OECD Series on Adverse Outcome Pathways No. 22

Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation

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OECD

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AOP No. 155 in the AOP-Wiki platform

Foreword

This Adverse Outcome Pathway (AOP) on Deiodinase 2 inhibition leading to increased mortality via reduced posterior 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].

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Abstract

This AOP describes the sequence of events leading from deiodinase inhibition to increased mortality via reduced posterior swim bladder inflation. Disruption of the thyroid hormone system is increasingly being recognized as an important toxicity pathway that can cause many adverse outcomes, including developmental abnormalities. Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of thyroid hormone (TH) action. Type II deiodinase (DIO2) has thyroxine (T4) as a preferred substrate and is mostly important for converting T4 to the more biologically active triiodothyronine (T3). Inhibition of DIO2 therefore reduces T3 levels. As in amphibians, the transition between the different developmental phases in fish, including maturation and inflation of the swim bladder, is mediated by THs (Brown et al., 1988; Liu and Chan, 2002). The swim bladder 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. This AOP describes how DIO2 inhibition results in reduced T3 levels, which prohibit normal inflation of the posterior chamber of the swim bladder in the embryonic phase. The posterior chamber is important for regulating buoyancy and thus for swimming performance (Robertson et al., 2007). Reduced swimming performance reduces chances of survival due to a decreased ability to forage and avoid predators. 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 (Jomaa et al., 2014; Cavallin et al., 2017; Stinckens et al., 2018) and on knockdown/knockout and TH supplementation studies in zebrafish embryos where the DIO2 gene is inactivated (Walpita et al., 2009, 2010; Heijlen et al., 2014; Bagci et al., 2015; Houbrechts et al., 2016).

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).Other than the difference in deiodinase (DIO) isoform, the current AOP is identical to the corresponding AOP leading from DIO1 inhibition to increased mortality via posterior swim bladder inflation (https://aopwiki.org/aops/157). The overall importance of DIO1 versus DIO2 in fish is not exactly clear. DIO2 inhibitors are often also inhibitors of DIO1 (Stinckens et al. 2018). In the ToxCast DIO2 inhibition single concentration assay, 304 out of 1820 chemicals were positive and 177 of these were also positive for DIO1 inhibition (viewed on 5/7/2022). This complicates the distinction between the relative contribution of DIO1 and DIO2 inhibition to reduced swim bladder inflation. The current state of the art suggests that DIO2 is more important than DIO1 in regulating swim bladder inflation (Stinckens et al., 2018). Therefore the current AOP may be of higher biological relevance compared to AOP 157.

Table of contents

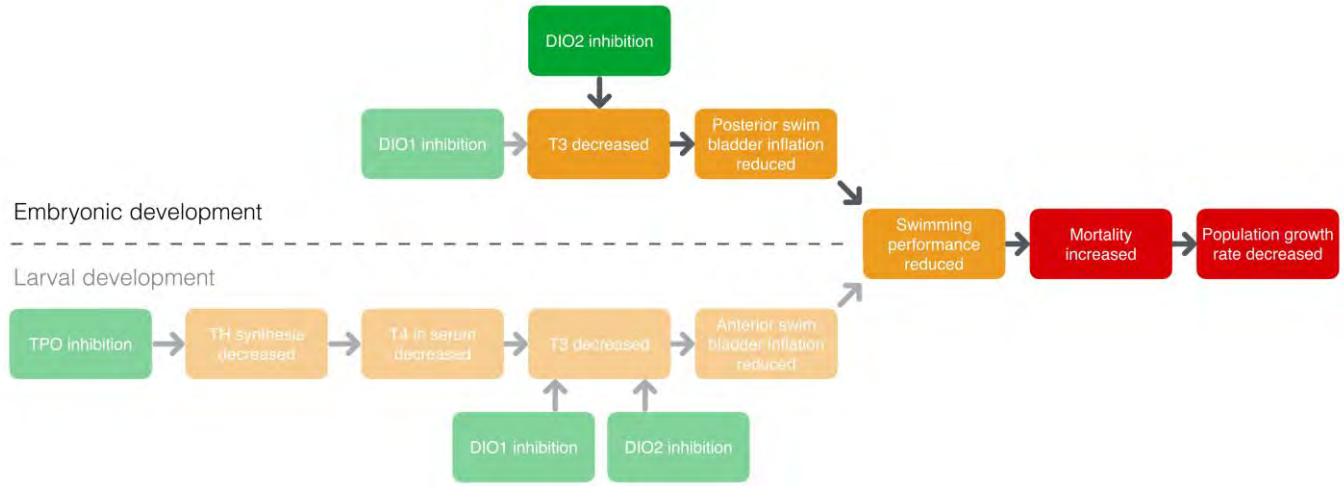
Background

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	1002	Inhibition, Deiodinase 2	Inhibition, Deiodinase 2
2	KE	1003	Decreased, Triiodothyronine (T3)	Decreased, Triiodothyronine (T3)
3	KE	1004	Reduced, Posterior swim bladder inflation	Reduced, Posterior swim bladder inflation
4	KE	1005	Reduced, Swimming performance	Reduced, Swimming performance
5	AO	351	Increased Mortality	Increased Mortality
6	AO	360	Decrease, Population growth rate	Decrease, Population growth rate

Key Event Relationships

Title	Relationship Type	Evidence	Quantitative Understanding
Inhibition, Deiodinase 2 leads to Decreased, Triiodothyronine (T3)	adjacent	Moderate	Low
Decreased, Triiodothyronine (T3) leads to Reduced, Posterior swim bladder inflation	adjacent	Moderate	Low
Reduced, Posterior swim bladder inflation leads to Reduced, Swimming performance	adjacent	Moderate	Low
Reduced, Swimming performance leads to Increased Mortality	adjacent	Moderate	Low
Increased Mortality leads to Decrease, Population growth rate	adjacent	Moderate	Moderate
Inhibition, Deiodinase 2 leads to Reduced, Posterior swim bladder inflation	non-adjacent	Moderate	Low
Reduced, Posterior swim bladder inflation leads to Increased Mortality	non-adjacent	High	Low

Stressors

Name	Evidence
iopanoic acid	High

Iopanoic acid is a well-known inhibitor of deiodinase 1, 2 and 3 and multiple studies have shown that exposure of fish early life stages to iopanoic acid results in reduced swim bladder inflation.

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 exactly understood

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Life stage: The current AOP is only applicable to early embryonic development, which is the period where the posterior swim bladder chamber inflates. In all life stages, the conversion of T4 into more biologically active T3 is essential. Inhibition of DIO2 therefore impacts swim bladder inflation in both early and late (https://aopwiki.org/aops/156) developmental life stages.

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. Knowledge could be expanded to physoclistous fish, such as the Japanese rice fish or medaka (Oryzias latipes) that has a single chambered swim bladder that inflates during early development.

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. In medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes of the sequence of events along this AOP. Sex differences are typically not investigated in tests using early life stages of fish and it is currently unclear whether sex-related differences are important 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 posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, 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 posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

Essentiality of the Key Events

Overall, the support for essentiality of the KEs is high since there is direct evidence from specifically designed experimental studies illustrating essentiality for several of the important KEs in the AOP. This includes ample evidence from knockdown studies in zebrafish that use targeted perturbation of key events and show downstream effects, and evidence from both chemical exposure with TH supplementation and knockdown with TH supplementation showing that blocking a KE prevents downstream KEs from occurring.

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

Data to support the quantitative understanding of this AOP is currently lacking.

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, deiodinase inhibition can be assessed using an in chemico assay, measurements of 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) (OECD TG 234), and assessments of posterior 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 T3 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 T3 levels within specific developmental stages. For example, clear changes in T3 levels have been observed in zebrafish at 14, 21 and 32 dpf (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: 1002: Inhibition, Deiodinase 2

Short Name: Inhibition, Deiodinase 2

Key Event Component

Process	Object	Action
catalytic activity	type II iodothyronine deiodinase	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	MolecularInitiatingEvent
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	MolecularInitiatingEvent
Aop:190 - Type II iodothyronine deiodinase (DIO2) inhibition leading to altered amphibian metamorphosis	MolecularInitiatingEvent

Stressors

Name
Iopanoic acid
Perfluorooctanoic acid

Biological Context:

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

DIO2 inhibitors are often also inhibitors of DIO1 (Olker et al., 2019; Stinckens et al. 2018). In the ToxCast DIO2 inhibition single concentration assay, 304 out of 1820 chemicals were positive and 177 of these were also positive for DIO1 inhibition (viewed on 5/7/2022). Olker et al. (2019) identified 20 DIO2-specific inhibitors using a human recombinant DIO2 enzyme (e.g., tetramethrin, elzasonan). In fact, many compounds inhibit all three DIO isoforms. Olker et al. (2019) identified 93 compounds that inhibit DIOs 1, 2 and 3.

- Iopanoic acid - A typical inhibitor of DIO2 (and DIO1 and 3) is iopanoic acid (IOP), which acts as a substrate of all three DIO isoforms (Renko et al., 2015)
- Perfluorooctanoic acid - Perfluorooctanoic acid (PFOA) is an inhibitor of DIO2 and DIO1 (Stinckens et al., 2018)

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
rat	Rattus norvegicus	Moderate	NCBI
human	Homo sapiens	High	NCBI
pigs	Sus scrofa	Moderate	NCBI
Oreochromis niloticus	Oreochromis niloticus	Moderate	NCBI
zebrafish	Danio rerio	Moderate	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI
African clawed frog	Xenopus laevis		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: Deiodination by DIO enzymes is known to exist in a wide range of vertebrates and invertebrates. This KE is plausibly applicable across vertebrates. Reports of inhibition of DIO2 activity are relatively scarce compared to DIO1. Studies reporting DIO2 inhibition have used human recombinant DIO2 enzyme (Olker et al., 2019), primary human astrocytes (Roberts et al., 2015), rat pituitary (Li et al., 2012), pig liver (Stinckens et al., 2018), Nile tilapia (Oreochromis niloticus) liver (Walpita et al., 2007). Evidence for fish (e.g., zebrafish and fathead minnow) is mostly indirect since

DIO enzyme activity is usually not measured in chemical exposure experiments. Houbrechts et al. (2016) showed decreased DIO2 activity in a DIO1-DIO2 knockdown zebrafish at the ages of 3 and 7 days post fertilization together with impaired swim bladder inflation, showing that the enzyme is present, the activity is measurable and impairing its activity has negative effects. Noyes confirmed decreased outer ring deiodination activity in fathead minnows exposed to decabromodiphenyl ether (BDE-209). Walpita et al. (2007) showed decreased DIO2 activity in the liver of Nile tilapia injected with dexamethasone. Stinckens et al. (2018) showed that chemicals with DIO inhibitory potential in pig liver impaired swim bladder inflation in zebrafish, a thyroid hormone regulated process. Six out of seven DIO1 inhibitors impaired posterior chamber inflation, but almost all of these compounds also inhibit DIO2. TCBPA, the only compound that inhibits DIO1 and not DIO2, had no effect on the posterior swim bladder. Based on these results, DIO2 seemed to be more important than DIO1.

In mammals, DIO2 is thought to control the intracellular concentration of T3, while DIO1 is thought to be more important in determining systemic T3 levels. The cells that express DIO2 locally produce T3 that can more rapidly access the thyroid receptors in the nucleus than T3 from plasma (Bianco et al., 2002). For example, DIO2 is highly expressed in the mammalian brain. However, this hypothesis has been challenged. For example, Maia et al. (2005) determined that in a normal physiological situation in humans the contribution of DIO2 to plasma T3 levels is twice that of DIO1. Only in a hyperthyroid state was the contribution of DIO1 higher than that of DIO2. A DIO1 knockout mouse showed normal T3 levels and a normal general phenotype and DIO1 was rather found to play a role in limiting the detrimental effects of conditions that alter normal thyroid function, including hyperthyroidism and iodine deficiency (Schneider et al., 2006). van der Spek et al. concluded that the primary role of DIO1 in vivo is to degrade inactivated TH (van der Spek et al., 2017).

The presence of DIO1 in the liver of teleosts has been a controversial issue and DIO1 function in teleostean and amphibian T3 plasma regulation is unclear (Finnson et al., 1999; Kuiper et al., 2006). In teleosts, DIO2 has a markedly higher activity level compared to other vertebrates and it is expressed in liver (Orozco and Valverde, 2005), suggesting its importance in determining systemic thyroid hormone levels. This could explain why DIO2 inhibition seems to be more important than DIO1 inhibition in determining the adverse outcome in zebrafish (Stinckens et al., 2018).

Life stage: Deiodinase activity is important for all vertebrate life stages. Already during early embryonic development, deiodinase activity is needed to regulate thyroid hormone concentrations and coordinate developmental processes. DIO2 shows more marked changes in expression around the time of the embryo-larval and larval-to-juvenile transition periods during zebrafish development, highlighting its importance for early life stages (Vergauwen et al., 2018).

Sex: This KE is plausibly applicable to both sexes. Deiodinases are important for TH homeostasis and identical in both sexes. Therefore inhibition of deiodinases is not expected to be sex-specific.

Key Event Description

Disruption of the thyroid hormone system is increasingly being recognized as an important toxicity pathway, as it can cause many adverse outcomes. Thyroid hormones do not only play an important role in the adult individual, but they are also critical during embryonic development. Thyroid hormones (THs) play an important role in a wide range of biological processes in vertebrates including growth, development, reproduction, cardiac function, thermoregulation, response to injury, tissue repair and homeostasis. Numerous chemicals are known to disturb thyroid function, for example by inhibiting

thyroperoxidase (TPO) or deiodinase (DIO), upregulating excretion pathways or modifying gene expression. The two major thyroid hormones are triiodothyronine (T3) and thyroxine (T4), both iodinated derivatives of tyrosine. Most TH actions depend on the binding of T3 to its nuclear receptors. Active and inactive THs are tightly regulated by enzymes called iodothyronine deiodinases (DIO). The activation occurs via outer ring deiodination (ORD), i.e. removing iodine from the outer, phenolic ring of T4 to form T3, while inactivation occurs via inner ring deiodination (IRD), i.e. removing iodine from the inner tyrosol ring of T4 or T3.

Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of TH action. All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain selenocysteine in their catalytic centre. Type I deiodinase is capable to convert T4 into T3, as well as to convert reverse T3 (rT3) to 3,3'-Diiodothyronine (3,3' T2), through outer ring deiodination. rT3, rather than T4, is the preferred substrate for DIO1. furthermore, DIO1 has a very high Km (µM range, compared to nM range for DIO2) (Darras and Van Herck, 2012). Type II deiodinase (DIO2) is only capable of ORD activity with T4 as a preferred substrate (i.e., activation of T4 to T3). DIO3 can inner ring deiodinate T4 and T3 to the inactive forms of THs, rT3 and 3,3'-T2 respectively. DIO2 is a transmembrane protein anchored to the endoplasmic reticulum and the active site faces the perinuclear cytosol. The relative contribution of the DIOs to thyroid hormone levels varies amongst species, developmental stages and tissues.

How it is Measured or Detected

At this time, there are no approved OECD or EPA guideline protocols for measurement of DIO inhibition. Deiodination is the major pathway regulating T3 bioavailability in mammalian tissues. In vitro assays can be used to examine inhibition of deiodinase 2 (DIO2) activity upon exposure to thyroid disrupting compounds.

Several methods for deiodinase activity measurements are available. A first in vitro assay measures deiodinase activities by quantifying the radioactive iodine release from iodine-labelled substrates, depending on the preferred substrates of the isoforms of deiodinases (Forhead et al., 2006; Pavelka, 2010; Houbrechts et al., 2016; Stinckens et al., 2018). Each of these assays requires a source of deiodinase which can be obtained for example using unexposed pig liver tissue (available from slaughterhouses) or rat liver tissue. Olker et al. (2019) on the other hand used an adenovirus expression system to produce the DIO2 enzyme and developed an assay for nonradioactive measurement of iodide released using the Sandell-Kolthoff method, a photometric method based on Ce4+ reduction (Renko et al., 2012). This assay was then used to screen the ToxCast Phase 1 chemical library. The specific synthesis of DIO2 through the adenovirus expression system provides an important advantage over other methods where activity of the different deiodinase isoforms needs to be distinguished in other ways, such as based on differences in enzyme kinetics.

Measurements of in vivo deiodinase activity in tissues collected from animal experiments are scarce. Noyes et al. (2011) showed decreased rate of outer ring deiodination (mediated by DIO1 and DIO2) in whole fish microsomes after exposure to BDE-209. After incubation with the substrate, thyroid hormone levels were measured using LC-MS/MS. Houbrechts et al. (2016) confirmed DIO2 deiodination activity in a DIO1-DIO2 knockdown zebrafish at the ages of 3 and 7 days post fertilization. Decreased T3 levels are often used as evidence of DIO inhibition, for example after exposure to iopanoic acid, in fish species

such as zebrafish (Stinckens et al., 2020) and fathead minnow (Cavallin et al., 2017). It should be noted that it is difficult to make the distinction between decreased T3 levels caused by outer ring deiodination mediated by DIO2 inhibition or DIO1 inhibition.

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List of Key Events in the AOP

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

Biological Context:

Level of Biological Organization
Tissue

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
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, whereas 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 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 system 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 minnow 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 hormones 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 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, 2020).

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Event: 1004: Reduced, Posterior swim bladder inflation

Short Name: Reduced, Posterior swim bladder inflation

Key Event Component

Process	Object	Action
swim bladder inflation	posterior chamber swim bladder	decreased

AOPs Including This Key Event

AOP Name	Role of event in AOP
DIO2i posterior swim bladder	KeyEvent
DIO1i posterior swim bladder	KeyEvent

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
medaka	Oryzias latipes	Medium	

Life Stage Applicability

Life Stage	Evidence
Embryo	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 (Wooley and Qin, 2010).

Much of the evidence for impaired posterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2018; Cavallin et al., 2017; Wang et al., 2020). Increasing evidence is becoming available on defects of swim bladder inflation in medaka (*Oryzias latipes*), a species with only one swim bladder chamber (Gonzalez-doncel et al., 2003; Dong et al., 2016; Kupsco et al., 2016; Mu et al., 2017; Pandelides et al., 2021). Exposure to T3, methimazole, heptafluorobutanoic acid (PFBA) and tris[1,3-dichloro-2-propyl] phosphate (TDCPP) inhibited inflation of the swim bladder in female medaka. Interestingly, for those females that developed a swim bladder, exposure to methimazole and all halogenated chemicals with the exception of PFBA, resulted in larger swim bladders (Godfrey et al., 2019). Horie et al. (2022) eludicated the timing of swim bladder inflation in medaka and compared effects on the swim bladder after exposure of zebrafish and medaka to PFBA and TDCPP. This KE is plausibly applicable across fish species with swim bladders, both physostomous and physoclistous.

Life stage: The posterior chamber inflates during a specific developmental time frame. In zebrafish, the posterior chamber inflates around 96-120 hpf which is 2-3 dph. In the fathead minnow, the posterior chamber inflates around 6 dpf. In medaka, the swim bladder inflates around 2 hours post hatch (hatching occurs around 8 dpf) (Horie et al., 2022). Therefore this KE is only applicable to the embryonic life stage.

Sex: This KE is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE. 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 posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, 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 posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current KE.

Key Event Description

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 96-120 h post fertilization (hpf) which is 2-3 days post hatch, and the anterior chamber inflates around 21 dpf (days post fertilization). In fathead minnow, the posterior and anterior chamber inflate around 6 and 14 dpf respectively.

The posterior chamber is formed from a bud originating from the foregut endoderm (Winata et al., 2009). The posterior chamber operates as a hydrostatic organ. The volume of gas in the adult swim bladder is continuously adjusted to regulate body density and buoyancy.

Many amphibians and frogs go through an embryo-larval transition phase marking the switch from endogenous feeding (from the yolk) to exogenous feeding. In zebrafish, embryonic-to-larval transition takes place around 96 hours post fertilization (hpf). As in amphibians, the transition between the different developmental phases includes maturation and inflation of the swim bladder (Liu and Chan, 2002).

Reduced inflation of the posterior chamber may manifest itself as either a complete failure to inflate the chamber or a reduced size of the chamber.

How it is Measured or Detected

In several fish species, inflation of the posterior chamber can easily be observed using a stereomicroscope because the larvae are still transparent during those early developmental stages. This is for example true for zebrafish and fathead minnow. Posterior chamber size can then be measured based on photographs with a calibrator.

When observing effects on swim bladder inflation, it is important to verify that reduced swim bladder inflation occurs at concentrations significantly lower than those causing mortality, since a wide variety of chemicals cause impaired posterior chamber inflation at exposure concentrations that also cause mortality (Stinckens et al., 2018).

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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 ID and Name	Event Type
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Acute Respiratory Distress Syndrome (ARDS) and Multiple Organ Dysfunction (MOD)	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
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: 1026: Inhibition, Deiodinase 2 leads to Decreased, Triiodothyronine (T3)

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

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: Deiodinases are important for the activation of T4 to T3 across vertebrates. Therefore, this KER is plausibly applicable across vertebrates. There appear to be differences among vertebrate classes relative to the role of the different deiodinase isoforms in regulating thyroid hormone levels. Maia et al. (2005) determined that in a normal physiological situation in humans the contribution of DIO2 to plasma T3 levels is twice that of DIO1. A DIO2 knockout (KO) mouse however showed a very mild gross phenotype with only mild growth retardation in males (Schneider et al., 2001). It seemed that by blocking the negative feedback system, DIO2 KO resulted in increased levels of T4 and TSH and in normal rather than decreased T3 levels compared to WT. Potential differences in the role of the deiodinase isoforms in the negative feedback system and the final consequences for TH levels across vertebrates is currently not entirely clear. These differences make it difficult to exactly evaluate the importance of DIO2 in regulating serum/tissue T3 levels across vertebrates. Mol et al. (1998) concluded that deiodinases in teleosts were more similar to mammalian deiodinases than had been generally accepted, based on the similarities in susceptibility to inhibition and the agreement of the Km values.

Life stage: Deiodinases are important for the activation of T4 to T3 across all life stages.

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

The two major thyroid hormones are thyroxine (T4) and the more biologically active triiodothyronine (T3), both iodinated derivatives of tyrosine. Active and inactive THs are tightly regulated by enzymes called iodothyronine deiodinases (DIO). The activation occurs via outer ring deiodination (ORD), i.e. removing iodine from the outer, phenolic ring of T4 to form T3, while inactivation occurs via inner ring deiodination (IRD), i.e. removing iodine from the inner tyrosol ring of T4 or T3.

Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of TH action. All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain selenocysteine in their catalytic centre. Type I deiodinase is capable of converting T4 into T3, as well as to convert rT3 to the inactive thyroid hormone 3,3’ T2, through outer ring deiodination. rT3, rather than T4, is the preferred substrate for DIO1. furthermore, DIO1 has a very high Km (µM range, compared to nM range for DIO2) (Darras and Van Herck, 2012). Type II deiodinase (DIO2) is only capable of ORD activity with T4 as a preferred substrate (i.e., activation of T4 tot T3). DIO3 can inner ring deiodinate T4 and T3 to the inactive forms of THs, reverse T3, (rT3) and 3,3’-T2 respectively. (Darras and Van Herck, 2012) DIO2 and DIO3 expression customize the timing and intensity of TH signalling in an organ/tissue-specific way (Russo et al 2021).

Evidence Supporting this KER

Inhibition of DIO2 activity is widely accepted to directly decrease T3 levels, since the conversion of T4 to T3 is inhibited. The importance of DIO2 inhibition in altering serum and/or tissue T3 levels depends on the relative role of different deiodinases in regulating serum versus tissue T3 levels and in negative feedback within the HPT axis. Both aspects appear to vary among vertebrate taxa.

Biological Plausibility

Inhibition of DIO2 activity is widely accepted to directly decrease T3 levels, since the conversion of T4 to T3 is inhibited.

Empirical Evidence

- Houbrechts et al. (2016) developed a zebrafish Dio2 knockout and confirmed both the absence of the full length Dio2 protein in the liver and the dramatical decrease of T4 activating enzyme activity in liver, brain and eyes. Finally, they found decreased levels of T3 in liver, brain and eyes.
- Winata et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in DIO1+2 and DIO2 knockdown zebrafish. These effects were rescued after T3 supplementation but not by T4 supplementation, confirming that decreased T3 levels were at the basis of the observed effects.
- 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). Transcriptional analysis showed that especially DIO2, but also DIO3 mRNA levels (in some treatments), were increased in 10 to 21 day old larvae exposed to IOP as of the age of 6 days. This suggests that IOP effectively inhibited DIO2 and DIO3 in the larvae and that mRNA levels increased as a compensatory response. The authors also observed pronounced decreases of whole-body T3 concentrations and increases of whole-body T4 concentrations.
- Stinckens et al. (2020) showed that IOP reduced whole-body T3 levels in zebrafish in 21 and 32 day old larvae that had been exposed starting from fertilization.
- While DIO1 has a high Km and rT3 is its preferred substrate, DIO2 has a low Km and T4 is its preferred substrate, indicating that DIO2 is more important than DIO1 in converting T4 to T3 in a physiological situation across species (Darras and Van Herck, 2012).

Uncertainties and Inconsistencies

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.

The importance of DIO2 inhibition in altering serum or tissue T3 levels depends on the relative role of different deiodinases in regulating serum versus tissue T3 levels and in negative feedback within the HPT axis. Both aspects appear to vary among vertebrate taxa. The high level of DIO2 activity and its expression in the liver of teleosts are unique among vertebrates (Orozco and Valverde, 2005). It is thought that DIO2 is important for local T3 production in several tissues but also contributes to circulating T3, especially in fish and amphibians (Darras et al., 2015).

Deiodinase 2 inhibition may not always directly lead to decreased T3 levels as there may be age-specific, exposure window- specific, and exposure duration-specific effects that may deviate from that dynamic. Differences in feedback mechanisms may be an important contributor. In DIO2 knockout mice

it seemed that the negative feedback system was blocked resulting in increased levels of T4 and TSH and in normal rather than decreased T3 levels compared to WT.

In the study of Cavallin et al. (2017) fathead minnow embryos were exposed to IOP, a model iodothyronine deiodinase inhibitor that is assumed to inhibit all three deiodinase enzymes (DIO1,2,3). The authors observed increased whole-body T3 concentrations in 4 and 6 day old embryos, while they observed decreased T3 concentrations in 10 to 21 day old larvae exposed to IOP as of the age of 6 days. One possible explanation for the elevated T3 concentrations may be the potential impact of IOP exposure on DIO3. DIO3 is an inactivating enzyme that removes iodine from the inner ring of both T4 and T3, resulting in reverse T3 (rT3) and 3,5-diiodo-L- thyronine (T2), respectively (Bianco and Kim, 2006). Maternal sources of thyroid hormones are known to include both T4 and T3 (Power et al., 2001; Walpita et al., 2007). Consequently, reduced conversion of maternal T3 to inactive forms may be one plausible explanation for the increase. Another explanation may result from the role of deiodinases in the negative feedback system of the HPT axis. Inhibition of deiodinase (unclear which isoforms) may block the negative feedback system and result in increased release of T4. Increased levels of T4 were indeed observed by Cavallin et al. (2017).

Quantitative Understanding of the Linkage

Since in fish enzyme activity and thyroid hormone levels are rarely measured in the same study, quantitative understanding of this linkage is limited.

Known Feedforward/Feedback loops influencing this KER

Thyroid hormone levels are regulated via negative feedback, in part via regulation of the expression of all three DIO isoforms in response to deviating TH levels. This feedback mechanism influences this KER. Additionally, deiodinases regulate the activity of thyroid hormones, not only in serum and target organs, but also in the thyroid gland. On top of that, deiodinases themselves are mediators of the negative feedback system that results in increased TSH levels when the levels of T4 (and also T3) in serum are low (Schneider et al., 2001), resulting in an even more complicated impact on this KER. Increased TSH levels then stimulate increased T4 release from the thyroid gland, resulting in a compensatory increase of serum T4 levels. In DIO2 knockout mice it seemed that the negative feedback system was blocked resulting in increased levels of T4 and TSH and in normal rather than decreased T3 levels compared to WT. By inhibiting DIO1 using a PTU exposure, Schneider et al. (2001) showed that DIO2 played a role in the increased TSH levels in response to T3 or T4 injection in mice.

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Relationship: 1027: Decreased, Triiodothyronine (T3) leads to Reduced, Posterior swim bladder inflation

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 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Embryo	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).

Much of the evidence for impaired posterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2018; Cavallin et al., 2017; Wang et al., 2020). Increasing evidence is becoming available on defects of swim bladder inflation in medaka (Oryzias

latipes), a species with only one swim bladder chamber (Gonzalez-doncel et al., 2003; Dong et al., 2016; Kupsco et al., 2016; Mu et al., 2017; Pandelides et al., 2021). Exposure to T3, methimazole, heptafluorobutanoic acid (PFBA) and tris[1,3-dichloro-2-propyl] phosphate (TDCPP) inhibited inflation of the swim bladder in female medaka. Interestingly, for those females that developed a swim bladder, exposure to methimazole and all halogenated chemicals with the exception of PFBA, resulted in larger swim bladders (Godfrey et al., 2019). Horie et al. (2022) eludicated the timing of swim bladder inflation in medaka and compared effects on the swim bladder after exposure of zebrafish and medaka to PFBA and TDCPP. This KER is plausibly applicable across fish species with swim bladders, both physostomous and physoclistous.

Life stage: This KER is only applicable to early embryonic development, which is the period where the posterior swim bladder chamber inflates. The relationship between reduced T3 levels and reduced posterior chamber inflation is not applicable to older larvae that successfully inflated the posterior chamber but show impaired anterior chamber inflation after chronic exposure to low concentrations of thyroid hormone system disruptors. In 32 day old zebrafish exposed to methimazole, propylthiouracil, 2- mercaptobenzothiazole or iopaonic acid (Stinckens et al., 2016, 2020) as well as in 14-21 day old fathead minnows exposed to iopaonic acid (Cavallin et al., 2017), a clear inverse relationship was found. With decreasing whole-body T3 concentrations, posterior chamber volume increased, suggesting a possible compensatory mechanism for the observed decrease in anterior chamber volume. As a result, the sum of both chamber surfaces, reflecting the total amount of gas, was equal to controls for most treatments (Stinckens et al., 2016; Stinckens et al., 2020).

Sex: This KER is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KER. 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 posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, 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 posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in this KER.

Key Event Relationship Description

Reduced T3 levels prohibit local TH action in the target tissues. The site of decreased T3 in this case is the swim bladder. Since swim bladder development and/or inflation is regulated by thyroid hormones, this results in impaired posterior chamber inflation.

Evidence Supporting this KER

There is convincing evidence that decreased T3 levels result in impaired posterior chamber inflation, but the underlying mechanisms are not completely understood. The quantitative understanding is currently very limited because T3 levels and posterior inflation are seldom measured in the same study. 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 posterior chamber is part of the embryonic-to-larval transition in fish, together with structural and functional maturation of the mouth and gastrointestinal tract, and resorption of the yolk sac (Liu and Chan, 2002). Marelli et al. (2016) showed that thyroid hormone receptor alpha and beta are both expressed in swim bladder tissue of zebrafish at 5 days post fertilization, corresponding to the timing of posterior inflation. This time point has additionally been shown to coincide with increased T3 and T4 levels (Chang et al., 2012), suggesting that posterior inflation is under thyroid hormone regulation.

Empirical Evidence

- Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass (Morone saxatilis) lead to significant increases in both swim bladder inflation and survival (Brown et al., 1988).
- Dong et al. (2013) and Thisse et al. (2003) showed localized expression of DIO1 and DIO2 in the swim bladder tissue of 96 and 120 hpf zebrafish larvae, suggesting that local activation of thyroid hormones (i.e. conversion of T4 to T3) is required in swim bladder tissue around that time period.
- Marelli et al. (2016) used morpholinos to block translation of thryoid hormone receptor alpha or beta in zebrafish. They found that thyroid hormone receptor alpha and beta knockdowns failed to inflate the posterior chamber of the swim bladder by 120 hpf, indicating that the action of T3 is needed for proper inflation of the posterior chamber. High T3 doses partially rescued the negative impact in partially resistant mutants, further confirming the importance of T3 in this process.
- Stinckens et al. (2018) showed that effects on posterior chamber inflation in zebrafish could be predicted based on in chemico DIO2 inhibition potential with only few false positives and false negatives. While T3 levels were not determined in this study, DIO2 inhibition is expected to result in decreased T3 levels.
- Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of DIO1+2 in zebrafish resulted in impairment of the inflation of the posterior chamber of the swim bladder. DIO1 and 2 knockdown is expected to result in reduced T3 levels. Indeed, Walpita et al. (2009, 2010) showed that T3 supplementation effectively rescued the effects of DIO1 and 2 knockdown, while T4 supplementation did not.
- de Vrieze et al. (2014) found that knockdown of monocarboxylate transporter 8 (mct8) in zebrafish resulted in a dose- dependent impairment of posterior chamber inflation. Since this transporter is known to transport thyroid hormones across cell membranes, this supports the importance of thyroid hormones in regulating posterior chamber inflation.

- Shi et al. (2019) found that exposure of adult zebrafish to 6:2 chlorinated polyfluorinated ether sulfonate (F-53B), an alternative to perfluorooctanesulfonate (PFOS), decreased T3 levels in both male and female zebrafish. Additionally, F-53B was maternally transferred to the offspring. Decreased T3 levels together with impaired posterior chamber inflation was observed in the F1 offspring. Although the assumed site of T3 decrease is in the swim bladder tissue itself, most fish early life stage studies only quantify whole-body T3 levels which does not allow for making the distinction between systemic and local T3 levels.
- 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 from fertilization until the age of 5 days.
- Exogenous T3 or T4 supplementation partly rescued PFECA-induced posterior swim bladder malformation, confirming the causal relationship between reduced T3 levels and reduced posterior chamber inflation.
- Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. This confirms that T3 plays an important role in posterior swim bladder inflation.

Uncertainties and Inconsistencies

The mechanism through which altered TH levels result in impaired posterior chamber inflation still needs to be elucidated. It is currently unclear which aspect of swim bladder development and inflation is affected by TH disruption. Based on the developmental stages of the posterior chamber, several hypotheses could explain effects on posterior chamber inflation due to disrupted TH levels. A first hypothesis includes effects on the budding of the posterior chamber inflation. Secondly, the effect on posterior chamber inflation could also be caused by disturbing the formation and growth of the three tissue layers of this organ. It has been reported that the Hedgehog signalling pathway plays an essential role in swim bladder development and is required for growth and differentiation of cells of the swim bladder. The Wnt/β-catenin signalling pathway is required for the organization and growth of all three tissue layers (Yin et al., 2011, 2012, Winata 2009, Kress et al., 2009). Both signalling pathways have been related to THs in amphibian and rodent species (Kress et al., 2009; Plateroti et al., 2006; Stolow and Shi, 1995). Molla et al. (2019) showed that insulin-like growth factor (IGF-1) plays a role in swim bladder inflation/maturation in zebrafish. 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. Several other hypotheses include effects on the successful initial inflation of the posterior chamber, effects on lactic acid production that is required for the maintenance of the swim bladder volume, or effects on the production of surfactant that is crucial to maintain the surface tension necessary for swim bladder inflation.

Another uncertainty lies in the systemic versus local changes in T3 levels and the relative importance of the different T4 activating iodothyronine deiodinases (DIO1, DIO2) in regulating swim bladder inflation. Stinckens et al. (2018) showed that exposure of zebrafish embryos to seven strong DIO1 inhibitors (measured using in chemico enzyme inhibition assays), six out of seven compounds impaired posterior chamber inflation, but almost all of these compounds also inhibit DIO2. Tetrachlorobisphenol A (TCBPA), the only compound that inhibits DIO1 and not DIO2, had no effect on the posterior swim bladder. Exposure to strong DIO2 inhibitors on the other hand affected posterior chamber inflation and/or surface area in all cases. These results suggest that DIO2 enzymes may play a more important role in swim bladder inflation compared to DIO1 enzymes. In the ToxCast DIO2 inhibition single concentration assay, 304 out of 1820 chemicals were positive and 177 of these were also positive for DIO1 inhibition (viewed on 5/7/2022). This complicates the distinction between the relative contribution

of DIO1 and DIO2 inhibition to reduced swim bladder inflation. It has been previously suggested that DIO2 is the major contributor to TH activation in developing zebrafish embryos (Darras et al., 2015; Walpita et al., 2010). It has been shown that a morpholino knockdown targeting DIO1 mRNA alone did not affect embryonic development in zebrafish, while knockdown of DIO2 delayed progression of otic vesicle length, head-trunk angle and pigmentation index (Houbrechts et al., 2016; Walpita et al., 2010, 2009). DIO1 inhibition may only become essential in hypothyroidal circumstances, for example when DIO2 is inhibited or in case of iodine deficiency, in zebrafish (Walpita et al., 2010) and mice (Galton et al., 2009; Schneider et al., 2006).

As reported by Bagci et al. (2015) and Heijlen et al. (2014), posterior chamber inflation was impaired in DIO3 knockdown zebrafish. Heijlen et al. (2014) additionally reported histologically abnormal tissue layers in the swim bladder of DIO3 knockdown zebrafish. DIO3 is a thyroid hormone inactivating enzyme, which would result in higher levels of T3. Wei et al. (2018) showed that exposure to bisphenol S in adult zebrafish decreased T4 levels and increased T3 levels, and these changes in thyroid hormone levels were transferred to the offspring, in which impaired swim bladder inflation was observed. This indicates that not only too low, but also too high T3 levels, impact posterior chamber inflation. The underlying mechanism is currently unknown.

In the study of Cavallin et al. (2017) fathead minnow embryos were exposed to IOP, a model iodothyronine deiodinase inhibitor that is assumed to inhibit all three deiodinase enzymes (DIO1,2,3). The authors observed increased whole-body T3 concentrations in 4 and 6 day old embryos, together with impaired posterior chamber inflation. Transcript levels of DIO1, 2 and 3 remained unaltered and thus offered no proof of a compensatory mechanism that could explain these results.

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, posterior swim bladder chamber inflation, which occurs early during development, appears to be less sensitive to inhibition of TH synthesis than to inhibition of the conversion of T4 to T3 (Stinckens et al., 2016, 2018; Nelson et al., 2016). There have however been a few reports of reduced posterior inflation upon inhibition of TH synthesis (Liu and Chan, 2002). It must however be noted that these observations could reflect delayed inflation due to a general delay in development rather than a direct effect on the swim bladder. Longer observations would have to clarify this.

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Relationship: 1028: Reduced, Posterior 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 posterior swim bladder inflation	adjacent	Moderate	Low
Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Link
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI
bluefin tuna	Thunnus thynnus	Moderate	NCBI
Dicentrarchus labrax	Dicentrarchus labrax	Moderate	NCBI
Perca flavescens	Perca flavescens	Moderate	NCBI
Salmo salar	Salmo salar	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	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 a posterior chamber. Evidence exists for a wide variety of freshwater and marine fish species.

Life stage: This KER is only applicable to early embryonic development, which is the period where the posterior swim bladder chamber inflates. To what extent fish can survive and swim with partly inflated swim bladders during later life stages is unknown.

Sex: This KE/KER is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In Medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In 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. 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 posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, 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 posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

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 posterior swim bladder inflation and reduced swimming performance, is moderate.

Biological Plausibility

The posterior chamber of the swim bladder has a function in regulating the buoyancy of fish (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, as has been suggested previously (Bagci et al., 2015; Hagenaars et al., 2014). Fish without a functional swim bladder are severely disadvantaged, making the likelihood of surviving smaller. Stoyek et al. (2011) showed that the posterior

chamber volume is maintained at a stable level at varying pressures corresponding to varying depths through gas exchange with the anterior chamber.

Empirical Evidence

Buoyancy is one of the primary mechanisms of fish to regulate behaviour, swimming performance and energy expenditure. There is extensive evidence of a link between reduced posterior chamber inflation and reduced swimming performance:

- Stewart and Gee (1981) showed that fathead minnows swimming from still water to a current resorbed gas to fill the swim bladder and tailor buoyancy precisely to the level were swimming is most efficient.
- Lindsey et al., 2010 reported that zebrafish 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. Furthermore, they reported that the range of swimming depth varies with stages of swim bladder development.
- Czesny et al., 2005 reported that yellow perch larvae without inflated swim bladders capture free-swimming prey poorly and expend more energy on feeding and maintaining their position within the water column, due to impacted swimming behaviour. Kurata et al., 2014 observed that Bluefin tuna larvae present at the bottom of a tank, incapable of swimming upwards, had significantly lower swim bladder inflation.
- Chatain (1994) associated sea bass larvae with non-inflated swim bladders with numerous complications, such as spinal deformities and lordosis and reduced growth rates, adding to the impact on swimming behaviour.
- An increasing incidence of swim bladder non-inflation has also been reported in Atlantic salmon. Affected fish had severely altered balance and buoyancy, observed through a specific swimming behaviour, as the affected fish were swimming upside down in an almost vertical position (Poppe et al., 1977).
- Permanent DIO 2 deficiency in zebrafish was shown to result in reduced posterior chamber inflation and disturbed locomotor activity (Houbrechts et al., 2016).
- Michiels et al. (2017) showed that both for controls and zebrafish embryos exposed to an environmental sample, the swimming distance was significantly lower in larvae that failed to inflate the posterior chamber compared to larvae from the same treatment that had inflated posterior chambers.
- Exposure of zebrafish embryos to thyroid disrupting compounds resulted in an effect on posterior chamber inflation as well as on the swimming distance in the larval stage (Stinckens et al., unpublished).
- 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, it is plausible to assume that uninflated posterior chambers limited the ability to swim and find food.
- Hagenaars et al. (2014) showed that zebrafish embryos exposed to 4.28 mg/L PFOS had lower swimming speeds when the posterior chamber was not inflated. It should be noted that almost all larvae with a non-inflated swimbladder had a spinal curvature and it could therefore not statistically be determined whether the reduced swimming speed was due to a spinal curvature, a non-inflated swim bladder or the interaction of both.

- Knockdown of deiodinase 3 (expected to lead to hyperthyroidism) in zebrafish was shown to result in both impaired inflation of the posterior chamber and reduced swimming activity and escape response (Heijlen et al., 2014; Bagci et al., 2015).
- Massei et al. (in preparation) showed that impaired swim bladder inflation and reduced swimming activity of 5 day old zebrafish larvae were correlated after exposure to narcotics.

Uncertainties and Inconsistencies

Robertson et al., (2007) reported that the swim bladder only becomes functional as a buoyancy regulator when it is fully developed into a double-chambered swim bladder. This implies that effects on posterior chamber inflation would not directly result in effects on swimming capacity. However, it was also reported that gas in the swim bladder increases the buoyancy of zebrafish larvae already just after initial inflation, while it would be actively controlled only after 28–30 d post hatch. Therefore, an effect on swimming capacity is still likely.

Exposure of zebrafish embryos to 6-propylthiouracil (PTU) resulted in an effect on posterior chamber inflation, but did not result in a direct effect on the swimming distance in the larval stage (Stinckens et al., unpublished). Vergauwen et al. (2015) reported decreased swimming activity as well as impaired posterior chamber inflation after exposure to phenanthrene, a non-polar narcotic, but there was no significant difference between swimming activity of larvae with our without inflated posterior chamber within the same treatment. Possibly, the impact of baseline toxicity on respiration and energy metabolism was more important in decreasing swimming activity compared to impaired inflation of the posterior chamber.

It has been difficult to unambiguously attribute reduced swimming activity to impaired inflation of the posterior chamber, since swimming activity can be altered via different modes of action including altered energy metabolism, altered brain development and thus swimming behaviour. For example, the swimming activity of zebrafish larvae was reduced after 5 days of exposure to 2-mercaptobenzothiazole (MBT), while they had inflated posterior chambers.

Quantitative Understanding of the Linkage

The quantitative understanding of the linkage between impaired posterior chamber inflation and effect on swimming behaviour is limited.

Response-response relationship

Relations between reduced swim bladder inflation and reduced swimming performance are currently based on a binary observation of swim bladder inflation. Several studies have shown that larvae with inflated swim bladders have higher swiming activity compared to larvae that failed to inflate the swim bladder. No direct relationship between swim bladder surface (quantitative measure of swim bladder inflation) and swimming performance has been reported yet.

Time-scale

The data of Michiels et al. (2017) and Stinckens et al. (unpublished) on swim bladder inflation and swimming activity have been collected on the same day. The process of posterior chamber inflation normally occurs during a specific developmental time frame, resulting in limited flexibility to explore

temporal concordance. Based on the biologically plausible direct importance of swim bladder functionality to swimming performance, no lag is expected.

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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	Link
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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Stinckens, E., Vergauwen, L., Blackwell, B.R., Anldey, G.T., Villeneuve, D.L., Knapen, D., 2020. Effect of Thyroperoxidase and Deiodinase Inhibition on Anterior Swim Bladder Inflation in the Zebrafish. Environmental Science & Technology 54, 6213-6223.

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: 1042: Inhibition, Deiodinase 2 leads to Reduced, Posterior swim bladder inflation

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Embryo	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).

Much of the evidence for impaired posterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2018; Cavallin et al., 2017; Wang et al., 2020), but this KE is plausibly applicable across fish species with swim bladders, both physostomous and physoclistous.

Sex: This KE/KER is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In Medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In 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. 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 posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, 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 posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

Life stage: This KER is only applicable to early embryonic development, which is the period where the posterior swim bladder chamber inflates.

Key Event Relationship Description

The two major thyroid hormones are thyroxine (T4) and the more biologically active triiodothyronine (T3), both iodinated derivatives of tyrosine. Active and inactive THs are tightly regulated by enzymes called iodothyronine deiodinases (DIO). The activation occurs via outer ring deiodination (ORD), i.e. removing iodine from the outer, phenolic ring of T4 to form T3, while inactivation occurs via inner ring deiodination (IRD), i.e. removing iodine from the inner tyrosol ring of T4 or T3.

Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of TH action. All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain selenocysteine in their catalytic centre. Type I deiodinase is capable of converting T4 into T3, as well as to convert rT3 to the inactive thyroid hormone 3,3’ T2, through outer ring deiodination. rT3, rather than T4, is the preferred substrate for DIO1. furthermore, DIO1 has a very high Km (µM range, compared to nM range for DIO2) (Darras and Van Herck, 2012). Type II deiodinase (DIO2) is only capable of ORD activity with T4 as a preferred substrate (i.e., activation of T4 tot T3). DIO3 can inner ring deiodinate T4 and T3 to the inactive forms of THs, reverse T3, (rT3) and 3,3’-T2 respectively. (Darras and Van Herck, 2012)

Inhibition of DIO2 therefore results in decreased T3 levels. Since swim bladder development and/or inflation is regulated by thyroid hormones, this results in impaired posterior chamber inflation.

Evidence Supporting this KER

There is convincing evidence that inhibition of DIO activity, either through specific knockdown or through chemical exposure, results in impaired posterior chamber inflation, but the underlying mechanisms are not completely understood, including the relative importance of DIO1 and DIO2. Based on current evidence, it seems that DIO2 is more important in regulating posterior chamber inflation. Due to the difficulty of measuring DIO activity in small fish embryos, quantitative linkages and temporal concordance have been difficult to establish. The quantitative understanding is currently based on a relationship between the classification of chemicals according to their in chemico DIO inhibitory potential (using a threshold and uncertainty zone) on the one hand, and occurence of in vivo effects on posterior chamber inflation on the other hand. Predictions based on this relationship have been proven highly successful. Therefore the evidence supporting this KER can be considered moderate.

Biological Plausibility

Inhibition of DIO 2 activity is widely accepted to reduce the conversion of T4 to the more biologically active T3. 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 posterior swim bladder chamber is part of the embryonic-to-larval transition in fish, together with structural and functional maturation of the mouth and gastrointestinal tract, and resorption of the yolk sac. Together with empirical evidence, it is plausible to assume that posterior swim bladder inflation is under thyroid hormone regulation but scientific understanding is incomplete. It follows that disrupted conversion of T4 to T3 is likely to interfere with normal inflation of the posterior swim bladder chamber.

Empirical Evidence

Deiodinases are critical for normal development. Several defects have already been reported in cases where the TH hormone balance is disturbed. Winata et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in DIO1+2 and DIO2 knockdown fish. These effects were rescued after T3 supplementation, indicating the importance of T4 to T3 conversion by deiodinases.

Substantial evidence for the link between deiodinase inhibition and impaired posterior chamber inflation is available:

- Chang et al., (2012) established a base-line for TH levels during zebrafish development and observed peaks in whole-body T3 content at 5 dpf when the posterior chamber of the swim bladder inflates.
- Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of DIO1+2 in zebrafish resulted in impairment ofthe inflation of the posterior chamber of the swim bladder.
- Permanent DIO 2 deficiency in zebrafish was shown to result in reduced posterior chamber inflation (Houbrechts et al., 2016). DIO1 and DIO2 mRNA has also been shown to be present in zebrafish swim bladder tissue at 96 hpf using whole mount insitu hybridization (Heijlen et al., 2013; Dong et al., 2013), suggesting a tissue-specific role of T3 in the inflation process of the posterior chamber.
- Propylthiouracil (PTU) decreased serum T3 levels in the rat (Frumess and Larsen, 1975) and resulted in effects on posterior chamber inflation in zebrafish (Jomaa et al., 2014; Stinckens et al., 2018). It should be noted that there are some uncertainties related to the species-specific susceptibility of DIO1 to inhibition by PTU, as teleostean DIO1 seems to be lesssensitive to inhibition by PTU (Orozco and Valverde, 2005; Kuiper et al., 2006; Orozco et al., 2012).
- Stinckens et al. (2018) showed that effects on posterior chamber inflation in zebrafish could be predicted based on in chemico DIO2 inhibition potential with only few false positives and false

negatives.

- After exposure of fathead minnows (Pimephales promelas) to the non-specific deiodinase inhibitor IOP from 1-6 dpf, Incidence and length of inflated posterior swim bladders were significantly reduced (Cavallin et al., 2017).
- While DIO1 has a high Km and rT3 is its preferred substrate, DIO2 has a low Km and T4 is its preferred substrate, indicatingthat DIO2 is more important than DIO1 in converting T4 to T3 in a physiological situation (Darras and Van Herck, 2012). It follows that DIO2 inhibition is likely more important than DIO1 inhibition in reducing posterior chamber inflation.

Uncertainties and Inconsistencies

The mechanism through which altered TH levels result in impaired posterior chamber inflation still needs to be elucidated.

It is currently unclear which aspect of swim bladder development and inflation is affected by TH disruption. Based on the developmental stages of the posterior chamber, several hypotheses could explain effects on posterior chamber inflation due to disrupted TH levels. A first hypothesis includes effects on the budding of the posterior chamber inflation. Secondly, the effect on posterior chamber inflation could also be caused by disturbing the formation and growth of the three tissue layers of this organ. It has been reported that the Hedgehog signalling pathway plays an essential role in swim bladder development and is required for growth and differentiation of cells of the swim bladder. The Wnt/β-catenin signalling pathway is required for the organization and growth of all three tissue layers (Yin et al., 2011, 2012, Winata 2009, Kress et al., 2009). Both signalling pathways have been related to THs in amphibian and rodent species (Kress et al., 2009; Plateroti et al., 2006; Stolow and Shi, 1995). Several other hypotheses include effects on the successful initial inflation of the posterior chamber, effects on lactic acid production that is required for the maintenance of the swim bladder volume, or effects on the production of surfactant that is crucial to maintain the surface tension necessary for swim bladder inflation.

Another uncertainty lies in the relative importance of the different T4 activating iodothyronine deiodinases (DIO1, DIO2) in regulating swim bladder inflation. Stinckens et al. (2018) showed that when exposing zebrafish embryos to seven strong DIO1 inhibitors (measured using in chemico enzyme inhibition assays), six out of seven compounds impaired posterior chamber inflation, but almost all of these compounds also inhibit DIO2. Tetrachlorobisphenol A (TCBPA), the only compound that inhibits DIO1 and not DIO2, had no effect on the posterior swim bladder. Exposure to strong DIO2 inhibitors on the other hand affected posterior chamber inflation and/or surface area in all cases. These results suggest that DIO2 enzymes may play a more important role in swim bladder inflation compared to DIO1 enzymes. In the ToxCast DIO2 inhibition single concentration assay, 304 out of 1820 chemicals were positive and 177 of these were also positive for DIO1 inhibition (viewed on 5/7/2022). This complicates the distinction between the relative contribution of DIO1 and DIO2 inhibition to reduced swim bladder inflation. It has been previously suggested that DIO2 is the major contributor to TH activation in developing zebrafish embryos (Darras et al., 2015; Walpita et al., 2010). It has been shown that a morpholino knockdown targeting DIO1 mRNA alone did not affect embryonic development in zebrafish, while knockdown of DIO2 delayed progression of otic vesicle length, head-trunk angle and pigmentation index (Houbrechts et al., 2016; Walpita et al., 2010, 2009). DIO1 inhibition may only become essential in hypothyroidal circumstances, for example when DIO2 is inhibited or in case of iodine deficiency, in zebrafish (Walpita et al., 2010) and mice (Galton et al., 2009; Schneider et al., 2006).

Heijlen et al. (2015) reported histologically abnormal tissue layers in the swim bladder of DIO3 knockdown zebrafish. As reported in Bagci et al. (2015) and Heijlen et al. (2014), posterior chamber inflation was impaired in DIO3 knockdown zebrafish. DIO3 is a thyroid hormone inactivating enzyme, which would result in higher levels of T3. This indicates that not only too low, but also too high T3 levels, impact posterior chamber inflation. The underlying mechanism is currently unknown.

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Relationship: 2213: Reduced, Posterior swim bladder inflation 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	Non-adjacent	High	Low
Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Non-adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Taxonomic: The literature provides strong support for the relevance of this KER for physoclistous fish (e.g., yellow perch, Japanese Medaka) whose inflation occurs at a critical time in development when the fish must gulp air to inflate its swim bladder before the pneumatic duct closes. The relevance to physostomes (such as zebrafish and fathead minnows) that maintain an open pneumatic duct into adulthood is less apparent. The latter likely have greater potential to inflate the swim bladder at some point in development, even if early larval inflation is impaired. However, it is plausible that structural damage that prevented inflation of the organ in a phystostome would be expected to cause similar effects.

Life stage: This KER is applicable to early embry-larval development, which is the period where the posterior swim bladder chamber inflates and larvae start to freely feed. To what extent fish can survive with partly inflated swim bladders during later life stages is unknown.

Sex: This KER is probably not sex-dependent since both females and males rely on the posterior swim bladder chamber to regulate buyoancy. Furthermore, 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 posterior chamber inflates around 5 days post fertilization, when sex differentiation has not started yet, sex differences are expected to play a minor role.

Key Event Relationship Description

Because of its roles in energy sparing and swimming performance, it is expected that failure to inflate the swim bladder would create increased oxygen and energy demands leading to decreased growth, which in turn leads to decreased probability of survival.

Evidence Supporting this KER

There is strong evidence for a link between reduced posterior chamber inflation and increased mortality across different fish species.

Biological Plausibility

The posterior chamber of the swim bladder has a function in regulating the buoyancy of fish (Roberston et al., 2007). Fish rely on the lipid and gas content in their body to regulate their position within the water column. Efficient regulation of buoyancy is energy sparing and allows for fish to expend less energy in maintaining and changing positions in the water column. Because of its roles in energy sparing and swimming performance, it is expected that failure to inflate the swim bladder would create increased oxygen and energy demands leading to decreased growth, which in turn leads to decreased probability of survival. In particular, these impacts would be expected in non-laboratory environments where fish must expend energy to capture food and avoid predators and where available food is limited. Additionally, fish without a functional swim bladder are severely disadvantaged in terms of foraging and avoiding predators, making the likelihood of surviving smaller.

Empirical Evidence

- Czesny et al. (2005) demonstrated that swim bladder non-inflation was associated with multiple phenotypic and behavioral outcomes that would be expected to adversely impact survival.
    - Yellow perch with non-inflated swim bladders grew more slowly than those with inflated swim bladders, both in the laboratory and in the field.
    - Yellow perch with non-inflated swim bladders always captured prey less efficiently than those with inflated swim bladders of the same size class.
    - Yellow perch with non-inflated swim bladders suffered from increased predation risk.
    - Yellow perch with non-inflated swim bladders experienced significantly increased mortality

and lower time to mortality in a foodless environment compared to those with inflated swim bladders, indicating greater energy expenditure.
    - Yellow perch with non-inflated swim bladders had significantly greater oxygen consumption than fish of the same size class with inflated swim bladders, again indicating greater energy expenditure.
    - The authors hypothesized that failed swim bladder inflation occurs frequently in natural systems, but these individuals rarely survive in a natural environment where food resources are limited.
    - Note: yellow perch are a physoclistous species in which initial inflation can only occur during a narrow window of development in which the pneumatic duct is still connected to the gut, allowing the fish to gulp air and inflate its swim bladder. Once the pneumatic duct closes, normal inflation is no longer possible.
- In aquaculture systems, failure to inflate the swim bladder has been shown to reduce growth rates and cause high mortalities in a wide range of species (reviewed by Woolley and Qin, 2010).
- Pond-cultured walleye with non-inflated swim bladders were found to be smaller (weight and length) than fish with inflated swim bladders. There was also association with deformities (e.g., lordosis) that were expected to impair survival (Kindschi and Barrows, 1993).
- Review of failed swim bladder inflation in wild perch and 26 other physoclistous species showed that fish whose swim bladders failed to inflate had higher mortality, reduced growth, and increased incidence of spinal malformations stereotypical of persistent upward swimming (Egloff, 1996).
- Chatain (1994) reported that sea bream (Sparus auratus) and sea bass (Dicentrarchus labrax) with non-inflated swim bladders were 20-30% less in weight than those with inflated swim bladders and more susceptible to stress-induced mortality (e.g., associated with handling, hypoxia, etc.). It was suggested this was due to both increased energetic demands and decreased feeding efficiency.
- Marty et al. 1995 measured increased oxygen consumption in Japanese medaka (Oryzias latipes) with non-inflated swim bladders compared to those whose swim bladders had inflated.
- In zebrafish (Danio rerio) whose smim bladder inflation was prevented by holding in a closed chamber (preventing air gulping to inflate the swim bladder), larval survival was significantly less than that of fish held in open chambers whose swim bladders could inflate. There was also increased incidence of spinal curvature in the closed chamber fish whose swim bladders were prevented from inflating (Goolish and Oukutake, 1999).
- Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass (Morone saxatilis) lead to significant increases in both swim bladder inflation and survival (Brown et al., 1988).
- In striped bass, (Morone saxatilis) failure to inflate the swimbladder was reported to results in dysfunctional buoyancy control, deformities, and poor larval survival and growth (Martin-Robichaud and Peterson, 2008).
- 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, it is plausible to assume that uninflated posterior chambers limited the ability to swim and find food.
- MeHg and HgCl2 exposure in medaka caused failure to inflate the swim bladder among other malformations, and also caused increased mortality. (Dong et al., 2016)
- Medaka embryos treated either with hypoxia or with a mixture of polyaromatic hydrocarbons showed higher occurrences of swim bladder non-inflation and decreased survival. (Mu et al., 2017)
- Triphenyltin (TPT) exposure in zebrafish embryos induced a high percentage of uninflated swim bladders and all affected larvae died within 9 dph. (Horie et al., 2021)

Uncertainties and Inconsistencies

Some studies showed an absence of increased mortality after impaied posterior chamber inflation but this is probably caused by the fact that observation was limited to short term effects (e.g., Wang et al., 2020). Observations of absence of mortality often performed at 96/120 hpf in zebrafish, which is immediately after posterior chamber inflation.

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Annex 1: Weight of evidence evaluation table

AOP 155: Deiodinase 2 inhibition leading to increased mortality via posterior 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 1026: Inhibition, Deiodinase 2 (KE 1002) leads to Decreased, Triiodothyronine (T3) in serum (KE 1003)	**Moderate** Inhibition of DIO2 activity is widely accepted to directly decrease T3 levels, since the conversion of T4 to T3 is inhibited. 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. The importance of DIO2 inhibition in altering serum T3 levels depends on the relative role of different deiodinases in regulating serum versus tissue T3 levels and in negative feedback within the HPT axis, where some uncertainties remain.			
Relationship 1027: Decreased, Triiodothyronine (T3) in serum (KE 1003) leads to Reduced, Posterior swim bladder inflation (KE 1004)	**Moderate** 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 posterior swim bladder chamber is part of the embryonic-to-larval transition in fish, together with structural and functional maturation of the mouth and gastrointestinal tract, and resorption of the yolk sac. Together with empirical evidence, it is plausible to assume that posterior swim bladder inflation is under thyroid hormone regulation but scientific understanding is incomplete.			
Relationship 1028: Reduced, Posterior swim bladder inflation (KE 1004) leads to Reduced, Swimming performance (KE 1005)	**Moderate** The posterior chamber of the swim bladder has a function in regulating the buoyancy of fish. It is highly plausible that impaired inflation impacts swimming performance. There is a lot of evidence of such a relationship but it has been difficult to unambiguously attribute reduced swimming activity to impaired inflation of the posterior chamber, since swimming activity can be altered via different modes of action.			
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 1042: Inhibition, Deiodinase 2 (KE 1002) leads to Reduced, Posterior swim bladder inflation (KE 1004)	**Moderate** Inhibition of DIO 2 activity is widely accepted to reduce the conversion of T4 to the more biologically active T3. 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 posterior swim bladder chamber is part of the embryonic-to-larval transition in fish, together with structural and functional maturation of the mouth and gastrointestinal tract, and resorption of the yolk sac. Together with empirical evidence, it is plausible to assume that posterior swim bladder inflation is under thyroid hormone regulation but scientific understanding is incomplete. It follows that disrupted conversion of T4 to T3 is likely to interfere with normal inflation of the posterior swim bladder chamber.			
Non-adjacent relationship 2213: Reduced, Posterior swim bladder inflation (KE 1004) leads to Increased mortality (KE 351)	**High** The posterior chamber of the swim bladder has a function in regulating the buoyancy of fish. Fish rely on the lipid and gas content in their body to regulate their position within the water column. Efficient regulation of buoyancy is energy sparing and allows for fish to expend less energy in maintaining and changing positions in the water column. Because of its roles in energy sparing and swimming performance, it is expected that failure to inflate the swim bladder would create increased oxygen and energy demands leading to decreased growth, which in turn leads to decreased probability of survival. In particular, these impacts would be expected in non-laboratory environments where fish much expend energy to capture food and avoid predators and where available food is limited. Additionally, fish without a functional swim bladder are severely disadvantaged in terms of foraging and avoiding predators, making the likelihood of surviving smaller. There is ample evidence showing that impaired posterior chamber inflation reduces survival.			

AOP 155: Deiodinase 2 inhibition leading to increased mortality via posterior 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 1002 (MIE): Inhibition, deiodinase 2	Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of Dio1+2 in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Permanent Dio2 knockout also impaired swim bladder inflation and locomotor activity in zebrafish (Houbrechts et al., 2016). 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. This confirms that DIO2 is essential for causing downstream effects. These effects were rescued after T3 supplementation but not after T4 supplementation, confirming the importance of T4 to T3 conversion by Dio2 and the essentiality of DIO2 inhibition for causing downstream effects (Walpita et al., 2009, 2010).			
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 (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.			
KE 1004: Reduced, posterior swim bladder inflation	Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass (Morone saxatilis) lead to significant increases in both swim bladder inflation and survival (Brown et al., 1988), confirming the essentiality of posterior swim bladder inflation for the occurrence of the downstream key event 'reduced young of year survival'.			
KE 1005: Reduced, swimming performance	Experimental blocking of this KE is difficult to achieve.			
KE 351: Increased mortality	By definition, increased mortality reduces population size.			
AOP as a whole	**High** Overall, the support for essentiality of the KEs is high since there is direct evidence from specifically designed experimental studies illustrating essentiality for several of the important KEs in the AOP. This includes ample evidence from knockdown studies in zebrafish that use targeted perturbation of key events and show downstream effects, and evidence from both chemical exposure with TH supplementation and knockdown with TH supplementation showing that blocking a KE prevents downstream KEs from occurring.			

	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 1026: Inhibition, Deiodinase 2 (KE 1002) leads to Decreased, Triiodothyronine (T3) in serum (KE 1003)	**Low** Although direct measurements of both KEs in the same organisms are not available in fish, several studies have shown that chemicals able to inhibit DIO2 in vitro, reduce T3 levels. The relative importance of DIO2 versus DIO1 is uncertain, but available evidence suggests that DIO2 is more important.			
Relationship 1027: Decreased, Triiodothyronine (T3) in serum (KE 1003) leads to Reduced, Posterior swim bladder inflation (KE 1004)	**Moderate** Many studies showed that chemicals reducing TH synthesis or activation inhibit proper posterior chamber inflation but studies reporting measurements of both endpoints are rare. 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 1028: Reduced, Posterior swim bladder inflation (KE 1004) leads to Reduced, Swimming performance (KE 1005)	**Moderate** There is ample evidence of a link between reduced posterior chamber inflation and reduced swimming performance. This link has been studied both from an aquaculture perspective as well as in chemical exposure experiments. Evidence of dose concordance is limited. Temporal concordance is difficult to establish since swim bladder inflation can only occur at a specific time point.			
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 1042: Inhibition, Deiodinase 2 (KE 1002) leads to Reduced, Posterior swim bladder inflation (KE 1004)	**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 DIO2 in vitro, reduce posterior chamber inflation. The relative importance of DIO1 versus DIO2 is uncertain, but available evidence suggests that DIO2 is more important. The mechanism through which DIO2 inhibition results in impaired posterior chamber inflation is uncertain.			
Non-adjacent relationship 2213: Reduced, Posterior swim bladder inflation (KE 1004) leads to Increased mortality (KE 351)	**Moderate** There is a extensive evidence of a link between reduced posterior chamber inflation and increased mortality based on studies in freshwater and marine fish species. Uncertainties are related to the dependence of the linkage on the circumstances such as food availability and predation.			

							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£	320 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

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