxonomic: Organogenesis of the swim bladder begins with an evagination from the gut. In physostomous fish, a connection between the swim bladder and the gut is retained. In physoclystous fish, once initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Woolley and Qin, 2010). This AOP is currently mainly based on experimental evidence from studies on zebrafish and fathead minnows, physostomous fish with a two-chambered swim bladder. This AOP is not applicable to fish that do not have a second swim bladder chamber that inflates during larval development, e.g., the Japanese rice fish or medaka (*Oryzias latipes*).

Sex: All key events in this AOP are plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear

whether sex-related differences are important in determining the magnitude of the changes across the sequence of events in this AOP. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). 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. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role in the current AOP. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

Essentiality of the Key Events

Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation from a study where a similar MIE was targeted: Chopra et al. (2019) showed that knockdown of dual oxidase, another enzyme that is important for TH synthesis since it provides hydrogen peroxide for iodide oxidation, reduced anterior swim bladder inflation. It should be noted that dual oxidase also plays a role in oxidative stress. Additionally, there is indirect evidence from deiodinase knockdowns supporting the downstream part of the AOP linking decreased T3 levels to reduced swim bladder inflation (targeted at posterior chamber inflation, not specifically at anterior chamber inflation, see AOPs 155-158). There is also evidence that alleviation of the effect on anterior chamber inflation reduces the effect on swimming performance.

Weight of Evidence Summary

Biological plausibility: see Table. Overall, the weight of evidence for the biological plausibility of the KERs in the AOP is moderate since there is empirical support for an association between the sets of KEs and the KERs are plausible based on analogy to accepted biological relationships, but scientific understanding is not completely established.

Empirical support: see Table. Overall, the empirical support for the KERs in the AOP is moderate since dependent changes in sets of KEs following exposure to several specific stressors has been demonstrated, with limited evidence for dose and temporal concordance and some uncertainties.

Quantitative Consideration

There is some level of quantitative understanding that can form the basis for development of a quantitative AOP. Quantitative relationships between reduced T4 and reduced T3, and between reduced T3 and reduced anterior chamber inflation were established. The latter is particularly critical for linking impaired swim bladder inflation to TH disruption.

Considerations for Potential Applications of the AOP

A growing number of environmental pollutants are known to adversely affect the thyroid hormone system, and major gaps have been identified in the tools available for the identification, and the hazard and risk assessment of these thyroid hormone disrupting chemicals. Villeneuve et al. (2014) discussed the relevance of swim bladder inflation as a potential key event and endpoint of interest in fish tests. Knapen et al. (2020) provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. While the AOP is only applicable to fish, some of the upstream KEs are relevant across vertebrates. The taxonomic domain of applicability call of the KEs can be found on the respective pages. A suite of assays covering all the essential biological processes involved in the underlying toxicological pathways can be implemented in a tiered screening and testing approach for thyroid hormone disruption in fish, using the levels of assessment of the OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals as a guide. Specifically, for this AOP, thyroperoxidase inhibition can be assessed using an in chemico assay, measurements of T4 and T3 levels could be added to the Fish Embryo Acute Toxicity (FET) test (OECD TG 236), the Fish Early Life Stage Toxicity (FELS) Test (OECD TG210) and the Fish Sexual Development Test (FSDT), and assessments of anterior chamber inflation and swimming performance could be added to the FELS Test and FSDT.

Thyroid hormone system disruption causes multiple unspecific effects. Addition of TH measurements could aid in increasing the diagnostic capacity of a battery of endpoints since they are specific to the TH system. A battery of endpoints would ideally include the MIE, the AO and TH levels as the causal link. It is also in this philosophy that TH measurements are currently being considered as one of the endpoints in project 2.64 of the OECD TG work plan, "Inclusion of thyroid endpoints in OECD fish Test Guidelines". While thyroid hormone measurements showed low levels of variation and were highly predictive of downstream effects in dedicated experiments to support this AOP, more variability may be present in other studies. Because of the rapid development in fish, it is important to compare thyroid hormone levels within specific developmental stages. For example, clear changes in thyroid hormone levels have been observed in zebrafish at 5, 14, 21 and 32 dpf (Stinckens et al., 2016; Stinckens et al., 2020) and in fathead minnows at 4, 6, 10, 14, 18 and 21 dpf (Nelson et al., 2016; Cavallin et al., 2017) using liquid chromatography tandem mass spectrometry (LC−MS/MS).

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Appendix 1 - MIE, KEs and AO

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 Name	Role of event in AOP
TPO Inhibition and Altered Neurodevelopment	Molecular Initiating Event
Thyroid peroxidase- follicular adenoma/carcinoma	Molecular Initiating Event
TPOi anterior swim bladder	Molecular Initiating Event
TPO inhib alters metamorphosis	Molecular Initiating Event
TPO inhibition and impaired fertility	Molecular Initiating Event
TPOi retinal layer structure	Molecular Initiating Event
TPOi eye size	MolecularInitiatingEvent
TPOi photoreceptor patterning	MolecularInitiatingEvent

Stressors

Name
2(3H)-Benzothiazolethione
2-mercaptobenzothiazole
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 methimazole (MMI) and 6-propylthiouracil (PTU, also a deiodinase inhibitor), 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	Link
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
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

Term	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 monoiodotyrosine (MIT) and diiodotyrosine (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 deiodinases is not expected to be sex-specific.

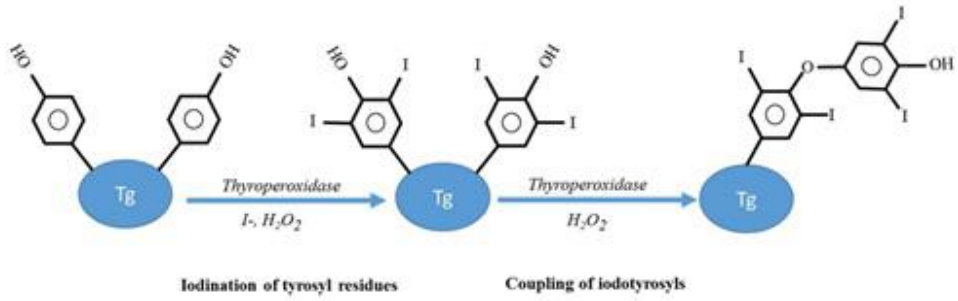
Key Event Description

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). Ususally just the first step of this series of events is covered by assays that measure TPO inhibition. Therefore, in the context of this AOP we are using TPO inhibition 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 (isoflavones such as genistein) 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). Recombinant sources of TPO have also been used (e.g. Schmutzler et al., 2007; Dong et al., 2020)

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

AOP ID and Name	Event Type
Aop:110 - Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	Key Event

Stressors

Name
Propylthiouracil
Methimazole

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 Graves' 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 the transport protein 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). Due to the maternal factor, the life stage specific impact of TPO inhibition after exposure to environmental chemicals is complex (Ramhoj et al., 2022).

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	Key Event
Aop:54 - Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment	Key Event
Aop:8 - Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	Key Event
Aop:65 - XX Inhibition of Sodium Iodide Symporter and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	Key Event
Aop:134 - Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals	Key Event
Aop:152 - Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity	Key Event
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:175 - Thyroperoxidase inhibition leading to altered amphibian metamorphosis	Key Event
Aop:176 - Sodium Iodide Symporter (NIS) Inhibition leading to altered amphibian metamorphosis	Key Event
Aop:194 - Hepatic nuclear receptor activation leading to altered amphibian metamorphosis	Key Event
Aop:366 - Competitive binding to thyroid hormone carrier protein transthyretin (TTR) leading to altered amphibian metamorphosis	Key Event
Aop:367 - Competitive binding to thyroid hormone carrier protein thyroid binding globulin (TBG) leading to altered amphibian metamorphosis	Key Event
Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure	Key Event
Aop:364 - Thyroperoxidase inhibition leading to increased mortality via decreased eye size	Key Event
Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning	Key Event
Aop:119 - Inhibition of thyroid peroxidase leading to follicular cell adenomas and	Key Event

AOP ID and Name	Event Type
carcinomas (in rat and mouse)	
Aop:110 - Inhibition of iodide pump activity leading to follicular cell adenomas and carcinomas (in rat and mouse)	Key Event
Aop:162 - Enhanced hepatic clearance of thyroid hormones leading to thyroid follicular cell adenomas and carcinomas in the rat and mouse	Key Event

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

Term	Scientific Term	Evidence	Links
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, reverse T3 (rT3), and 3,3'-Diiodothyronine (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) which 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. 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)

Short Name: Decreased, Triiodothyronine (T3)

Key Event Component

Process	Object	Action
decreased triiodothyronine 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	Key Event
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Key Event
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:189 - Type I iodothyronine deiodinase (DIO1) inhibition leading to altered amphibian metamorphosis	Key Event
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure	Key Event
Aop:364 - Thyroperoxidase inhibition leading to increased mortality via decreased eye size	Key Event
Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning	Key Event

Biological Context

Level of Biological Organization
Tissue

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 T3 and T4 that are mostly bound to transport proteins in blood as well as T3 and T4 in tissues. 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 come from rat studies and have been measured mostly in serum. 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 decreased THs is still regarded as a measurable key event causatively linked to downstream adverse outcomes.

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). Such measurements in fish early life stages are usually based on whole animal samples and do not allow for distinguishing between systemic and tissue TH alterations.

THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in amphibian and lamprey 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 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: THs are essential in all life stages, but decreases of TH levels are not applicable to all developmental phases. 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. THs 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 TH 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 TH 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 and T3 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 free 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 storage which ensures 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). Initially, 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). Four types of thyroid hormone signaling have been defined (Anyetei-Anum et al., 2018): type 1 is the canonical pathway in which liganded TR binds directly to DNA; type 2 describes liganded TR tethered to chromatin-associated proteins, but not bound to DNA directly; type 3 suggests that liganded TR can exert its function without recruitment to chromatin in either the nucleus or cytoplasm; and type 4 proposes that thyroid hormone acts at the plasma membrane or in the cytoplasm without binding TR, a mechanism of action that is emerging as a key component of thyroid hormone signaling.

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 regulate the production of thyroid hormones. Less T3 (the biologically more active TH) than T4 is produced by the thyroid gland. 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 to date. The monocarboxylate transporters (Mct8, Mct10) and the anion-transporting polypeptide (OATP1c1) show the highest degree of affinity towards TH (Jansen et al., 2005) and mutations in these genes have pathophysiological effects in humans (Bernal et al., 2015). Unlike humans with an MCT8 deficiency, MCT8 knockout mice do not have neurological impairment. One explanation for this discrepancy could be differences in expression of the T4 transporter OATP1C1 in the blood–brain barrier. This shows that cross-species differences in the importance of specific transporters may occur.

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) in serum, or in tissues. 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 TH synthesis comes from measurements of whole-body TH levels and using LC-MS techniques (Hornung et al., 2015) are increasingly used to accurately quantify whole-body TH levels (Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020).

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Event: 1007: Reduced, Anterior swim bladder inflation

Short Name: Reduced, Anterior swim bladder inflation

Key Event Component

Process	Object	Action
swim bladder inflation	anterior chamber swim bladder	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
swim bladder

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Teleost fish can be divided in two groups according to swim bladder morphology: physoclistous (e.g., yellow perch, sea bass, striped bass) and physostomous (e.g., zebrafish and fathead minnow). Physostomous fish retain a duct between the digestive tract and the swim bladder during adulthood allowing them to gulp air at the surface to fill the swim bladder. In contrast, in physoclistous fish, once initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Woolley and Qin, 2010). The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2016; Nelson et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020). While zebrafish and fathead minnows are physostomous fish with a two-chambered swim bladder, the Japanese rice fish or medaka (*Oryzias latipes*) is a physoclistous fish with a single chambered swim bladder that inflates during early development. The key event 'reduced anterior chamber inflation' is not applicable to such fish species. Therefore, the current key event is plausibly applicable to physostomous fish in general.

Life stage: The anterior chamber inflates during a specific developmental time frame. In zebrafish, the anterior chamber inflates around 21 days post fertilization (dpf) which is during the larval stage. In the fathead minnow, the anterior chamber inflates around 14 dpf, also during the larval stage. Therefore this KE is only applicable to the larval life stage.

Sex: This KE plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). 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. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current KE.

Key Event Description

The swim bladder of bony fish is evolutionary homologous to the lung (Zheng et al., 2011). The teleost swim bladder is a gas-filled structure that consists of two chambers, the posterior and anterior chamber. In zebrafish, the posterior chamber inflates around 96 h post fertilization (hpf) which is 2 days post hatch, and the anterior chamber inflates around 21 dpf. In fathead minnow, the posterior and anterior chamber inflate around 6 and 14 dpf respectively. Inflation of the anterior swim bladder chamber is part of the larval-to-juvenile transition in fish, together with the development of adult fins and fin rays, ossification of the axial

skeleton, formation of an adult pigmentation pattern, scale formation, maturation and remodeling of organs including the lateral line, nervous system, gut and kidneys (McMenamin and Parichy, 2013).

The anterior chamber is formed by evagination from the cranial end of the posterior chamber (Robertson et al., 2007). Dumbarton et al. (2010) showed that the anterior chamber of zebrafish has particularly closely packed and highly organized bundles of muscle fibres, suggesting that contraction of these muscles would reduce swim bladder volume. While it had previously been suggested that the posterior chamber had a more important role as a hydrostatic organ, this implies high importance of the anterior chamber for buoyancy. The anterior chamber has an additional role in hearing (Bang et al., 2002). Weberian ossicles (the Weberian apparatus) connect the anterior chamber to the inner ear resulting in an amplification of sound waves. Reduced inflation of the anterior chamber may manifest itself as either a complete failure to inflate the chamber or reduced size of the chamber. Reduced size is often associated with a deviating morphology.

How it is Measured or Detected

In several fish species, inflation of the anterior chamber can be observed using a stereomicroscope because the larvae are still transparent during the larval stage. This is for example true for zebrafish and fathead minnow. Anterior chamber size can then be measured based on photographs with a calibrator.

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Event: 1005: Reduced, Swimming performance

Short Name: Reduced, Swimming performance

Key Event Component

Process	Object	Action
aquatic locomotion		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	Key Event
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Key Event
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Key Event
Aop:242 - Inhibition of lysyl oxidase leading to enhanced chronic fish toxicity	Key Event
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	Key Event

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	Moderate
Juvenile	Moderate
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Importance of swimming performance for natural behaviour is generally applicable to fish and tho other taxa that rely on swimming to support vital behaviours.

Life stage: Importance of swimming performance for natural behaviour is generally applicable across all free-swimming life stages, i.e., post-embryonic life stages.

Sex: Importance of swimming performance for natural behaviour is generally applicable across sexes.

Key Event Description

Adequate swimming performance in fish is essential for behaviour such as foraging, predator avoidance and reproduction.

How it is Measured or Detected

For fish larvae, automated observation and tracking systems are commercially available and increasingly used for measuring swimming performance including distance travelled, duration of movements, swimming speed, etc. This kind of measurements is often included in publications describing effects of chemicals in zebrafish larvae (Hagenaars et al., 2014; Stinckens et al., 2016; Vergauwen et al., 2015).

For juvenile and adult fish, measurements of swim performance vary. However, in some circumstances, swim tunnels have been used to measure various data (Fu et al., 2013).

Little and Finger (1990) discussed swimming behavior as an indicator of sublethal toxicity in fish.

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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

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	Adverse Outcome
Aop:96 - Axonal sodium channel modulation leading to acute mortality	Adverse Outcome
Aop:104 - Altered ion channel activity leading impaired heart function	Adverse Outcome
Aop:113 - Glutamate-gated chloride channel activation leading to acute mortality	Adverse Outcome
Aop:160 - Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality	Adverse Outcome
Aop:161 - Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality	Adverse Outcome
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	Adverse Outcome
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	Adverse Outcome
Aop:186 - unknown MIE leading to renal failure and mortality	Adverse Outcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	Adverse Outcome
Aop:320 - Binding of viral S-glycoprotein to ACE2 receptor leading to acute respiratory distress associated mortality	Adverse Outcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure	Adverse Outcome
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading	Adverse

AOP ID and Name	Event Type
to Acute Respiratory Distress Syndrome (ARDS) and Multiple Organ Dysfunction (MOD)	Outcome
Aop:364 - Thyroperoxidase inhibition leading to increased mortality via decreased eye size	Adverse Outcome
Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning	Adverse Outcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	Adverse Outcome
Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure	Adverse Outcome
Aop:410 - Repression of Gbx2 expression leads to defects in developing inner ear and consequently to increased mortality	Key Event

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 growth rate

Short Name: Decrease, Population growth rate

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)	Adverse Outcome
Aop:25 - Aromatase inhibition leading to reproductive dysfunction	Adverse Outcome
Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction	Adverse Outcome
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	Adverse Outcome
Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior	Adverse Outcome
Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	Adverse Outcome
Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	Adverse Outcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release	Adverse Outcome
Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I /metaphase I transition	Adverse Outcome
Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction	Adverse Outcome
Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint	Adverse Outcome
Aop:292 - Inhibition of tyrosinase leads to decreased population in fish	Adverse Outcome
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	Adverse Outcome
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	Adverse Outcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	Adverse Outcome
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	Adverse Outcome
Aop:336 - DNA methyltransferase inhibition leading to population decline (1)	Adverse Outcome
Aop:337 - DNA methyltransferase inhibition leading to population decline (2)	Adverse Outcome

AOP ID and Name	Event Type
Aop:338 - DNA methyltransferase inhibition leading to population decline (3)	Adverse Outcome
Aop:339 - DNA methyltransferase inhibition leading to population decline (4)	Adverse Outcome
Aop:340 - DNA methyltransferase inhibition leading to transgenerational effects (1)	Adverse Outcome
Aop:341 - DNA methyltransferase inhibition leading to transgenerational effects (2)	Adverse Outcome
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	Adverse Outcome
Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline	Adverse Outcome
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	Adverse Outcome
Aop:299 - Excessive reactive oxygen species production leading to population decline via reduced fatty acid beta-oxidation	Adverse Outcome
Aop:311 - Excessive reactive oxygen species production leading to population decline via mitochondrial dysfunction	Adverse Outcome
Aop:216 - Excessive reactive oxygen species production leading to population decline via follicular atresia	Adverse Outcome
Aop:238 - Excessive reactive oxygen species production leading to population decline via lipid peroxidation	Adverse Outcome
Aop:326 - Thermal stress leading to population decline (3)	Adverse Outcome
Aop:325 - Thermal stress leading to population decline (2)	Adverse Outcome
Aop:324 - Thermal stress leading to population decline (1)	Adverse Outcome
Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure	Adverse Outcome
Aop:349 - Inhibition of 11β-hydroxylase leading to decresed population trajectory	Adverse Outcome
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory	Adverse Outcome
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	Adverse Outcome
Aop:386 - Increased reactive oxygen species production leading to population decline via inhibition of photosynthesis	Adverse Outcome
Aop:387 - Increased reactive oxygen species production leading to population decline via mitochondrial dysfunction	Adverse Outcome
Aop:388 - DNA damage leading to population decline via programmed cell death	Adverse Outcome
Aop:389 - Oxygen-evolving complex damage leading to population decline via inhibition of photosynthesis	Adverse Outcome
Aop:364 - Thyroperoxidase inhibition leading to increased mortality via decreased eye size	Adverse Outcome
Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning	Adverse Outcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	Adverse Outcome

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.

References

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
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 increased mortality via altered retinal layer structure	adjacent	High	Low
Thyroperoxidase inhibition leading to increased mortality via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to increased mortality 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 I125, 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.

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.

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
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 increased mortality via altered retinal layer structure	adjacent	Moderate	Low
Thyroperoxidase inhibition leading to increased mortality via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to increased mortality 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		

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 More recently, modeling of TH in the rat fetus demonstrates the quantitative relationship between TH synthesis and serum T4 concentrations (Hassan et al., 2017). 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.

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%.

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)

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 increased mortality via altered retinal layer structure	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to increased mortality via decreased eye size	adjacent		
Thyroperoxidase inhibition leading to increased mortality 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 reported 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.

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

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).
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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: 1035: Decreased, Triiodothyronine (T3) leads to Reduced, Anterior swim bladder inflation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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 anterior swim bladder inflation	adjacent	Moderate	Moderate
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Teleost fish can be divided in two groups according to swim bladder morphology: physoclistous (e.g., yellow perch, sea bass, striped bass, medaka) and physostomous (e.g., zebrafish and fathead minnow). Physostomous fish retain a duct between the digestive tract and the swim bladder during adulthood allowing them to gulp air at the surface to fill the swim bladder. In contrast, in physoclistous fish, once initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Woolley and Qin, 2010). The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2016; Nelson et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020). While zebrafish and fathead minnows are physostomous fish with a two-chambered swim bladder, the Japanese rice fish or medaka (*Oryzias latipes*) is a physoclistous fish with a single chambered swim bladder that inflates during early development. This KER is not applicable to such fish species. Therefore, the current key event is plausibly applicable to physostomous fish in general.

Life stage: The anterior chamber inflates during a specific developmental time frame. In zebrafish, the anterior chamber inflates around 21 days post fertilization (dpf) which is during the larval stage. In the fathead minnow, the anterior chamber inflates around 14 dpf, also during the larval stage. Therefore this KER is only applicable to the larval life stage.

Sex: This KER plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KER. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). 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. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current KER.

Key Event Relationship Description

Thyroid hormones are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition and larval-to-juvenile transition, including anterior chamber inflation in fish. Reduced T3 levels prohibit local TH action in the target tissues. Since swim bladder development and/or inflation is regulated by thyroid hormones, this results in impaired anterior chamber inflation.

Evidence Supporting this KER

There is convincing evidence that decreased T3 levels result in impaired anterior chamber inflation, but the underlying mechanisms are not completely understood. A very convincing linear quantitative relationship between reduced T3 levels and reduced anterior chamber volume was shown in zebrafish across exposure to a limited set of three compounds. Therefore the evidence supporting this KER can be considered moderate.

Biological Plausibility

Thyroid hormones are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition (Liu and Chan, 2002) and larval-to-juvenile transition (Brown et al., 1997) in fish. Inflation of the anterior swim bladder chamber is part of the larval-to-juvenile transition in fish, together with the development of adult fins and fin rays, ossification of the axial skeleton, formation of an adult pigmentation pattern, scale formation, maturation and remodeling of organs including the lateral line, nervous system, gut and kidneys (Brown, 1997; Liu and Chan, 2002; McMenamin and Parichy, 2013).

Empirical Evidence

Dedicated studies with two different experimental setups have been conducted to investigate the link between reduced T3 levels and reduced anterior chamber inflation:

1. Studies applying larval exposures initiated after posterior chamber inflation

- In a study in which larval fathead minnows (*Pimephales promelas*) were exposed to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole (MBT), T3 concentrations measured at 14dpf were reduced at the same concentration (1 mg/L) that significantly reduced anterior swim bladder inflation at the same time-point (Nelson et al. 2016).
- In the study of Cavallin et al. (2017) fathead minnow larvae were exposed to IOP, a model iodothyronine deiodinase inhibitor that is assumed to inhibit all three deiodinase enzymes (DIO1,2,3). The authors observed pronounced decreases of whole body T3 concentrations and increases of whole body T4 concentrations, together with impaired inflation of the anterior swim bladder chamber. More specifically, inflation was delayed and the size of the swim bladder chamber was reduced until the end of the exposure experiment.

Since exposures were started after inflation of the posterior chamber, these studies show that DIO inhibition can directly affect anterior chamber inflation.

2. Studies applying continuous exposure initiated immediately after fertilization and thus including both posterior and anterior chamber inflation

- In the study of Stinckens et al. (2020) exposure concentrations were chosen where the posterior chamber inflates. A strong correlation between reduced T3 levels and reduced anterior chamber inflation was observed in zebrafish exposed to iopanoic acid, a deiodinase inhibitor, as well as methimazole and propylthiouracil, both thyroperoxidase inhibitors, from fertilization until the age of 32 days. Anterior chamber inflation was delayed and a number of larvae did not manage to inflate the anterior chamber by the end of the 32 day exposure period. Additionally, exposed fish that had inflated the swim bladder had reduced anterior chamber sizes.

Uncertainties and Inconsistencies

- Since in fish early life stages THs are typically measured on a whole-body level, it is currently uncertain whether TH levels changes occur at the serum and/or tissue level.
- The mechanism underlying the link between reduced T3 and reduced anterior chamber inflation remains unclear, but several hypotheses exist (Stinckens et al., 2020). For example, altered gas distribution between chambers could be the result of impaired development of smooth muscle fibers, delayed and/or impaired evagination of the anterior chamber, impaired anterior budding through altered Wnt and hedgehog signalling, etc. Reinwald et al. (2021) showed that T3 and propylthiouracil treatment of zebrafish embryos altered expression of genes involved in muscle contraction and functioning in an opposing fashion. The authors suggested impaired muscle function as an additional key event between decreased T3 levels and reduced swim bladder inflation.
- Increased T3 levels also seem to result in reduced swim bladder inflation. For example, Li et al. (2011) reported impairment of swim bladder inflation in Chinese rare minnows (Gobiocypris rarus) exposed to exogenous T3.

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Relationship: 1034: Reduced, Anterior swim bladder inflation leads to Reduced, Swimming performance

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low
Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Importance of proper functioning of the swim bladder for supporting natural swimming behaviour can be plausibly assumed to be generally applicable to fish possessing an anterior chamber. Evidence exists for the role of the posterior chamber in swimming performance comes from a wide variety of freshwater and marine fish species. Evidence for the specific role of the anterior chamber is however less abundant.

Life stage: In zebrafish, the anterior chamber inflates around 21 days post fertilization (dpf) which is during the larval stage. In the fathead minnow, the anterior chamber inflates around 14 dpf, also during the larval stage. Therefore this KER is only applicable to the larval life stage. To what extent fish can survive and swim with partly inflated swim bladders during later life stages is unknown.

Sex: This KER plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KER. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). 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. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current KER.

Key Event Relationship Description

Effects on swim bladder inflation can alter swimming performance and buoyancy of fish, which is essential for predator avoidance, energy sparing, migration, reproduction and feeding behaviour, resulting in increased mortality.

Evidence Supporting this KER

The weight of evidence supporting a direct linkage between these two KEs, i.e. reduced anterior swim bladder inflation and reduced swimming performance, is weak.

Biological Plausibility

The anterior chamber of the swim bladder has a function in regulating the buoyancy of fish, by altering the volume of the swim bladder (Roberston et al., 2007). Fish rely on the lipid and gas content in their body to regulate their position within the water column, with the latter being more efficient at increasing body buoyancy. Therefore, fish with functional swim bladders have no problem supporting their body (Brix 2002), while it is highly likely that impaired inflation severely impacts swimming performance. Fish with no functional swim bladder can survive, but are severely disadvantaged, making the likelihood of surviving smaller.

Several studies in zebrafish and fathead minnow showed that a smaller AC was associated with a larger posterior chamber (Nelson et al., 2016; Stinckens et al., 2016; Cavallin et al., 2017, Stinckens et al., 2020) suggesting a possible compensatory mechanism. As shown by Stoyek et al. (2011) however, the AC volume is highly dynamic under normal conditions due to a series of regular corrugations running along the chamber wall, and is in fact the main driver for adjusting buoyancy while the basic PC volume remains largely invariable. Therefore, it is plausible to assume that functionality of the swim bladder is affected when AC inflation is incomplete, even when the PC appears to fully compensate the gas volume of the swim bladder.

Empirical Evidence

- Lindsey et al. (2010) showed that zebrafish started swimming deeper down in the water column upon inflation of the anterior chamber, confirming a role of the anterior chamber in supporting swimming performance.
- After exposure to 2-mercaptobenzothiazole, a TPO inhibitor, from 0 to 32 days post fertilization (dpf) in zebrafish, the swimming activity of fish was impacted starting at 26 dpf if the inflation of the anterior chamber of the swim bladder was impaired or had no normal structure/size (Stinckens et al., 2016).
- Methimazole (MMI) and propylthiouracil (PTU), two thyroperoxidase inhibitors, and iopanoic acid (IOP), a deiodinase inhibitor, each reduced both anterior chamber inflation and swimming distance in zebrafish exposed from fertlization until the age of 32 days (Stinckens et al., 2020). Stinckens et al. (2020) showed a specific, direct link between reduced anterior chamber inflation and reduced swimming performance.
    - First, after 21 d of exposure to 111 mg/L propylthiouracil around 30% of anterior chambers were not inflated and swimming distance was reduced, while by 32 days post fertilization all larvae had inflated their anterior chamber (although chamber surface was still smaller) and the effect on swimming distance had disappeared.
    - The most direct way to assess the role of anterior chamber inflation in swimming performance, however, is to compare larvae with and without inflated anterior chamber at the same time point and within the same experimental treatment. Both in the propylthiouracil exposure at 21 days post fertilization and in the iopanoic acid exposure at 21 and 32 days post fertilization, swimming distance was clearly reduced in larvae lacking an inflated anterior chamber, while the swimming distance of larvae with inflated anterior chamber was equal to that of controls.
    - Exposure concentrations were selected where the posterior chamber inflates. Even though the posterior chamber was generally larger when anterior chamber inflation was reduced, this did not remove the effect on swimming performance, confirming a direct link between proper anterior chamber inflation and swimming performance.
    - No morphological effects were observed, but in some treatments reduced length and/or condition factor was observed. However, reduced swimming performance after 32 days of IOP exposure to medium concentrations was not accompanied by reduced length or condition factor. Therefore, at least in this study no evidence was found that the effect on swimming performance was an indirect consequence of effects other than reduced swim bladder inflation.
- It has also been reported that larvae that fail to inflate their swim bladder use additional energy to maintain buoyancy (Lindsey et al., 2010, Goodsell et al. 1996), possibly contributing to reduced swimming activity.

Uncertainties and Inconsistencies

After exposure to 100 mg/L methimazole, 95% of the zebrafish larvae failed to inflate their anterior chamber at 32 dpf and swimming distance was reduced (Stinckens et al., 2020). On the other hand, there was no effect of impaired anterior chamber inflation on swimming distance in the methimazole exposure of 50 mg/L. Also, inflated but smaller anterior chambers did not result in a decreased swimming performance in this study. A similar result, where non-inflated anterior chambers did not consistently lead to reduced swimming performance, was previously found after exposure to 2-mercaptobenzothiazole (Stinckens et al., 2016). In summary, the precise relationship between these two KEs is not easy to determine and may be different for different chemicals. This is in part due to the complexity of the swim bladder system and the difficulty of distinguishing effects resulting from altered anterior chamber inflation from those resulting from altered posterior chamber inflation. Additionally, swimming capacity can be affected via other processes which may

or may not depend on the HPT axis, such as general malformations, decreased cardiorespiratory function, energy metabolism and growth.

As Robertson et al., (2007) reported, the swim bladder only starts regulating buoyancy actively from 32 dpf onward in zebrafish, possibly explaining the lack of effect on swimming capacity in some cases.

The anterior chamber is also important for producing and transducing sound through the Weberian Apparatus (Popper, 1974; Lechner and Ladich, 2008). It is highly plausible that impaired inflation or size of the anterior swim bladder could lead to increased mortality as hearing loss would affect their ability to respond to their surrounding environment, thus impacting ecological relevant endpoints such as predator avoidance or prey seeking (Wisenden et al., 2008; Fay, 2009).

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Relationship: 2212: Reduced, Swimming performance leads to Increased Mortality

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of evidence	Quantitative Understanding
Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	adjacent	Moderate	Low
Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low
Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	adjacent	Moderate	Low
Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate
Juvenile	Moderate
Larvae	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Importance of swimming performance on survival is generally applicable to all hatched fish across life stages and sexes and to other taxa that rely on swimming to support vital behaviours.

Key Event Relationship Description

Reduced swimming performance is likely to affect essential endpoints such as predator avoidance, feeding behaviour and reproduction in taxa that rely on swimming to support these vital behaviours. These parameters are biologicaly plausible to affect survival, especially in a non-laboratory environment where food is scarce and predators are abundant.

Evidence Supporting this KER

A direct relationship between reduced swimming performance and reduced survival is difficult to establish. There is however a lot of indirect evidence linking reduced swim bladder inflation to reduced survival (https://aopwiki.org/relationships/2213), which can be plausibly assumed to be related to reduced swimming performance.

For example, all zebrafish larvae that failed to inflate the posterior chamber after exposure to 2 mg/L iopanoic acid (IOP), died by the age of 9 dpf (Stinckens et al., 2020). Since larvae from the same group that were able to inflate the posterior chamber survived and the test was performed in the laboratory in optimal conditions, it is plausible to assume that the cause of death was the inability to swim and find food due to the failure to inflate the posterior swim bladder chamber.

Biological Plausibility

Reduced swimming performance is likely to affect essential endpoints such as predator avoidance, feeding behaviour and reproduction. These parameters are biologicaly plausible to affect survival, especially in a non-laboratory environment where food is scarce and predators are abundant.

Empirical Evidence

A direct relationship between reduced swimming performance and reduced survival is difficult to establish. There is however a lot of indirect evidence linking reduced swim bladder inflation to reduced survival (see non-adjacent KER 1041), which can be plausibly assumed to be related to reduced swimming performance.

For example, all zebrafish larvae that failed to inflate the posterior chamber after exposure to 2 mg/L iopanoic acid (IOP), died by the age of 9 dpf (Stinckens et al., 2020). Since larvae from the same group that were able to inflate the posterior chamber survived and the test was performed in the laboratory in optimal conditions, it is plausible to assume that the cause of death was the inability to swim and find food due to the failure to inflate the posterior swim bladder chamber.

Uncertainties and Inconsistencies

A direct relationship between reduced swimming performance and reduced survival is difficult to establish in a laboratory environment where food is abundant and there are no predators.

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage is currently limited.

Time-scale

Reduced swimming performance is not expected to immediately lead to mortality. Depending on the extent of the reduction in swimming performance and depending on the cause of death (e.g., starvation due to the inability to find food, being caught by a predator) the lag time may vary.

As an example, Stinckens et al. (2020) found that zebrafish larvae that failed to inflate the swim bladder at 5 dpf and did not manage to inflate it during the days afterwards died by the age of 9 dpf. Since zebrafish initiate exogenous feeding around 5 dpf when the yolk is almost completely depleted, there was a lag period of around 4 days after which reduced feeding resulted in mortality. Obviously, in a laboratory setup there is no increased risk of being caught by a predator.

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Relationship: 2013: Increased Mortality leads to Decrease, Population growth rate

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 linked data from behavioural assays 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. Expsoed 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 decrerase 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 increased mortality via altered retinal layer structure	non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

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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Relationship: 1039: T4 in serum, Decreased leads to Reduced, Anterior swim bladder inflation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Teleost fish can be divided in two groups according to swim bladder morphology: physoclistous (e.g., yellow perch, sea bass, striped bass) and physostomus (e.g., zebrafish and fathead minnow). Physostomus fish retain a duct between the digestive tract and the swim bladder during adulthood allowing them to gulp air at the surface to fill the swim bladder. In contrast, in physoclistous fish, once initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Woolley and Qin, 2010). The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2016; Nelson et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020). While zebrafish and fathead minnows are physostomous fish with a two-chambered swim bladder, the Japanese rice fish (Oryzias latipes) is a physoclistous fish with a single chambered swim bladder that inflates during early development. This KER is not applicable to such fish species. Therefore, the current key event is plausibly applicable to physostomous fish in general.

Life stage: The anterior chamber inflates during a specific developmental time frame. In zebrafish, the anterior chamber inflates around 21 days post fertilization (dpf) which is during the larval stage. In the fathead minnow, the anterior chamber inflates around 14 dpf, also during the larval stage. Therefore this KER is only applicable to the larval life stage.

Sex: This KE/KER plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE/KER. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable

heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). 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. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

Key Event Relationship Description

Reduced T4 levels in serum prohibit local production of active T3 hormone by deiodinases expressed in the target tissues. There is evidence suggesting that anterior swim bladder inflation relies on increased thyroid hormone levels at this specific developmental time point.

Evidence Supporting this KER

There is convincing evidence that decreased T4 levels result in impaired anterior chamber inflation, but the underlying mechanisms are not completely understood. A convincing linear quantitative relationship between reduced T4 levels and reduced anterior chamber volume was shown in zebrafish across exposure to a limited set of three compounds. Therefore the evidence supporting this KER can be considered moderate.

Biological Plausibility

Thyroid hormones are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition (Liu and Chan, 2002) and larval-to-juvenile transition (Brown et al., 1997) in fish. The formation of the anterior chamber coincides with the second transition phase (Winata et al., 2009) and with a peak in T4 synthesis (Chang et al., 2012) suggesting that anterior inflation is under thyroid hormone regulation. Since most of the more biologically active T3 originates from the conversion of T4, decreased circulatory T4 levels are plausibly linked to reduced anterior chamber inflation.

Empirical Evidence

- Chang et al. (2012) observed an increase of whole body T4 concentrations in zebrafish larvae at 21 days post fertilization, corresponding to the timing of anterior swim bladder inflation.
- Nelson et al. (2016) showed reduced whole body T4 levels at 6 days post fertilization and delayed anterior inflation (lower proportion of inflation at 14 dpf compared to controls) after exposure of fathead minnow embryos to 2-mercaptobenzothiazole. All anterior chambers eventually inflated but their size was reduced and morphology deviated.
- Stinckens et al. (2016) showed reduced whole body T4 levels both at 5 (before anterior inflation) and 32 days post fertilization (after anterior inflation) when zebrafish were exposed to 2-mercaptobenzothiazole (a thyroperoxidase inhibitor) from 0 to 32 days post fertilization. A large percentage of MBT-exposed fish had an uninflated anterior swim bladder, although some recovery was observed over time.

- Stinckens et al. (2016) further showed a significant correlation between whole body T4 levels and anterior chamber volume, with reduced T4 levels leading to smaller anterior chambers.
- Stinckens et al. (2020) established a significant correlation between reduced whole body T4 levels and reduced anterior chamber volume in 32 day old zebrafish across two compound exposures. This includes methimazole and propylthiouracil, two inhibitors of TH synthesis. Godfrey et al. (2017) also observed impaored anterior chamber inflation after exposure of zebrafish to Methimazole. Stinckens et al. (2020) continued the follow-up until 32 dpf and observed no recovery.
- Chopra et al. (2019) found that a nonsense mutation of the duox gene, coding for the enzyme dual oxidase, another enzyme that is important for TH synthesis since it provides hydrogen peroxide for iodide oxidation, resulted in decreased intrafollicular T4 levels and impaired anterior chamber inflation until at least 54 dpf in zebrafish. It should be noted that dual oxidase is not only involved in thyroid hormone synthesis, but also in the production of reactive oxygen species (ROS) (Flores et al. 2010; Niethammer et al. 2009). In zebrafish, ROS can also be induced e.g. by copper (Zhou et al. 2016), which has also been shown to impair swim bladder development (Xu et al. 2017). Impaired production of ROS after dual oxidase knockdown may contribute to an impairment of swim bladder development.

Uncertainties and Inconsistencies

Reduced anterior chamber inflation upon disruption of the thyroid hormone system is in most cases, but not always, accompanied by reduced whole body T3 levels. Stinckens et al. (2016) found a consistent relationship between reduced whole body T4 levels, but not T3 levels, and reduced anterior chamber inflation after exposure to 2-mercaptobenzothiazole (MBT). Possibly, local T4 levels in the swim bladder tissue were too low to allow for enough local activation to T3. This relates to the general uncertainty on serum versus tissue TH levels. Alternatively, differences in timing between T3/T4 measurements (at 120hpf and 32dpf), the moment when there is a need for T3 to inflate the swim bladder (unknown but probably in between 120hpf and 32dpf) and the observation of the phenotype (32dpf), could lead to the hypothesis that T3 concentration was reduced in between the two measurements. There is also a possibility that the effect of MBT on anterior chamber inflation is not directly caused by decreased thyroid hormone levels, but rather by another mechanism such as oxidative stress. MBT is known to elevate the production of reactive oxygen species (ROS) levels in fish cells (Zeng et al., 2016). In general, chemicals may have multiple modes of action and effects on autophagy, ROS, cardiac function may impact swim bladder inflation.

The mechanism through which reduced T4 hormone concentrations in serum result in anterior chamber inflation impairment is not yet understood. The anterior chamber is formed by evagination from the cranial end of the posterior chamber (Robertson et al., 2007, Winata et al., 2009). Several hypotheses could explain effects on anterior chamber inflation due to reduced T4 levels:

- Evagination from the posterior chamber could be impaired. Villeneuve et al. (unpublished results) showed that although the anterior bud was present after exposure to a deiodinase 2 inhibitor, the anterior chamber did not inflate.
- The formation of the tissue layers of the anterior swim bladder could be affected, although Villeneuve et al. (unpublished results) observed intact tissue layers of the anterior swim bladder after exposure to a deiodinase 2 inhibitor.
- The anterior chamber is inflated with gas from the posterior chamber through the communicating duct. Impaired gas exchange between the two chambers could be at the basis of impaired anterior inflation. Both Nelson et al. (2016) and Stinckens et al. (2016) found that posterior chambers were larger when anterior chambers were smaller or not inflated at all. The sum of the areas of the posterior and anterior chambers remained constant independent of inflation of the anterior chamber (Stinkens et al., 2016). These results suggest retention of the gas in the posterior chamber.

- Since gas exchange relies on a functional communicating duct between the posterior and anterior chamber, and the communicating duct is known to progressively narrow and eventually close during development, a dysfunctional communicating duct or a closure prior to anterior inflation could inhibit inflation. However, Villeneuve et al. (unpublished results) showed that the communicating duct was anatomically intact and open after exposure to iopanoic acid (a deiodinase 2 inhibitor), still leading to impaired anterior inflation.
- Lactic acid production which is essential for producing gas to fill the swim bladder could be affected, although the observation that the total amount of gas in both chambers is not affected when anterior inflation is impaired seems to contradict this (Stinckens et al., 2016).
- Possibly there is an effect on the production of surfactant, which is crucial to maintain the surface tension necessary for swim bladder inflation.
- Reinwald et al. (2021) showed that T3 and propylthiouracil treatment of zebrafish embryos altered expression of genes involved in muscle contraction and functioning in an opposing fashion. The authors suggested impaired muscle function as an additional key event between decreased T3 levels and reduced swim bladder inflation.

In some cases indirect effects may play a role in the impact of chemical exposure or genetic knockdown/knockout on swim bladder inflation. For example, dual oxidase also plays a role in oxidative stress.

Quantitative Understanding of the Linkage

Stinckens et al. (2016) showed a significant linear quantitative relationship between whole body T4 levels and anterior chamber volume (measured as surface in 2D images) in 32 day old juvenile zebrafish, with reduced T4 levels leading to smaller anterior chambers after continuous exposure to 2-mercaptobenzothiazole, a thyroid hormone synthesis inhibitor.

Stinckens et al. (2020, supplementary information) established a significant linear quantitative relationship between reduced T4 levels and reduced anterior chamber volume (measured as surface in 2D images) in 32 day old juvenile zebrafish across two compound exposures. This includes methimazole and propylthiouracil, two inhibitors of TH synthesis.

Known Feedforward/Feedback loops influencing this KER

Reduced anterior chamber inflation upon disruption of the thyroid hormone system is consistently accompanied by reduced whole body T4 levels when fish are exposed to thyroid hormone synthesis inhibitors. however, when fish are exposed to deiodinase inhibitors, in the absence of feedback processes, stable T4 levels would be expected. Stable T4 levels were indeed observed in 14, 21 and 32 day old zebrafish exposed to iopanoic acid, a deiodinase inhibitor. While Cavallin et al. (2017) found a consistent relationship between reduced whole body T3 levels, and reduced anterior chamber inflation after exposure of fathead minnows to iopanoic acid, they observed increased T4 levels. Possibly, the inhibition of the conversion of T4 to T3 resulted in a compensatory mechanism that increased T4 levels. This was accompanied by increases of deiodinase 2 and 3 mRNA levels, which also indicate a compensatory response to deiodinase inhibition.

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Annex 1: Weight of evidence evaluation table

AOP 159: Thyroperoxidase inhibition leading to increased mortality via anterior swim bladder inflation - Weight of evidence evaluation

	Defining Question	**High (Strong)**	**Moderate**	**Low (Weak)**
1. Support for Biological Plausibility of KERs	Is there a mechanistic relationship between KE_{up} and KE_{down} consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs , but the structural or functional relationship between them is not understood.
Relationship: 309 Thyroperoxidase, Inhibition (KE 279) leads to TH synthesis, Decreased (KE 277)	**High** The role and importance of thyroperoxidase (TPO) in thyroid hormone synthesis across vertebrates is well established. TPO is the only enzyme capable of de novo synthesis of TH. Therefore, inhibition of TPO activity is widely accepted to directly impact TH synthesis.			
Relationship: 305 TH synthesis, Decreased (KE 277) leads to T4 in serum, Decreased (KE 281)	**High** It is commonly accepted that decreased thyroid hormone synthesis leads to decreased serum T4 levels.			
Non-adjacent relationship: 366 Thyroperoxidase, Inhibition (KE 279) leads to T4 in serum, Decreased (KE 281)	**High** The role of thyroperoxidase in the synthesis of thyroid hormones that are then released to the blood is well established.			
Relationship 2038: T4 in serum, Decreased (KE 281) leads to Decreased, Triiodothyronine (T3) in serum (KE 1003)	**Moderate** When serum thyroxine (T4) levels are decreased, less T4 is available for conversion to the more biologically active triiodothyronine (T3). Since in fish early life stages THs are typically measured on a whole body level, it is currently uncertain whether T3 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. While there is empirical support for the association between decreased serum T4 and decreased serum T3 levels in fish, the 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. The role of taxonomic differences in this relationship is currently unclear.			
Relationship 1035: Decreased, Triiodothyronine (T3) in serum (KE 1003) leads to Reduced, Anterior swim bladder inflation (KE 1007)	**Moderate** Thyroid hormones, especially the more biologically active T3, are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition and larval-to-juvenile transition in fish. Inflation of the anterior swim bladder chamber is part of the larval-to-juvenile transition in fish, together with the development of adult fins and fin rays, ossification of the axial skeleton, formation of an adult pigmentation pattern, scale formation, maturation and remodeling of organs including the lateral line, nervous system, gut and kidneys. Together with empirical evidence, it is plausible to assume that anterior inflation is under thyroid hormone regulation but scientific understanding is incomplete.			
Relationship 1034: Reduced, Anterior swim bladder inflation (KE 1007) leads to Reduced, Swimming performance (KE 1005)	**Moderate** Next to a role in hearing, the anterior chamber of the swim bladder has a function in regulating the buoyancy of fish. Stoyek et al. (2011) showed that the anterior chamber volume is highly dynamic under normal conditions due to a series of regular corrugations running along the chamber wall, and is in fact the main driver for adjusting buoyancy while the basic posterior chamber volume remains largely invariable. Therefore, it is plausible to assume that functionality of the swim bladder is affected when anterior chamber inflation is incomplete, even when the posterior chamber appears to fully compensate the gas volume of the swim bladder.			
Relationship 2212: Reduced, Swimming performance (KE 1005) leads to Increaed mortality (KE 351)	**Moderate** Reduced swimming performance is likely to affect essential endpoints such as predator avoidance, feeding behaviour and reproduction. These parameters are biologically plausible to affect survival. Apart from some indirect evidence, it has been difficult to clearly establish this relationship in the laboratory. It may only become apparent in a non-laboratory environment where food is scarce and predators are abundant.			
Relationship 2013: Increased mortality (KE 351) leads to Decrease, Population trajectory (KE 360)	**High** It is widely accepted that mortality increases, the population trajectory will eventually decrease.			
Non-adjacent relationship 1039:	**Moderate**			

T4 in serum, Decreased (KE 281), leads to Reduced, Anterior swim bladder inflation (KE 1007)	Thyroid hormones are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition and larval-to-juvenile transition in fish. Inflation of the anterior swim bladder chamber is part of the larval-to-juvenile transition in fish, together with the development of adult fins and fin rays, ossification of the axial skeleton, formation of an adult pigmentation pattern, scale formation, maturation and remodeling of organs including the lateral line, nervous system, gut and kidneys. Together with empirical evidence, it is plausible to assume that anterior inflation is under thyroid hormone regulation but scientific understanding is incomplete. Since most of the more biologically active T3 originates from the conversion of T4, decreased circulatory T4 levels are plausibly linked to reduced anterior chamber inflation.

AOP 159: Thyroperoxidase inhibition leading to increased mortality via anterior swim bladder inflation - Weight of evidence evaluation

2. Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE 279 (MIE): Thyroperoxidase, inhibition	There is evidence of recovery of serum T4 levels after cessation of exposure to a TPO inhibitor in rats (Cooper et al., 1982; 1983; AOP 42), but not in fish.			
KE 277: Thyroid hormone synthesis, decreased	There is evidence of recovery of serum T4 levels in athyroid mice following grafting of in-vitro derived follicles (Antonica et al., 2012; AOP 42). Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation.			
KE 281: Thyroxine (T4) in serum, decreased	There is ample evidence of recovery of phenotypes after cessation of exposure to TPO inhibitors and subsequent T4 recovery in mammals (Cooke et al., 1993; Goldey et al., 1995; Sawin et al., 1998; Axelstad et al., 2008; Shibutani et al., 2009; Lasley and Gilbert, 2011; AOP 42), but not in fish.			
KE 1003: Decreased triiodothyronine (T3) in serum	There is ample evidence confirming the essentiality of decreased T3 levels for the occurrence of reduced posterior chamber inflation, confirming a direct link between T3 levels and the swim bladder system in general. (1) from zebrafish knockdown/knockout studies: • Knockdown of deiodinase 1 and 2 (Bagci et al., 2015; Heijlen et al., 2013, 2014), knockdown of TH transporter MCT8 (de Vrieze et al., 2014), knockdown of thryoid hormone receptor alpha or beta (Marelli et al., 2016), and permanent knockout of deiodinase 2 (Houbrechts et al., 2016) in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Marelli et al. (2016) additionally showed that high T3 doses partially rescued the negative impact in mutants with partially resistant thyroid hormone receptors. • Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. These effects were rescued after T3 supplementation, but not after T4 supplementation. While swim bladder inflation was not among the assessed endpoints in this study, this generally confirms the essentiality of decreased T3 in causing downstream effects upon disruption of DIO1 and 2 function (Walpita et al., 2009, 2010). (2) from chemical exposures: • Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids and exogeneous T3 or T4 supplementation partly rescued this effect. • Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass lead to significant increases in posterior swim bladder inflation (Brown et al., 1988). Similarly, Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. Less information is available about the essentiality of reduced T3 levels for reduced anterior chamber inflation. • Chopra et al. (2019) provided indirect evidence showing that knockdown of dual oxidase - expected to lead to reduced T4 and T3 levels since dual oxidase is important for thyroid hormone synthesis - reduced anterior swim bladder inflation. It should be noted that dual oxidase also plays a role in oxidative stress. Proving essentiality of reduced T3 levels for reduced anterior chamber inflation is further complicated by the complexity of the swim bladder system and the difficulty of distinguishing effects resulting from altered anterior chamber inflation from those resulting from altered posterior chamber inflation.			
KE 1007: Reduced, anterior swim bladder inflation	Stinckens et al. (2020) showed that at the time point where control zebrafish inflate the anterior chamber, larvae exposed to PTU have a lower frequency of inflated anterior chambers together with reduced swimming distance. Later during the exposure the frequency of non-inflated anterior chambers decreased and the effect on swimming distance disappeared confirming the essentiality of reduced anterior chamber inflation for the downstream effect on swimming performance.			
KE 1005: Reduced, swimming performance	Experimental blocking of this KE is difficult to achieve.			
KE 351: Increased mortality	By definition, increased mortality is essential for reduced population size.			
AOP as a whole	**Moderate** Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation from a study where a similar MIE was targeted: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation. Additionally, there is indirect evidence from deiodinase knockdowns supporting the downstream part of the AOP linking decreased T3 levels to reduced swim bladder inflation (targeted at posterior chamber inflation, not specifically at anterior chamber inflation). There is also evidence that alleviation of the effect on anterior chamber inflation reduces the effect on swimming performance.			

	It should be noted that dual oxidase is not only involved in thyroid hormone synthesis, but also in the production of reactive oxygen species (ROS) (Flores et al. 2010; Niethammer et al. 2009). In zebrafish, ROS can also be induced e.g. by copper (Zhou et al. 2016), which has also been shown to impair swim bladder development (Xu et al. 2017). Impaired production of ROS after dual oxidase knockdown may contribute to an impairment of swim bladder development.

	Defining Questions	High (Strong)	Moderate	Low (Weak)
3. Empirical Support for KERs	Does empirical evidence support that a change in KEup leads to an appropriate change in KEdown? Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup > than that for KEdown? Inconsistencies?	if there is dependent change in both events following exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance) and no or few data gaps or conflicting data	if there is demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with the expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc.	if there are limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all), and/or lacking evidence of temporal or dose-response concordance, or identification of significant inconsistencies in empirical support across taxa and species that don't align with the expected pattern for the hypothesised AOP
Relationship: 309 Thyroperoxidase, Inhibition (KE 279) leads to TH synthesis, Decreased (KE 277)	**Low** Direct measurements of both KEs in the same study are not available in fish, but studies have shown that known TPO inhibitors reduce TH synthesis in the thyroid follicles and alter thyroid follicle histology in fish.			
Relationship: 305 TH synthesis, Decreased (KE 277) leads to T4 in serum, Decreased (KE 281)	**Low** Direct measurements of both KEs in the same study are not available in fish, but separate studies have shown that known TPO inhibitors reduce TH synthesis in the thyroid follicles, alter thyroid follicle histology and reduce T4 in fish.			
Non-adjacent relationship: 366 Thyroperoxidase, Inhibition (KE 279) leads to T4 in serum, Decreased (KE 281)	**Moderate** Although direct measurements of both KEs in the same organisms are not available in fish, several studies have shown that chemicals able to inhibit TPO in vitro, reduce T4 levels. In rare cases, increased T4 levels have been observed after longer exposures to TPO inhibitors, which is probably due to compensatory feedback mechanisms.			
Relationship 2038: T4 in serum, Decreased (KE 281) leads to Decreased, Triiodothyronine (T3) in serum (KE 1003)	**Moderate** Several studies have shown both T4 and T3 decreases upon exposure to chemicals that inhibit TH synthesis including a strong correlation between T4 and T3 levels and evidence of time and dose concordance. In some cases T4 and T3 levels do not change in the same direction. This can mostly be explained by feedback mechanisms. 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.			
Relationship 1035: Decreased, Triiodothyronine (T3) in serum (KE 1003) leads to Reduced, Anterior swim bladder inflation (KE 1007)	**Moderate** Several studies showed both T3 decreases and reduced inflation of the anterior chamber with some evidence of dose concordance. Uncertainties mainly relate to the mechanism through which altered TH levels result in impaired posterior chamber inflation. Temporal concordance is difficult to establish since swim bladder inflation can only occur at a specific time point.			
Relationship 1034: Reduced, Anterior swim bladder inflation (KE 1007) leads to Reduced, Swimming performance (KE 1005)	**Moderate** There is extensive evidence of a link between reduced anterior chamber inflation and reduced swimming performance including some evidence of dose concordance. Temporal concordance is specifically supported by the study of Stinckens et al. (2020): First, after 21 d of exposure to 111 mg/L propylthiouracil around 30% of anterior chambers were not inflated and swimming distance was reduced, while by 32 days post fertilization all larvae had inflated their anterior chamber (although chamber surface was still smaller) and the effect on swimming distance had disappeared.			
Relationship 2212: Reduced, Swimming performance (KE 1005) leads to Increaed mortality (KE 351)	**Low** A direct relationship between reduced swimming performance and increased mortality has been difficult to establish. There is however a lot of indirect evidence linking reduced swim bladder inflation to increased mortality (see non-adjacent KER 2213), which can be plausibly assumed to be related to reduced swimming performance.			

Relationship 2013: Increased mortality (KE 351) leads to Decrease, Population trajectory (KE 360)	**Moderate** Survival rate is an obvious determinant of population size and is therefore included in population modeling. The extent to which increased mortality may impact population sizes in a realistic, environmental exposure scenario depends on the circumstances. Under some conditions, reduced larval survival may be compensated by reduced predation and increased food availability, and therefore not result in population decline.
Non-adjacent relationship 1039: T4 in serum, Decreased (KE 281), leads to Reduced, Anterior swim bladder inflation (KE 1007)	**Moderate** Several studies show both T4 decrease and reduced anterior chamber inflation including significant linear relationships between T4 levels and anterior chamber surface and some support for dose concordance.

							dose and temporal concordance									uncertainties, inconsistencies			
reference	species	chemical	expected MIE	exposure period	time point	concentrations tested	TPO inhibition	DIO1 inhibition	DIO2 inhibition	TH synthesis decreased	T4 in serum decreased	T3 in serum decreased	posterior swim bladder chamber inflation reduced	anterior swim bladder chamber inflation reduced	swimming performance reduced	increased mortality	decreased tpo mRNA	decreased dio1 mRNA	serum T4 increased	serum T3 increased
Cavallin et al. (2017)	fathead minnow	iopanoic acid	DIO1 and 2 inhibition	0-6dpf	4 dpf	0.6, 1.9, 6.0 mg/L	n/a	n/a	n/a	n/a	n/a	-£	n/a	n/a	n/a	-			0.6, 1.9, 6.0 mg/L£	6 mg/L£
Cavallin et al. (2017)	fathead minnow	iopanoic acid	DIO1 and 2 inhibition	0-6dpf	6 dpf	0.6, 1.9, 6.0 mg/L	n/a	-*	-*	n/a	n/a	-£	6 mg/L	n/a	n/a	-			-£	1.9, 6.0 mg/L£
Cavallin et al. (2017)	fathead minnow	iopanoic acid	DIO1 and 2 inhibition	6-21 dpf	10 dpf	0.6, 1.9, 6.0 mg/L	n/a	-*	-*	n/a	n/a	0.6, 1.9, 6.0 mg/L£	n/a	n/a	n/a	-			0.6, 1.9, 6.0 mg/L£	-£
Cavallin et al. (2017)	fathead minnow	iopanoic acid	DIO1 and 2 inhibition	6-21 dpf	14 dpf	0.6, 1.9, 6.0 mg/L	n/a	-*	0.6, 1.9, 6.0 mg/L*	n/a	n/a	0.6, 1.9, 6.0 mg/L£	n/a	0.6, 1.9, 6.0 mg/L	n/a	-			1.9, 6.0 mg/L£	-£
Cavallin et al. (2017)	fathead minnow	iopanoic acid	DIO1 and 2 inhibition	6-21 dpf	18 dpf	0.6, 1.9, 6.0 mg/L	n/a	-*	0.6, 1.9, 6.0 mg/L*	n/a	n/a	0.6, 1.9, 6.0 mg/L£	n/a	0.6, 1.9, 6.0 mg/L	n/a	-			0.6, 1.9, 6.0 mg/L£	-£
Cavallin et al. (2017)	fathead minnow	iopanoic acid	DIO1 and 2 inhibition	6-21 dpf	21 dpf	0.6, 1.9, 6.0 mg/L	n/a	-*	0.6, 1.9, 6.0 mg/L*	n/a	n/a	0.6, 1.9, 6.0 mg/L£	n/a	0.6, 1.9, 6.0 mg/L	n/a	6 mg/L			0.6, 1.9, 6.0 mg/L£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-168 hpf	120 hpf	0.1, 0.35, 0.56, 0.7, 0.88, 1.75, 3.5, 7 mg/L	n/a	n/a	n/a	n/a	0.35, 0.7 mg/L£ (0.1 mg/L no	-	-	n/a	0.35, 0.56, 0.7, 0.88, 1.75, 3.	3.5, 7 mg/L			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	20 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	21 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	22 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	23 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	24 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	25 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	26 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	0.35 mg/L	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	27 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	28 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	29 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	0.35 mg/L	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	30 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	0.35 mg/L	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	31 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.35 mg/L	n/a	-			-£	-£
Stinckens et al. (2016)	zebrafish	2-mercaptobenzothiazole	TPO inhibition	0-32 dpf	32 dpf	0.1, 0.35 mg/L	n/a	n/a	n/a	n/a	0.35 mg/L£	-£	n/a	0.35 mg/L	n/a	-			-£	-£
Nelson et al. (2016)	fathead minnow	2-mercaptobenzothiazole	TPO inhibition	0-21 dpf	6 dpf	0.25, 0.5, 1 mg/L	-	n/a	n/a	n/a	1 mg/L£	-£	-	n/a	n/a	-			-£	-£
Nelson et al. (2016)	fathead minnow	2-mercaptobenzothiazole	TPO inhibition	0-21 dpf	14 dpf	0.25, 0.5, 1 mg/L	0.5, 1 mg/L*	n/a	n/a	0.5, 1 mg/L£	n/a	1 mg/L£	n/a	0.5, 1 mg/L	n/a	-			n/a	-£
Nelson et al. (2016)	fathead minnow	2-mercaptobenzothiazole	TPO inhibition	0-21 dpf	21 dpf	0.25, 0.5, 1 mg/L	1 mg/L*	n/a	n/a	0.5, 1 mg/L£	-£	-£	n/a	0.5, 1 mg/L	n/a	-			0.25, 0.5, 1 mg/L£	-£
Wei et al. (2018)	zebrafish	bisphenol S	unknown	adults	F1 96 hpf	1, 10, 100 µg/L	n/a	n/a	n/a	n/a	1, 10, 100 µg/L£		1, 10, 100 µg/L	n/a	1, 10, 100 µg/L				-	1, 10, 100 µg/L£
Crane et al. (2005)	fathead minnow	ammonium perchlorate	NIS inhibition	0-28 dpf	28 dpf	1, 10, 100 mg/L	n/a	n/a	n/a	1, 10, 100 mg/L§	-£	-£	n/a	n/a	n/a	-			100 mg/L	-
Crane et al. (2006)	fathead minnow	methimazole	TPO inhibition	0-84 dpf	28 dpf	32, 100, 320 µg/L	n/a	n/a	n/a	n/a	32, 100 µg/L£	-£	n/a	n/a	n/a	32, 100 µg/L			-£	-£
Crane et al. (2006)	fathead minnow	methimazole	TPO inhibition	0-84 dpf	56 dpf	32, 100, 320 µg/L	n/a	n/a	n/a	n/a	-£	100 µg/L£	n/a	n/a	n/a	32, 100 µg/L			320 µg/L£	-£
Crane et al. (2006)	fathead minnow	methimazole	TPO inhibition	0-84 dpf	84 dpf	32, 100, 320 µg/L	n/a	n/a	n/a	n/a	-	-	n/a	n/a	n/a	32, 100 µg/L			-	-
Stinckens et al. (2020)	zebrafish	methimazole	TPO inhibition	0-32 dpf	21 dpf	50, 100 mg/L	n/a	n/a	n/a	n/a	50, 100 mg/L£	50, 100 mg/L£	-	50, 100 mg/L	n/a					
Stinckens et al. (2020)	zebrafish	methimazole	TPO inhibition	0-32 dpf	32 dpf	50, 100 mg/L	n/a	n/a	n/a	n/a	50, 100 mg/L£	50, 100 mg/L£	-	50, 100 mg/L	100 mg/L					
Stinckens et al. (2020)	zebrafish	propylthiouracil	TPO inhibition	0-32 dpf	14 dpf	37, 111 mg/L	n/a	n/a	n/a	n/a	37, 111 mg/L£	111 mg/L£	-	n/a	111 mg/L					
Stinckens et al. (2020)	zebrafish	propylthiouracil	TPO inhibition	0-32 dpf	21 dpf	37, 111 mg/L	n/a	n/a	n/a	n/a	37, 111 mg/L£	111 mg/L£	-	37, 111 mg/L	111 mg/L					
Stinckens et al. (2020)	zebrafish	propylthiouracil	TPO inhibition	0-32 dpf	32 dpf	37, 111 mg/L	n/a	n/a	n/a	n/a	37, 111 mg/L£	37, 111 mg/L£	-	37, 111 mg/L	-					
Stinckens et al. (2020)	zebrafish	iopanoic acid	DIO1 and 2 inhibition	0-32 dpf	9 dpf	2 mg/L	n/a	n/a	n/a	n/a	n/a	n/a	2 mg/L	n/a	n/a	2 mg/L				
Stinckens et al. (2020)	zebrafish	iopanoic acid	DIO1 and 2 inhibition	0-32 dpf	14 dpf	0.35, 1 mg/L	n/a	n/a	n/a	n/a	-£	-£	-	n/a	1, 2 mg/L					
Stinckens et al. (2020)	zebrafish	iopanoic acid	DIO1 and 2 inhibition	0-32 dpf	21 dpf	0.35, 1 mg/L	n/a	n/a	n/a	n/a	-£	0.35, 1 mg/L£	-	0.35, 1, 2 mg/L	0.35, 1, 2 mg/L					
Stinckens et al. (2020)	zebrafish	iopanoic acid	DIO1 and 2 inhibition	0-32 dpf	32 dpf	0.35, 1, 2 mg/L	n/a	n/a	n/a	n/a	-£	0.35, 1, 2 mg/L£	-	0.35, 1, 2 mg/L	0.35, 1, 2 mg/L					
Wang et al. (2020)	zebrafish	perfluorooctanoic acid (PFOA)	DIO1 and 2 inhibition	0-5 dpf	5 dpf	0, 50, 100, 150, 200, 2502, 300, 350, 400, 450, 500 mg/L	-*	-	125, 250, 500 mg/L*	-	250, 500 mg/L£	250, 500 mg/L£	200, 250, 300, 350, 400, 450	n/a	n/a	300, 400, 450, 500 mg/L	-	500 mg/L	-£	-£
Wang et al. (2020)	zebrafish	PFO3OA	unknown	0-5 dpf	5 dpf	0, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400 mg/L	1200, 2200 mg/L*	-*	600, 1200, 2200 mg/L*	-	600, 1200, 2200 mg/L£	1200, 2200 mg/L£	800, 1000, 1200, 1400, 1600	n/a	n/a	-	-	-	-£	-£
Wang et al. (2020)	zebrafish	PFO4DA	unknown	0-5 dpf	5 dpf	0, 30, 45, 60, 90, 120, 150, 180, 210, 240 mg/L	-*	240 mg/L*	-*	-	60, 120, 240 mg/L£ (lower co	60, 120, 240 mg/L£ (lower co	45, 60, 90, 120, 150, 180, 21	n/a	n/a	-	-	-	-£	-£
Wang et al. (2020)	zebrafish	PFO5DoDA	unknown	0-5 dpf	5 dpf	0, 5, 10, 15, 20, 25, 30, 35, 40 mg/L	-*	-*	10, 20, 40 mg/L*	-	10, 20, 40 mg/L£	10, 20, 40 mg/L£	20, 25, 30, 35, 40 mg/L§	n/a	n/a	-	10 mg/L	-	-£	-£
Rehberger et al. (2018)	zebrafish	propylthiouracil	TPO inhibition	0-5 dpf	5 dpf	0, 2.5, 10, 25, 50 mg/L	n/a	n/a	n/a	10, 25, 50 mg/L	n/a	n/a	n/a	n/a	n/a	n/a				

Legend

n/a: not measured

* based on increased mRNA levels of the target as indirect measurement of MIE

§ based on thyroid histopathology

£ based on whole body measurement

§ based on visual evaluation of graphs because no statistics have been reported

References

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