OECD Series on Adverse Outcome Pathways No. 23

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

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OECD

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AOP No. 156 in the AOP-Wiki platform

Foreword

This Adverse Outcome Pathway (AOP) on Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Party of the National Coordinators for the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA).

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

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

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

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

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

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Abstract

This AOP describes the sequence of events leading from deiodinase inhibition to increased mortality via reduced anterior 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 disruption of developmental processes. 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. 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. Thyroid hormones are critical in regulating developmental processes and thyroid hormone disruption can interfere with normal development. Swim bladder inflation is known to be under TH control (Brown et al., 1988; Liu and Chan, 2002). Many fish species have a swim bladder which is a gas-filled organ that typically consists of two chambers (Robertson et al., 2007). The posterior chamber inflates during early development in the embryonic phase, while the anterior chamber inflates during late development in the larval phase. Both the posterior and the anterior chamber have an important role in regulating buoyancy, and the anterior chamber has an additional role in hearing (Robertson et al., 2017). This AOP describes how inhibition of DIO2 reduces levels of T3, thereby prohibiting proper inflation of the anterior chamber. Due to its role in regulating buoyancy, this results in reduced swimming performance. Since reduced swimming performance results in a decreased ability to forage and avoid predators, this reduces chances of survival. The final adverse outcome is a decrease of the population growth rate. Since many AOPs eventually lead to this more general adverse outcome at the population level, the more specific and informative adverse outcome at the organismal level, increased mortality, is used in the AOP title. Support for this AOP is mainly based on chemical exposures in zebrafish and fathead minnows (Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020).

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 anterior swim bladder inflation (https://aopwiki.org/aops/158). The overall importance of DIO1 versus DIO2 in fish is not exactly clear. 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). 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. Six out of seven DIO1 inhibitors impaired posterior chamber inflation, but almost all of these compounds also inhibit DIO2 (Stinckens et al., 2018)). 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. Therefore the current AOP may be of higher biological relevance compared to AOP 158. Starting from reduced T3 levels, this AOP is identical to the AOP leading from thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159).

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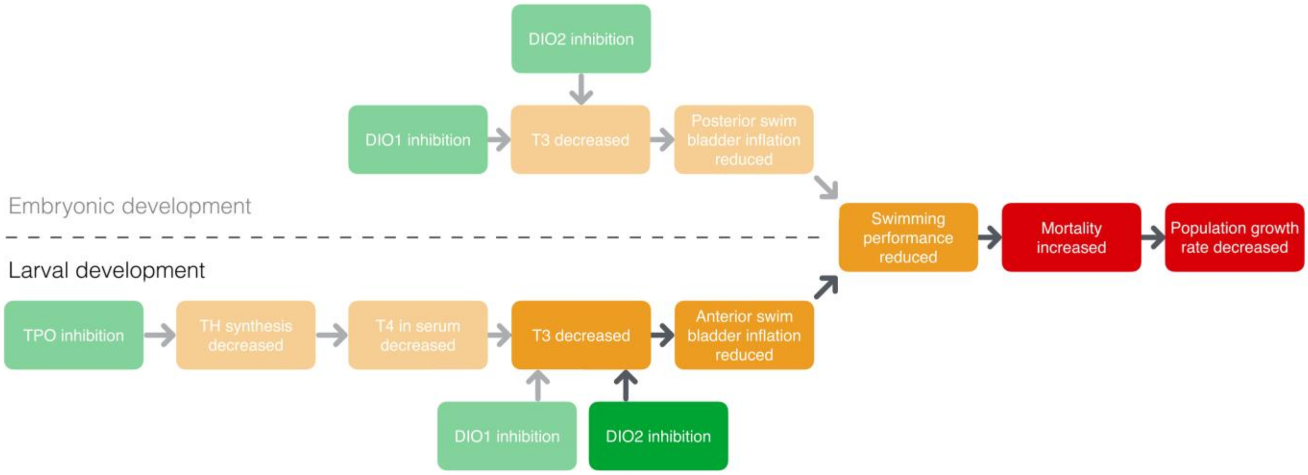
Background

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

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

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

Graphical Representation



Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1002	Inhibition, Deiodinase 2	Inhibition, Deiodinase 2
2	KE	1003	Decreased, Triiodothyronine (T3)	Decreased, Triiodothyronine (T3)
3	KE	1007	Reduced, Anterior swim bladder inflation	Reduced, Anterior 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

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Deiodinase 2	adjacent	Decreased, Triiodothyronine (T3)	Moderate	Low
Decreased, Triiodothyronine (T3)	adjacent	Reduced, Anterior swim bladder inflation	Moderate	Moderate
Reduced, Anterior swim bladder inflation	adjacent	Reduced, Swimming performance	Moderate	Low
Reduced, Swimming performance	adjacent	Increased Mortality	Moderate	Low
Increased Mortality	adjacent	Decrease, Population growth rate	Moderate	Moderate

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
Larvae	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	Moderate

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

Life stage: The current AOP is only applicable to larval development, which is the period where the anterior 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 (https://aopwiki.org/aops/155) and late developmental life stages.

Sex: All key events in this AOP are plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes across the sequence of events in this AOP. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role in the current AOP. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

Essentiality of the Key Events

Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is evidence from deiodinase knockdowns showing the link with reduced posterior chamber inflation and the essentiality for downstream effects, but anterior chamber inflation was not studied. There is additional indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation. It should be noted that dual oxidase also plays a role in oxidative stress. There is also evidence that alleviation of the effect on anterior chamber inflation reduces the effect on swimming performance.

Weight of Evidence Summary

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

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

Quantitative Consideration

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

Considerations for Potential Applications of the AOP

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

Thyroid hormone system disruption causes multiple unspecific effects. Addition of TH measurements could aid in increasing the diagnostic capacity of a battery of endpoints since they are specific to the TH system. A battery of endpoints would ideally include the MIE, the AO and TH levels as the causal link. It is also in this philosophy that TH measurements are currently being considered as one of the endpoints in project 2.64 of the OECD TG work plan, "Inclusion of thyroid endpoints in OECD fish Test Guidelines". While 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).

References

Brown, C.L., Doroshov, S.I., Nunez, J.M., Hadley, C., Vaneenennaam, J., Nishioka, R.S., Bern, H.A., 1988. MATERNAL TRIIODOTHYRONINE INJECTIONS CAUSE INCREASES IN SWIMBLADDER INFLATION AND SURVIVAL RATES IN LARVAL STRIPED BASS, MORONE-SAXATILIS. Journal of Experimental Zoology 248, 168-176.

Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry 36, 2942-2952.

Godfrey, A., Hooser, B., Abdelmoneim, A., Horzmann, K.A., Freemanc, J.L., Sepulveda, M.S., 2017. Thyroid disrupting effects of halogenated and next generation chemicals on the swim bladder development of zebrafish. Aquatic Toxicology 193, 228-235.

Knapen, D., Angrish, M.M., Fortin, M.C., Katsiadaki, I., Leonard, M., Margiotta-Casaluci, L., Munn, S., O'Brien, J.M., Pollesch, N., Smith, L.C., Zhang, X.W., Villeneuve, D.L., 2018. Adverse outcome pathway networks I: Development and applications. Environmental Toxicology and Chemistry 37, 1723-1733.

Knapen, D., Stinckens, E., Cavallin, J.E., Ankley, G.T., Holbech, H., Villeneuve, D.L., Vergauwen, L., 2020. Toward an AOP Network-Based Tiered Testing Strategy for the Assessment of Thyroid Hormone Disruption. Environmental Science & Technology 54, 8491-8499.

Liu, Y.W., Chan, W.K., 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. Differentiation 70, 36-45.

Maack, G., Segner, H., 2003. Morphological development of the gonads in zebrafish. Journal of Fish Biology 62, 895-906.

Nagabhushana A, Mishra RK. 2016. Finding clues to the riddle of sex determination in zebrafish. Journal of Biosciences. 41(1):145-155.

Olmstead AW, Villeneuve DL, Ankley GT, Cavallin JE, Lindberg-Livingston A, Wehmas LC, Degitz SJ. 2011. A method for the determination of genetic sex in the fathead minnow, pimephales promelas, to support testing of endocrine-active chemicals. Environmental Science & Technology. 45(7):3090-3095.

Robertson, G.N., McGee, C.A.S., Dumbarton, T.C., Croll, R.P., Smith, F.M., 2007. Development of the swimbladder and its innervation in the zebrafish, Danio rerio. Journal of Morphology 268, 967-985.

Stinckens, E., Vergauwen, L., Ankley, G.T., Blust, R., Darras, V.M., Villeneuve, D.L., Witters, H., Volz, D.C., Knapen, D., 2018. An AOP-based alternative testing strategy to predict the impact of thyroid hormone disruption on swim bladder inflation in zebrafish. Aquatic Toxicology 200, 1-12.

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.

Stinckens, E., Vergauwen, L., Schroeder, A., Maho, W., Blackwell, B., Witters, H., Blust, R., Ankley, G., Covaci, A., Villeneuve, D., Knapen, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part II: Zebrafish. Aquatic Toxicology 173, 204-217.

van Aerle R, Runnalls TJ, Tyler CR. 2004. Ontogeny of gonadal sex development relative to growth in fathead minnow. Journal of Fish Biology. 64(2):355-369.

Villeneuve, D., Angrish, M., Fortin, M., Katsiadaki, I., Leonard, M., Margiotta-Casaluci, L., Munn, S., O'Brien, J., Pollesch, N., Smith, L., Zhang, X., Knapen, D., 2018. Adverse Outcome Pathway Networks II: Network Analytics. Environ Toxicol Chem doi: 10.1002/etc.4124.

Villeneuve, D., Volz, D.C., Embry, M.R., Ankley, G.T., Belanger, S.E., Leonard, M., Schirmer, K., Tanguay, R., Truong, L., Wehmas, L., 2014. Investigating alternatives to the fish early-life stage test: a strategy for discovering and annotating adverse outcome pathways for early fish development. Environmental Toxicology and Chemistry 33, 158-169.

Woolley, L.D., Qin, J.G., 2010. Swimbladder inflation and its implication to the culture of marine finfish larvae. Reviews in Aquaculture 2, 181-190.

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	Molecular Event	Initiating
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Molecular Event	Initiating
Aop:190 - Type II iodothyronine deiodinase (DIO2) inhibition leading to altered amphibian metamorphosis	Molecular Event	Initiating

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). Another 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). 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	Links
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. Tetrachlorobisphenol A (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, reverse T3, (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) in a 96-well plate format. 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 decabromodiphenyl ether (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.

References

Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J., Larsen, P.R., 2002. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocrine Reviews 23, 38-89.

Cavallin JE, Ankley GT, Blackwell BR, Blanksma CA, Fay KA, Jensen KM, Kahl MD, Knapen D, Kosian PA, Poole ST et al. 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry. 36(11):2942-2952.

Darras, V.M., Van Herck, S.L.J., 2012. Iodothyronine deiodinase structure and function: from ascidians to humans. Journal of Endocrinology 215, 189-206.

Forhead, A.J., Curtis, K., Kaptein, E., Visser, T.J., Fowden, A.L., 2006. Developmental control of iodothyronine deiodinases by cortisol in the ovine fetus and placenta near term. Endocrinology 147, 5988-5994.

Houbrechts, A.M., Delarue, J., Gabriels, I.J., Sourbron, J., Darras, V.M., 2016. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. Endocrinology 157, 3668-3681.

Li, N.N., Jiang, Y.Q., Shan, Z.Y., Teng, W.P., 2012. Prolonged high iodine intake is associated with inhibition of type 2 deiodinase activity in pituitary and elevation of serum thyrotropin levels. British Journal of Nutrition 107, 674-682.

Noyes PD, Hinton DE, Stapleton HM. 2011. Accumulation and debromination of decabromodiphenyl ether (bde-209) in juvenile fathead minnows (pimephales promelas) induces thyroid disruption and liver alterations. Toxicological Sciences. 122(2):265-274.

Olker, J.H., Korte, J.J., Denny, J.S., Hartig, P.C., Cardon, M.C., Knutsen, C.N., Kent, P.M., Christensen, J.P., Degitz, S.J., Hornung, M.W., 2019. Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases. Toxicological Sciences 168, 430-442.

Orozco, A., Valverde, R.C., 2005. Thyroid hormone deiodination in fish. Thyroid 15, 799-813.

Pavelka, S., 2010. Radiometric enzyme assays: development of methods for extremely sensitive determination of types 1, 2 and 3 iodothyronine deiodinase enzyme activities. Journal of Radioanalytical and Nuclear Chemistry 286, 861-865.

Renko, K., Schache, S., Hoefig, C.S., Welsink, T., Schwiebert, C., Braun, D., Becker, N.P., Kohrle, J., Schomburg, L., 2015. An Improved Nonradioactive Screening Method Identifies Genistein and Xanthohumol as Potent Inhibitors of Iodothyronine Deiodinases. Thyroid 25, 962-968.

Roberts, S.C., Bianco, A.C., Stapleton, H.M., 2015. Disruption of Type 2 Iodothyronine Deiodinase Activity in Cultured Human Glial Cells by Polybrominated Diphenyl Ethers. Chemical Research in Toxicology 28, 1265-1274.

Stinckens, E., Vergauwen, L., Ankley, G.T., Blust, R., Darras, V.M., Villeneuve, D.L., Witters, H., Volz, D.C., Knapen, D., 2018. An AOP-based alternative testing strategy to predict the impact of thyroid hormone disruption on swim bladder inflation in zebrafish. Aquatic Toxicology 200, 1-12.

Stinckens E, Vergauwen L, Blackwell BR, Anldey GT, Villeneuve DL, Knapen D. 2020. Effect of thyroperoxidase and deiodinase inhibition on anterior swim bladder inflation in the zebrafish. Environmental Science & Technology. 54(10):6213-6223.

Vergauwen, L., Cavallin, J.E., Ankley, G.T., Bars, C., Gabriels, I.J., Michiels, E.D.G., Fitzpatrick, K.R., Periz-Stanacev, J., Randolph, E.C., Robinson, S.L., Saari, T.W., Schroeder, A.L., Stinckens, E., Swintek, J., Van Cruchten, S.J., Verbueken, E., Villeneuve, D.L., Knapen, D., 2018. Gene transcription ontogeny of hypothalamic-pituitary-thyroid axis development in early-life stage fathead minnow and zebrafish. General and Comparative Endocrinology 266, 87-100.

Walpita, C.N., Grommen, S.V., Darras, V.M., Van der Geyten, S., 2007. The influence of stress on thyroid hormone production and peripheral deiodination in the Nile tilapia (Oreochromis niloticus). Gen Comp Endocrinol 150, 18-25.

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

Biological Context

Level of Biological Organization
Tissue

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

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

Several studies have reported evidence of T3 decreases after exposure to TPO inhibitors and deiodinase inhibitors in early life stages of zebrafish (Stinckens et al., 2016; Stinckens et al., 2020; Wang et al., 2020) and fathead minnow (Nelson et al., 2016; Cavallin et al., 2017). Such measurements in fish early life stages are usually based on whole animal samples and do not allow for distinguishing between systemic and tissue TH alterations.THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in amphibian and lamprey metamorphoses (Manzon and Youson, 1997; Yaoita and Brown, 1990) as well as fish development, embryo-to-larval transition and larval-to-juvenile transition (Thienpont et al., 2011; Liu and Chan, 2002) is well established. Their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of TH in the different species may differ depending on the expression or function of specific proteins (e.g receptors or enzymes) that are related to TH function, and therefore extrapolation between species should be done with caution.

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

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

Key Event Description

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

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

There are major species differences in the predominant binding proteins and their affinities for THs (see section below on Taxonomic applicability). However, there is broad agreement that changes in 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 but 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 minnows are oviparous fish species in which maternal thyroid hormones are transferred to the eggs and regulate early embryonic developmental processes during external (versus intra-uterine in mammals) development (Power et al., 2001; Campinho et al., 2014; Ruuskanen and Hsu, 2018) until embryonic thyroid hormone synthesis is initiated. Maternal transfer of thyroid hormones, both T4 and

T3, to the eggs has been demonstrated in zebrafish (Walpita et al., 2007; Chang et al., 2012) and fathead minnows (Crane et al., 2004; Nelson et al., 2016).

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

How it is Measured or Detected

T3 and T4 can be measured as free (unbound) or total (bound + unbound) in serum, or in tissues. Free hormone are considered more direct indicators of T4 and T3 activities in the body. The majority of T3 and T4 measurements are made using either RIA or ELISA kits. In animal studies, total T3 and T4 are typically measured as the concentrations of free hormone are very low and difficult to detect.

Historically, the most widely used method in toxicology is RIA. The method is routinely used in rodent endocrine and toxicity studies. The ELISA method has become more routine in rodent studies. The ELISA method is a commonly used as a human clinical test method.

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

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

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

References

Anyetei-Anum, C.S., Roggero, V.R., Allison, L.A., 2018. Thyroid hormone receptor localization in target tissues. Journal of Endocrinology 237, R19-R34.

Bartalena L, Robbins J.Thyroid hormone transport proteins.Clin Lab Med. 1993 Sep;13(3):583-98.

Bassett JH, Harvey CB, Williams GR. (2003). Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol Cell Endocrinol. 213:1-11.

Bernal, J., Guadano-Ferraz, A., Morte, B., 2015. Thyroid hormone transporters-functions and clinical implications. Nature Reviews Endocrinology 11, 406-417.Bianco AC, Kim BW. (2006). Deiodinases: implications of the local control of thyroid hormone action. J Clin Invest. 116: 2571–2579.

Blanton ML, Specker JL. 2007. The hypothalamic-pituitary-thyroid (hpt) axis in fish and its role in fish development and reproduction. Crit Rev Toxicol. 37(1-2):97-115.

Brent GA. (2012). Mechanisms of thyroid hormone action. J Clin Invest. 122: 3035-3043.

Campinho MA, Saraiva J, Florindo C, Power DM. 2014. Maternal thyroid hormones are essential for neural development in zebrafish. Molecular Endocrinology. 28(7):1136-1149.

Cavallin JE, Ankley GT, Blackwell BR, Blanksma CA, Fay KA, Jensen KM, Kahl MD, Knapen D, Kosian PA, Poole ST et al. 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry. 36(11):2942-2952.

Chang J, Wang M, Gui W, Zhao Y, Yu L, Zhu G. 2012. Changes in thyroid hormone levels during zebrafish development. Zoological Science. 29(3):181-184.

Cheng SY, Leonard JL, Davis PJ. (2010).Molecular aspects of thyroid hormone actions. Endocr Rev. 31:139–170.

Crane HM, Pickford DB, Hutchinson TH, Brown JA. 2004. Developmental changes of thyroid hormones in the fathead minnow, pimephales promelas. General and Comparative Endocrinology. 139(1):55-60.

Davis PJ, Zhou M, Davis FB, Lansing L, Mousa SA, Lin HY. (2010). Mini-review: Cell surface receptor for thyroid hormone and nongenomic regulation of ion fluxes in excitable cells. Physiol Behav. 99:237–239.

Deal CK, Volkoff H. 2020. The role of the thyroid axis in fish. Frontiers in Endocrinology. 11.

DeVito M, Biegel L, Brouwer A, Brown S, Brucker-Davis F, Cheek AO, Christensen R, Colborn T, Cooke P, Crissman J, Crofton K, Doerge D, Gray E, Hauser P, Hurley P, Kohn M, Lazar J, McMaster S, McClain M, McConnell E, *Meier C, Miller R, Tietge J, Tyl R. (1999). Screening methods for thyroid hormone disruptors. Environ Health Perspect. 107:407-415.

Eales JG. (1997). Iodine metabolism and thyroid related functions in organisms lacking thyroid follicles: Are thyroid hormones also vitamins? Proc Soc Exp Biol Med. 214:302-317.

Eneqvist T, Lundberg E, Karlsson A, Huang SH, Santos CRA, Power DM, Sauer-Eriksson AE. 2004. High resolution crystal structures of piscine transthyretin reveal different binding modes for triiodothyronine and thyroxine. Journal of Biological Chemistry. 279(25):26411-26416.

Friesema EC, Jansen J, Milici C, Visser TJ. (2005). Thyroid hormone transporters. Vitam Horm. 70: 137–167.

Furlow JD, Neff ES. (2006). A developmental switch induced by thyroid hormone: Xenopus laevis metamorphosis. Trends Endocrinol Metab. 17:40–47.

Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, Zeöld A, Bianco AC. (2008). Cellular and molecular basis of deiodinase-regulated thyroid hormone signalling. Endocr Rev. 29:898–938.

Gilbert ME, Rovet J, Chen Z, Koibuchi N. (2012).Developmental thyroid hormone disruption: prevalence, environmental contaminants and neurodevelopmental consequences. Neurotoxicology. 33: 842-852.

Glinoer D. (2001).Potential consequences of maternal hypothyroidism on the offspring: evidence and implications. Horm Res. 55:109-114.

Hennemann G, Docter R, Friesema EC, de Jong M, Krenning EP, Visser TJ. (2001). Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. Endocr Rev. 22:451-476.

Hernandez-Mariano JA, Torres-Sanchez L, Bassol-Mayagoitia S, Escamilla-Nunez M, Cebrian ME, Villeda-Gutierrez EA, Lopez- Rodriguez G, Felix-Arellano EE, Blanco-Munoz J. 2017. Effect of exposure to p,p '-dde during the first half of pregnancy in the maternal thyroid profile of female residents in a mexican floriculture area. Environmental Research. 156:597-604.

Heyland A, Hodin J. (2004). Heterochronic developmental shift caused by thyroid hormone in larval sand dollars and its implications for phenotypic plasticity and the evolution of non-feeding development. Evolution. 58: 524-538.

Heyland A, Moroz LL. (2005). Cross-kingdom hormonal signaling: an insight from thyroid hormone functions in marine larvae. J Exp Biol. 208:4355-4361.

Hornung, M.W., Kosian, P.A., Haselman, J.T., Korte, J.J., Challis, K., Macherla, C., Nevalainen, E., Degitz, S.J., 2015. In Vitro, Ex Vivo, and In Vivo Determination of Thyroid Hormone Modulating Activity of Benzothiazoles. Toxicological Sciences 146, 254-264.

Hulbert A J. (2000). Thyroid hormones and their effects: A new perspective. Biol Rev. 75: 519-631.

Jansen J, Friesema EC, Milici C, Visser TJ. (2005). Thyroid hormone transporters in health and disease. Thyroid. 15: 757-768.

Larsen PR. (2009).Type 2 iodothyronine deiodinase in human skeletal muscle: new insights into its physiological role and regulation. J Clin Endocrinol Metab. 94:1893-1895.

Liu XS, Cai Y, Wang Y, Xu SH, Ji K, Choi K. 2019. Effects of tris(1,3-dichloro-2-propyl) phosphate (tdcpp) and triphenyl phosphate (tpp) on sex-dependent alterations of thyroid hormones in adult zebrafish. Ecotoxicology and Environmental Safety. 170:25-32.

Liu YW, Chan WK. 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. Differentiation. 70(1):36-45.

Manzon RG, Youson JH. (1997). The effects of exogenous thyroxine (T4) or triiodothyronine (T3), in the presence and absence of potassium perchlorate, on the incidence of metamorphosis and on serum T4 and T3 concentrations in larval sea lampreys (Petromyzon marinus L.). Gen Comp Endocrinol. 106:211-220.

Miller MD, Crofton KM, Rice DC, Zoeller RT. (2009).Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. Environ Health Perspect. 117:1033-1041.

Moeller LC, Dumitrescu AM, Seo H, Refetoff S. (2006). Thyroid hormone mediated changes in gene expression can be initiated by cytosolic action of the thyroid hormone receptor β through the phosphatidylinositol 3-kinase pathway. NRS. 4:1-4.

Nelson, K., Schroeder, A., Ankley, G., Blackwell, B., Blanksma, C., Degitz, S., Flynn, K., Jensen, K., Johnson, R., Kahl, M., Knapen, D., Kosian, P., Milsk, R., Randolph, E., Saari, T., Stinckens, E., Vergauwen, L., Villeneuve, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part I: Fathead minnow. Aquatic Toxicology 173, 192-203.

Obregon MJ, Mallol J, Escobar del Rey F, Morreale de Escobar G. (1981). Presence of l-thyroxine and 3,5,3-triiodo-l- thyronine in tissues from thyroidectomised rats. Endocrinology 109:908-913.

Oetting A, Yen PM. (2007). New insights into thyroid hormone action. Best Pract Res Clin Endocrinol Metab. 21:193–208.

Power DM, Llewellyn L, Faustino M, Nowell MA, Bjornsson BT, Einarsdottir IE, Canario AV, Sweeney GE. 2001. Thyroid hormones in growth and development of fish. Comp Biochem Physiol C Toxicol Pharmacol. 130(4):447-459.

Ruuskanen S, Hsu BY. 2018. Maternal thyroid hormones: An unexplored mechanism underlying maternal effects in an ecological framework. Physiological and Biochemical Zoology. 91(3):904-916.

Santos CRA, Power DM. 1999. Identification of transthyretin in fish (sparus aurata): Cdna cloning and characterisation. Endocrinology. 140(5):2430-2433.

Schussler, G.C. (2000). The thyroxine-binding proteins. Thyroid 10:141–149.

Stinckens E, Vergauwen L, Blackwell BR, Anldey GT, Villeneuve DL, Knapen D. 2020. Effect of thyroperoxidase and deiodinase inhibition on anterior swim bladder inflation in the zebrafish. Environmental Science & Technology. 54(10):6213-6223.

Stinckens, E., Vergauwen, L., Schroeder, A., Maho, W., Blackwell, B., Witters, H., Blust, R., Ankley, G., Covaci, A., Villeneuve, D., Knapen, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2- mercaptobenzothiazole part II: Zebrafish. Aquatic Toxicology 173, 204-217.

Thienpont B, Tingaud-Sequeira A, Prats E, Barata C, Babin PJ, Raldua D. 2011. Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. Environmental Science & Technology. 45(17):7525-7532

Visser WE, Friesema EC, Jansen J, Visser TJ. (2008). Thyroid hormone transport in and out of cells. Trends Endocrinol Metab. 19:50-56.

Walpita CN, Van der Geyten S, Rurangwa E, Darras VM. 2007. The effect of 3,5,3'-triiodothyronine supplementation on zebrafish (danio rerio) embryonic development and expression of iodothyronine deiodinases and thyroid hormone receptors. Gen Comp Endocrinol. 152(2-3):206-214.

Wang JX, Shi GH, Yao JZ, Sheng N, Cui RN, Su ZB, Guo Y, Dai JY. 2020. Perfluoropolyether carboxylic acids (novel alternatives to pfoa) impair zebrafish posterior swim bladder development via thyroid hormone disruption. Environment International. 134.

Webster GM, Venners SA, Mattman A, Martin JW. 2014. Associations between perfluoroalkyl acids (pfass) and maternal thyroid hormones in early pregnancy: A population-based cohort study. Environmental Research. 133:338-347.

Williams GR. (2008). Neurodevelopmental and neurophysiological actions of thyroid hormone. J Neuroendocrinol. 20:784–794.

Yamauchi K, Nakajima J, Hayashi H, Hara A. 1999. Purification and characterization of thyroid-hormone-binding protein from masu salmon serum - a homolog of higher-vertebrate transthyretin. European Journal of Biochemistry. 265(3):944-949.

Yamauchi K1, Ishihara A. Evolutionary changes to transthyretin: developmentally regulated and tissue-specific gene expression.FEBS J. 2009 Oct;276(19):5357-66.

Yaoita Y, Brown DD. (1990). A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. Genes Dev. 4:1917-1924.

Yen PM. (2001). Physiological and molecular basis of thyroid hormone action. Physiol Rev. 81:1097-1142.

Zhang J, Grundstrom C, Brannstrom K, Iakovleva I, Lindberg M, Olofsson A, Andersson PL, Sauer-Eriksson AE. 2018. Interspecies variation between fish and human transthyretins in their binding of thyroid-disrupting chemicals. Environmental Science & Technology. 52(20):11865-11874.´

Zoeller RT, Tan SW, Tyl RW. General background on the hypothalamic-pituitary-thyroid (HPT) axis. Crit Rev Toxicol. 2007 Jan- Feb;37(1-2):11-53.

Event: 1007: Reduced, Anterior swim bladder inflation

Short Name: Reduced, Anterior swim bladder inflation

Key Event Component

Process	Object	Action
swim bladder inflation	anterior chamber swim bladder	decreased

AOPs Including This Key Event

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

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
swim bladder

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

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

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

Sex: This KE/KER plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE/KER. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

Key Event Description

The swim bladder of bony fish is evolutionary homologous to the lung (Zheng et al., 2011). The teleost swim bladder is a gas-filled structure that consists of two chambers, the posterior and anterior chamber. In zebrafish, the posterior chamber inflates around 96 h post fertilization (hpf) which is 2 days post hatch, and the anterior chamber inflates around 21 dpf. In fathead minnow, the posterior and anterior chamber inflate around 6 and 14 dpf respectively. Inflation of the anterior swim bladder chamber is part of the larval-to-juvenile transition in fish, together with the development of adult fins and fin rays, ossification of the axial skeleton, formation of an adult pigmentation pattern, scale formation, maturation and remodeling of organs including the lateral line, nervous system, gut and kidneys (McMenamin and Parichy, 2013).

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

How it is Measured or Detected

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

References

Bang, P.I., Yelick, P.C., Malicko, J.J., Sewell, W.F. 2002. High-throughput behavioral screening method for detecting auditory response defects in zebrafish. Journal of Neuroscience Methods. 118, 177-187.

Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry 36, 2942-2952.

Dumbarton, T.C., Stoyek, M., Croll, R.P., Smith, F.M., 2010. Adrenergic control of swimbladder deflation in the zebrafish (Danio rerio). J. Exp. Biol. 213,2536–2546, http://dx.doi.org/10.1242/jeb.039792.

Godfrey, A., Hooser, B., Abdelmoneim, A., Horzmann, K.A., Freemanc, J.L., Sepulveda, M.S., 2017. Thyroid disrupting effects of halogenated and next generation chemicals on the swim bladder development of zebrafish. Aquatic Toxicology 193, 228-235.

McMenamin, S.K., Parichy, D.M., 2013. Metamorphosis in Teleosts. Animal Metamorphosis 103, 127-165.

Nagabhushana A, Mishra RK. 2016. Finding clues to the riddle of sex determination in zebrafish. Journal of Biosciences. 41(1):145- 155.

Nelson KR, Schroeder AL, Ankley GT, Blackwell BR, Blanksma C, Degitz SJ, Flynn KM, Jensen KM, Johnson RD, Kahl MD, Knapen D, Kosian PA, Milsk RY, Randolph EC, Saari T, Stinckens E, Vergauwen L, Villeneuve DL. 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole – Part I: fathead minnow. Aquatic Toxicology 173: 192-203.

Olmstead AW, Villeneuve DL, Ankley GT, Cavallin JE, Lindberg-Livingston A, Wehmas LC, Degitz SJ. 2011. A method for the determination of genetic sex in the fathead minnow, pimephales promelas, to support testing of endocrine-active chemicals. Environmental Science & Technology. 45(7):3090-3095.

Roberston, G.N., McGee, C.A.S., Dumbarton, T.C., Croll, R.P., Smith, F.M., 2007. Development of the swim bladder and its innervation in the zebrafish, Danio rerio. J. Morphol. 268, 967–985, http://dx.doi.org/10.1002/jmor.

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.

Stinckens, E., Vergauwen, L., Schroeder, A., Maho, W., Blackwell, B., Witters, H., Blust, R., Ankley, G., Covaci, A., Villeneuve, D., Knapen, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2- mercaptobenzothiazole part II: Zebrafish. Aquatic Toxicology 173, 204-217.

van Aerle R, Runnalls TJ, Tyler CR. 2004. Ontogeny of gonadal sex development relative to growth in fathead minnow. Journal of Fish Biology. 64(2):355-369.

Woolley, L.D., Qin, J.G., 2010. Swimbladder inflation and its implication to the culture of marine finfish larvae. Reviews in Aquaculture 2, 181-190.

Zheng, W., Wang, Z., Collins, J.E., Andrews, R.M., Stemple, D., Gong, Z. 2011. Comparative transcriptome analyses indicate molecular homology of zebrafish swim bladder and mammalian lung. PLoS One 6, http://dx.doi.org/10.1371/

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.

References

Fu C, Cao ZD, Fu SJ. 2013. The effects of caudal fin loss and regeneration on the swimming performance of three cyprinid fish species with different swimming capactities. The Journal of Experimental Biology 216:3164-3174. doi:10.1242/jeb.084244

Hagenaars, A., Stinckens, E., Vergauwen, L., Bervoets, L., Knapen, D., 2014. PFOS affects posterior swim bladder chamber inflation and swimming performanceof zebrafish larvae. Aquat. Toxicol. 157, 225–235.

Little EE, Finger SE. 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environmental Toxicology and Chemistry. 9(1):13-19.

Stinckens, E., Vergauwen, L., Schroeder, A.L., Maho, W., Blackwell, B., Witter, H.,Blust, R., Ankley, G.T., Covaci, A., Villenueve, D.L., Knapen, D., 2016. Disruption of thyroid hormone balance after 2-mercaptobenzothiazole exposure causes swim bladder inflation impairment—part II: zebrafish. Aquat. Toxicol. 173:204-17.

Vergauwen, Lucia; Nørgaard Schmidt, Stine; Maho, Walid; Stickens, Evelyn; Hagenaars, An; Blust, Ronny; Mayer, Philipp; Covaci, Adrian; Knapen, Dries. 2014. A high throughput passive dosing format for the Fish Embryo Acute Toxicity test. Chemosphere. 139: 9-17.

List of Adverse Outcomes in this AOP

Event: 351: Increased Mortality

Short Name: Increased Mortality

Key Event Component

Process	Object	Action
mortality		increased

AOPs Including This Key Event

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

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

Biological Context

Level of Biological Organization
Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

All living things are susceptible to mortality.

Key Event Description

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

How it is Measured or Detected

Mortality of animals is generally observed as cessation of the heart beat, breathing (gill or lung movement) and locomotory movements.

Mortality is typically measured by observation. Depending on the size of the organism, instruments such as microscopes may be used. The reported metric is mostly the mortality rate: the number of deaths in a given area or period, or from a particular cause.

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

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

Regulatory Significance of the AO

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

Event: 360: Decrease, Population growth rate

Short Name: Decrease, Population growth rate

Key Event Component

Process	Object	Action
population growth rate	population of organisms	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)	Adverse Outcome
Aop:25 - Aromatase inhibition leading to reproductive dysfunction	Adverse Outcome
Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction	Adverse Outcome
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	Adverse Outcome
Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior	Adverse Outcome
Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	Adverse Outcome
Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	Adverse Outcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release	Adverse Outcome
Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I /metaphase I transition	Adverse Outcome
Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction	Adverse Outcome
Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint	Adverse Outcome
Aop:292 - Inhibition of tyrosinase leads to decreased population in fish	Adverse Outcome
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	Adverse Outcome
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	Adverse Outcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	Adverse Outcome
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	Adverse Outcome
Aop:336 - DNA methyltransferase inhibition leading to population decline (1)	Adverse Outcome
Aop:337 - DNA methyltransferase inhibition leading to population decline (2)	Adverse Outcome

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

Biological Context

Level of Biological Organization
Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

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

Key Event Description

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

How it is Measured or Detected

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

Regulatory Significance of the AO

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

References

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

Appendix 2 - List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 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.

References

Bianco, A.C., Kim, B.W., 2006. Deiodinases: implications of the local control of thyroid hormone action. Journal of Clinical Investigation 116, 2571-2579.

Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry 36, 2942-2952.

Darras, V.M., Houbrechts, A.M., Van Herck, S.L.J., 2015. Intracellular thyroid hormone metabolism as a local regulator of nuclear thyroid hormone receptor-mediated impact on vertebrate development.

Darras, V.M., Van Herck, S.L.J., 2012. Iodothyronine deiodinase structure and function: from ascidians to humans. Journal of Endocrinology 215, 189-206.

Hernandez-Mariano JA, Torres-Sanchez L, Bassol-Mayagoitia S, Escamilla-Nunez M, Cebrian ME, Villeda-Gutierrez EA, Lopez-Rodriguez G, Felix-Arellano EE, Blanco-Munoz J. 2017. Effect of exposure to p,p '-dde during the first half of pregnancy in the maternal thyroid profile of female residents in a mexican floriculture area. Environmental Research. 156:597-604.

Houbrechts, A.M., Delarue, J., Gabriels, I.J., Sourbron, J., Darras, V.M., 2016. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. Endocrinology 157, 3668-3681.

Liu XS, Cai Y, Wang Y, Xu SH, Ji K, Choi K. 2019. Effects of tris(1,3-dichloro-2-propyl) phosphate (tdcpp) and triphenyl phosphate (tpp) on sex-dependent alterations of thyroid hormones in adult zebrafish. Ecotoxicology and Environmental Safety. 170:25-32.

Maia, A.L., Kim, B.W., Huang, S.A., Harney, J.W., Larsen, P.R., 2005. Type 2 iodothyronine deiodinase is the major source of plasma T-3 in euthyroid humans. Journal of Clinical Investigation 115, 2524-2533.

Mol, K.A., Van der Geyten, S., Burel, C., Kuhn, E.R., Boujard, T., Darras, V.M., 1998. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. Fish Physiology and Biochemistry 18, 253-266.

Orozco, A., Valverde, R.C., 2005. Thyroid hormone deiodination in fish. Thyroid 15, 799-813.

Power, D.M., Llewellyn, L., Faustino, M., Nowell, M.A., Bjornsson, B.T., Einarsdottir, I.E., Canario, A.V., Sweeney, G.E., 2001. Thyroid hormones in growth and development of fish. Comp Biochem Physiol C Toxicol Pharmacol 130, 447-459.

Schneider, M.J., Fiering, S.N., Pallud, S.E., Parlow, A.F., St Germain, D.L., Galton, V.A., 2001. Targeted disruption of the type 2 selenodeiodinase gene (D102) results in a phenotype of pituitary resistance to T-4. Molecular Endocrinology 15, 2137-2148.

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.

Walpita, C.N., Van der Geyten, S., Rurangwa, E., Darras, V.M., 2007. The effect of 3,5,3'-triiodothyronine supplementation on zebrafish (Danio rerio) embryonic development and expression of iodothyronine deiodinases and thyroid hormone receptors. Gen Comp Endocrinol 152, 206-214.

Webster GM, Venners SA, Mattman A, Martin JW. 2014. Associations between perfluoroalkyl acids (pfass) and maternal thyroid hormones in early pregnancy: A population-based cohort study. Environmental Research. 133:338-347.

Winata, C.L., Korzh, S., Kondrychyn, I., Korzh, V., Gong, Z. 2010. The role of vasulature and blood circulation in zebrafish swim bladder development. Dev. Biol. 10:3.

Winata, C.L., Korzh, S., Kondrychyn, I., Zheng, W., Korzh, V., Gong, Z. 2009. Development of zebrafish swimbladder: the requirement of Hedgehog signaling in specification and organization of the three tissue layers. Dev. Biol.331, 222–236, http://dx.doi.org/10.1016/j.ydbio.2009.04.035.

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

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

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

Sex: This KER plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KER. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current KER.

Key Event Relationship Description

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

Evidence Supporting this KER

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

Biological Plausibility

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

Empirical Evidence

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

1. Studies applying larval exposures initiated after posterior chamber inflation

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

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

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

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

Uncertainties and Inconsistencies

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

References

Brown, D.D., 1997. The role of thyroid hormone in zebrafish and axolotl development. Proceedings of the National Academy of Sciences of the United States of America 94, 13011-13016.

Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry 36, 2942-2952.

Godfrey, A., Hooser, B., Abdelmoneim, A., Horzmann, K.A., Freemanc, J.L., Sepulveda, M.S., 2017. Thyroid disrupting effects of halogenated and next generation chemicals on the swim bladder development of zebrafish. Aquatic Toxicology 193, 228-235.

Li W, Zha J, Yang L, Li Z, Wang Z. Regulation of thyroid hormone related genes mRNA expression by exogenous T_3 in larvae and adult Chinese rare minnow (Gobiocypris rarus). Environ Toxicol Pharmacol. 2011 Jan;31(1):189-97. doi: 10.1016/j.etap.2010.10.007.

Liu, Y.W., Chan, W.K., 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. Differentiation 70, 36-45.

McMenamin, S.K., Parichy, D.M., 2013. Metamorphosis in Teleosts. Animal Metamorphosis 103, 127-165.

Nagabhushana A, Mishra RK. 2016. Finding clues to the riddle of sex determination in zebrafish. Journal of Biosciences. 41(1):145-155.

Nelson KR, Schroeder AL, Ankley GT, Blackwell BR, Blanksma C, Degitz SJ, Flynn KM, Jensen KM, Johnson RD, Kahl MD, Knapen D, Kosian PA, Milsk RY, Randolph EC,Saari T, Stinckens E, Vergauwen L, Villeneuve DL. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part I: Fathead minnow. Aquat Toxicol. 2016 Apr;173:192-203. doi: 10.1016/j.aquatox.2015.12.024.

Olmstead AW, Villeneuve DL, Ankley GT, Cavallin JE, Lindberg-Livingston A, Wehmas LC, Degitz SJ. 2011. A method for the determination of genetic sex in the fathead minnow, pimephales promelas, to support testing of endocrine-active chemicals. Environmental Science & Technology. 45(7):3090-3095.

Reinwald H, Konig A, Ayobahan SU, Alvincz J, Sipos L, Gockener B, Bohle G, Shomroni O, Hollert H, Salinas G et al. 2021. Toxicogenomic fin(ger)prints for thyroid disruption aop refinement and biomarker identification in zebrafish embryos. Science of the Total Environment. 760.

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.

Stinckens, E., Vergauwen, L., Schroeder, A., Maho, W., Blackwell, B., Witters, H., Blust, R., Ankley, G., Covaci, A., Villeneuve, D., Knapen, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part II: Zebrafish. Aquatic Toxicology 173, 204-217.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. Journal of Experimental Biology 205, 711-718.

van Aerle R, Runnalls TJ, Tyler CR. 2004. Ontogeny of gonadal sex development relative to growth in fathead minnow. Journal of Fish Biology. 64(2):355-369.Zeng FX, Sherry JP, Bols NC. 2016. Evaluating the toxic potential of benzothiazoles with the rainbow trout cell lines, rtgill-w1 and rtl-w1. Chemosphere. 155:308-318.

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Larvae	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

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

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

Sex: This KER plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KER. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current KER.

Key Event Relationship Description

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

Evidence Supporting this KER

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

Biological Plausibility

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

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

Empirical Evidence

- Lindsey et al. (2010) showed that zebrafish started swimming deeper down in the water column upon inflation of the anterior chamber, confirming a role of the anterior chamber in supporting swimming performance.

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

Uncertainties and Inconsistencies

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

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

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

References

Brix O (2002) The physiology of living in water. In: Hart PJ, Reynolds J (eds) Handbook of Fish Biology and Fisheries, Vol. 1, pp. 70–96. Blackwell Publishing, Malden, USA.

Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry 36, 2942-2952.

Czesny, S.J., Graeb, B.D.S., Dettmersn, J.M., 2005. Ecological consequences of swimbladder noninflation for larval yellow perch. Trans. Am. Fish. Soc. 134,1011–1020, http://dx.doi.org/10.1577/T04-016.1.

Fay, R., 2009. Soundscapes and the sense of hearing of fishes. Integrative Zool. 4,26–32.

Goodsell, D.S., Morris, G.M., Olsen, A.J. 1996. Automated docking of fleixble ligands. Applications of Autodock. J. Mol. Recogonition, 9:1-5.

Lechner, W., Ladich, F., 2008. Size matters: diversity in swimbladders andWeberian ossicles affects hearing in catfishes. J. Exp. Biol. 211, 1681–1689.

Lindsey, B.W., Smith, F.M., Croll, R.P., 2010. From inflation to flotation: contribution of the swimbladder to whole-body density and swimming depth during development of the zebrafish (Danio rerio). Zebrafish 7, 85–96, http://dx.doi.org/10.1089/zeb.2009.0616.

Maack, G., Segner, H., 2003. Morphological development of the gonads in zebrafish. Journal of Fish Biology 62, 895-906.

Nagabhushana A, Mishra RK. 2016. Finding clues to the riddle of sex determination in zebrafish. Journal of Biosciences. 41(1):145-155.

Nelson, K., Schroeder, A., Ankley, G., Blackwell, B., Blanksma, C., Degitz, S., Flynn, K., Jensen, K., Johnson, R., Kahl, M., Knapen, D., Kosian, P., Milsk, R., Randolph, E., Saari, T., Stinckens, E., Vergauwen, L., Villeneuve, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part I: Fathead minnow. Aquatic Toxicology 173, 192-203.

Olmstead AW, Villeneuve DL, Ankley GT, Cavallin JE, Lindberg-Livingston A, Wehmas LC, Degitz SJ. 2011. A method for the determination of genetic sex in the fathead minnow, pimephales promelas, to support testing of endocrine-active chemicals. Environmental Science & Technology. 45(7):3090-3095.

Roberston, G.N., McGee, C.A.S., Dumbarton, T.C., Croll, R.P., Smith, F.M., 2007.Development of the swim bladder and its innervation in the zebrafish, Danio rerio. J. Morphol. 268, 967–985, http://dx.doi.org/10.1002/jmor.

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.

Stinckens, E., Vergauwen, L., Schroeder, A.L., Maho, W., Blackwell, B., Witter, H.,Blust, R., Ankley, G.T., Covaci, A., Villenueve, D.L., Knapen, D., 2016. Disruption of thyroid hormone balance after 2-mercaptobenzothiazole exposure causes swim bladder inflation impairment—part II: zebrafish. Aquat. Toxicol. 173:204-17.

Stoyek, M.R., Smith, F.M., Croll, R.P., 2011. Effects of altered ambient pressure on the volume and distribution of gas within the swimbladder of the adult zebrafish, Danio rerio. Journal of Experimental Biology 214, 2962-2972.

van Aerle R, Runnalls TJ, Tyler CR. 2004. Ontogeny of gonadal sex development relative to growth in fathead minnow. Journal of Fish Biology. 64(2):355-369.

Wisenden, B.D., Pogatschnik, J., Gibson, D., Bonacci, L., Schumacher, A., Willet, A.,2008. Sound the alarm: learned association of predation risk with novelauditory stimuli by fathead minnows (Pimephales promelas) and glowlighttetras (Hemigrammus erythrozonus) after single simultaneous pairings withconspecific chemical alarm cues. Environ. Biol. Fish 81, 141–147.

Woolley, L.D., Qin, J.G., 2010. Swimbladder inflation and its implication to theculture of marine finfish larvae. Rev. Aquac. 2, 181–190, http://dx.doi.org/10.1111/j.1753-5131.2010.01035.x.

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate
Juvenile	Moderate
Larvae	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

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

Key Event Relationship Description

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

Evidence Supporting this KER

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

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

Biological Plausibility

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

Empirical Evidence

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

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

Uncertainties and Inconsistencies

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

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage is currently limited.

Time-scale

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

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

References

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).

References

Alekseeva SM, Rudenko AI. 2018. Modeling of optimum fishing population. Marine Intellectual Technologies. 3(4):142-146.

Beaudouin, R., Goussen, B., Piccini, B., Augustine, S., Devillers, J., Brion, F., Pery, A.R., 2015. An individual-based model of zebrafish population dynamics accounting for energy dynamics. PloS one 10, e0125841.

Boreman J. 1997. Methods for comparing the impacts of pollution and fishing on fish populations. Transactions of the American Fisheries Society. 126(3):506-513.

Caswell, H., 2000. Matrix population models. Sinauer Sunderland, MA, USA.

Eng, M.L., Stutchbury, B.J.M. & Morrissey, C.A. Imidacloprid and chlorpyrifos insecticides impair migratory ability in a seed-eating songbird. Sci Rep 7, 15176 (2017)

Hazlerigg, C.R., Lorenzen, K., Thorbek, P., Wheeler, J.R., Tyler, C.R., 2012. Density-dependent processes in the life history of fishes: evidence from laboratory populations of zebrafish Danio rerio. PLoS One 7, e37550.

Jacobsen NS, Essington TE. 2018. Natural mortality augments population fluctuations of forage fish. Fish and Fisheries. 19(5):791- 797.

MacArthur, R., Wilson, E., 1967. The Theory of Island Biogeography. Princeton: Princeton Univ. Press. 203 p.

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

Miller, D.H., Clark, B.W., Nacci, D.E. 2020. A multidimensional density dependent matrix population model for assessing risk of stressors to fish populations. Ecotoxicology and environmental safety 201, 110786.

Pinceel, T., Vanschoenwinkel, B., Brendonck, L., Buschke, F., 2016. Modelling the sensitivity of life history traits to climate change in a temporary pool crustacean. Scientific reports 6, 29451.

Rearick, D.C., Ward, J., Venturelli, P., Schoenfuss, H., 2018. Environmental oestrogens cause predation-induced population decline in a freshwater fish. Royal Society open science 5, 181065.

Schäfers, C., Oertel, D., Nagel, R., 1993. Effects of 3, 4-dichloroaniline on fish populations with differing strategies of reproduction. Ecotoxicology and Ecophysiology, 133-146.

Stige, L.C., Rogers, L.A., Neuheimer, A.B., Hunsicker, M.E., Yaragina, N.A., Ottersen, G., Ciannelli, L., Langangen, Ø., Durant, J.M., 2019. Density-and size-dependent mortality in fish early life stages. Fish and Fisheries 20, 962-976.

Hazlerigg, C.R.E., Tyler, C.R., Lorenzen, K., Wheeler, J.R., Thorbek, P., 2014. Population relevance of toxicant mediated changes in sex ratio in fish: An assessment using an individual-based zebrafish (Danio rerio) model. Ecological Modelling 280, 76-88.

Stige, L.C., Rogers, L.A., Neuheimer, A.B., Hunsicker, M.E., Yaragina, N.A., Ottersen, G., Ciannelli, L., Langangen, O., Durant, J.M., 2019. Density- and size-dependent mortality in fish early life stages. Fish and Fisheries 20, 962-976.

Annex 1: Weight of evidence evaluation table

AOP 156: Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation - Weight of evidence evaluation

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

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, confirming a direct link between T3 levels and the swim bladder system in general. (1) from zebrafish knockdown/knockout studies: • Knockdown of deiodinase 1 and 2 (Bagci et al., 2015; Heijlen et al., 2013, 2014), knockdown of TH transporter MCT8 (de Vrieze et al., 2014), knockdown of thryoid hormone receptor alpha or beta (Marelli et al., 2016), and permanent knockout of deiodinase 2 (Houbrechts et al., 2016) in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Marelli et al. (2016) additionally showed that high T3 doses partially rescued the negative impact in mutants with partially resistant thyroid hormone receptors. • Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. These effects were rescued after T3 supplementation, but not after T4 supplementation. While swim bladder inflation was not among the assessed endpoints in this study, this generally confirms the essentiality of decreased T3 in causing downstream effects upon disruption of DIO1 and 2 function (Walpita et al., 2009, 2010). (2) from chemical exposures: • Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids and exogeneous T3 or T4 supplementation partly rescued this effect. • Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass lead to significant increases in posterior swim bladder inflation (Brown et al., 1988). Similarly, Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. Less information is available about the essentiality of reduced T3 levels for reduced anterior chamber inflation in particular. • Chopra et al. (2019) provided indirect evidence showing that knockdown of dual oxidase - expected to lead to reduced T4 and T3 levels since dual oxidase is important for thyroid hormone synthesis - reduced anterior swim bladder inflation. Proving essentiality of reduced T3 levels for reduced anterior chamber inflation is further complicated by the complexity of the swim bladder system and the difficulty of distinguishing effects resulting from altered anterior chamber inflation from those resulting from altered posterior chamber inflation.			
KE 1007: Reduced, anterior swim bladder inflation	Stinckens et al. (2020) showed that at the time point where control zebrafish inflate the anterior chamber, larvae exposed to PTU have a lower frequency of inflated anterior chambers together with reduced swimming distance. Later during the exposure the frequency of non-inflated anterior chambers decreased and the effect on swimming distance disappeared confirming the essentiality of reduced anterior chamber inflation for the downstream effect on swimming performance.			
KE 1005: Reduced, swimming performance	Experimental blocking of this KE is difficult to achieve.			
KE 351: Increased mortality	By definition, increased mortality is essential for reduced population size.			
AOP as a whole	**Moderate** Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is evidence from deiodinase knockdowns showing the link with reduced posterior chamber inflation and the essentiality for downstream effects, but anterior chamber inflation was not studied. There is additional indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation. It should be noted that dual oxidase also plays a role in oxidative stress. There is also evidence that alleviation of the effect on anterior chamber inflation reduces the effect on swimming performance.			

AOP 156: Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation - Weight of evidence evaluation

	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 1035: Decreased, Triiodothyronine (T3) in serum (KE 1003) leads to Reduced, Anterior swim bladder inflation (KE 1007)	**Moderate** Several studies showed both T3 decreases and reduced inflation of the anterior chamber with some evidence of dose concordance. Uncertainties mainly relate to the mechanism through which altered TH levels result in impaired posterior chamber inflation. Temporal concordance is difficult to establish since swim bladder inflation can only occur at a specific time point.			
Relationship 1034: Reduced, Anterior swim bladder inflation (KE 1007) leads to Reduced, Swimming performance (KE 1005)	**Moderate** There is extensive evidence of a link between reduced anterior chamber inflation and reduced swimming performance including some evidence of dose concordance. Temporal concordance is specifically supported by the study of Stinckens et al. (2020): First, after 21 d of exposure to 111 mg/L propylthiouracil around 30% of anterior chambers were not inflated and swimming distance was reduced, while by 32 days post fertilization all larvae had inflated their anterior chamber (although chamber surface was still smaller) and the effect on swimming distance had disappeared.			
Relationship 2212: Reduced, Swimming performance (KE 1005) leads to Increaed mortality (KE 351)	**Low** A direct relationship between reduced swimming performance and increased mortality has been difficult to establish. There is however a lot of indirect evidence linking reduced swim bladder inflation to increased mortality (see non-adjacent KER 2213), which can be plausibly assumed to be related to reduced swimming performance.			
Relationship 2013: Increased mortality (KE 351) leads to Decrease, Population trajectory (KE 360)	**Moderate** Survival rate is an obvious determinant of population size and is therefore included in population modeling. The extent to which increased mortality may impact population sizes in a realistic, environmental exposure scenario depends on the circumstances. Under some conditions, reduced larval survival may be compensated by reduced predation and increased food availability, and therefore not result in population decline.			

							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 cc	60, 120, 240 mg/L[£] (lower c	45, 60, 90, 120, 150, 180, 21	n/a	n/a	-	-	-	-[£]	-[£]
Wang et al. (2020)	zebrafish	PFO5DoDA	unknown	0-5 dpf	5 dpf	0, 5, 10, 15, 20, 25, 30, 35, 40 mg/L	-*	-*	10, 20, 40 mg/L*	-	10, 20, 40 mg/L[£]	10, 20, 40 mg/L[£]	20, 25, 30, 35, 40 mg/L[§]	n/a	n/a	-	10 mg/L	-	-[£]	-[£]
Rehberger et al. (2018)	zebrafish	propylthiouracil	TPO inhibition	0-5 dpf	5 dpf	0, 2.5, 10, 25, 50 mg/L	n/a	n/a	n/a	10, 25, 50 mg/L	n/a	n/a	n/a	n/a	n/a	n/a				

Legend

n/a: not measured

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

$ based on thyroid histopathology

£ based on whole body measurement

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

References

Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. Environmental Toxicology and Chemistry 36, 2942-2952.

Crane, H.M., Pickford, D.B., Hutchinson, T.H., Brown, J.A., 2005. Effects of ammonium perchlorate on thyroid function in developing fathead minnows, Pimephales promelas. Environmental Health Perspectives 113, 396-401.

Crane, H.M., Pickford, D.B., Hutchinson, T.H., Brown, J.A., 2006. The effects of methimazole on development of the fathead minnow, pimephales promelas, from embryo to adult. Toxicological Sciences 93, 278-285.

Nelson, K., Schroeder, A., Ankley, G., Blackwell, B., Blanksma, C., Degitz, S., Flynn, K., Jensen, K., Johnson, R., Kahl, M., Knapen, D., Kosian, P., Milsk, R., Randolph, E., Saari, T., Stinckens, E., Vergauwen, L., Villeneuve, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part I: Fathead minnow. Aquatic Toxicology 173, 192-203.

Rehberger, K., Baumann, L., Hecker, M., Braunbeck, T., 2018. Intrafollicular thyroid hormone staining in whole-mount zebrafish (Danio rerio) embryos for the detection of thyroid hormone synthesis disruption. Fish Physiology and Biochemistry 44, 997-1010.

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.

Stinckens, E., Vergauwen, L., Schroeder, A., Maho, W., Blackwell, B., Witters, H., Blust, R., Ankley, G., Covaci, A., Villeneuve, D., Knapen, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part II: Zebrafish. Aquatic Toxicology 173, 204-217.

Wang, J.X., Shi, G.H., Yao, J.Z., Sheng, N., Cui, R.N., Su, Z.B., Guo, Y., Dai, J.Y., 2020. Perfluoropolyether carboxylic acids (novel alternatives to PFOA) impair zebrafish posterior swim bladder development via thyroid hormone disruption. Environment International 134.

Wei, P.H., Zhao, F., Zhang, X.N., Liu, W.M., Jiang, G.B., Wang, H.F., Ru, S.G., 2018. Transgenerational thyroid endocrine disruption induced by bisphenol S affects the early development of zebrafish offspring. Environmental Pollution 243, 800-808.