# AOP ID and Title:

SNAPSHOT

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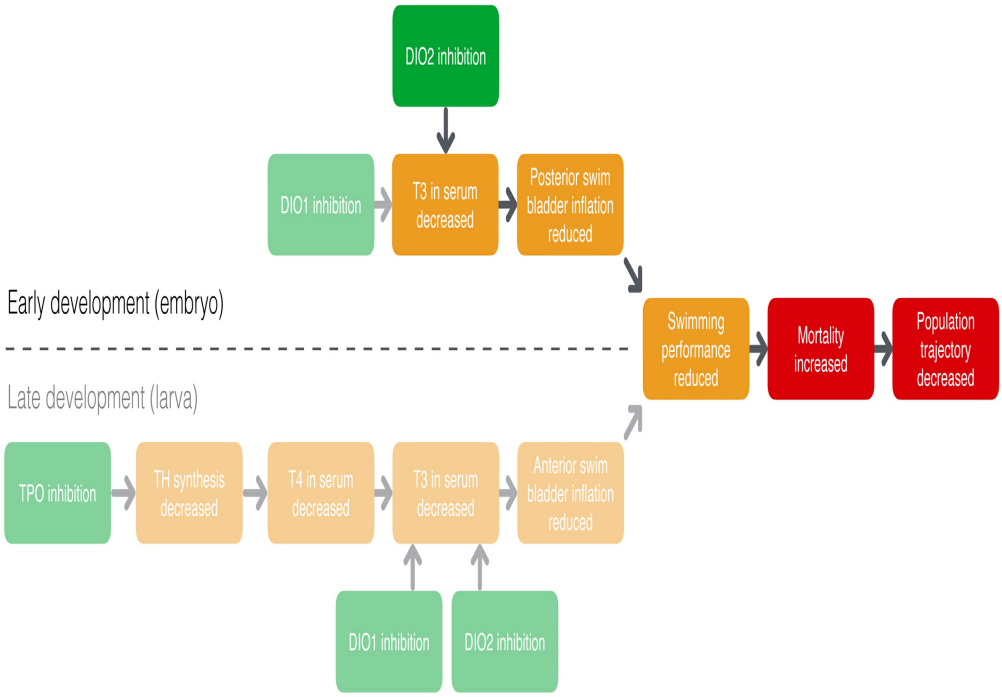
**AOP 155: Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation**

**Short Title: DIO2i posterior swim bladder**

## Graphical Representation

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## Status

| Author status | OECD status | OECD project | SAAOP status |
|---|---|---|---|
| Under development: Not open for comment. Do not cite | Under Development | 1.35 | Included in OECD Work Plan |

## Abstract

The AOP describes the effects of inhibition of deiodinase 2 on posterior swim bladder inflation leading to reduced young of year survival and population trajectory decline. The inhibition of deiodinase 2 (DIO2) is the molecular-initiating event (MIE), which results in decreased conversion of thyroxine (T4) to the biologically more active triiodothyronine (T3). As in amphibians, the transition between the different developmental phases in fish, including maturation and inflation of the swim bladder, has been shown to be mediated by THs (Brown et al., 1988; Liu and Chan, 2002).

Impaired swim bladder inflation results in reduced swimming performance (Stinckens et al. 2020; Hagenaars et al., 2014; Stinckens et al., 2016; Stinckens et al., 2018), an adverse outcome that can affect feeding behavior and predator avoidance, ultimately leading to lower survival probability and population trajectory decline (Czesny et al., 2005; Woolley and Qin, 2010; Villeneuve et al., 2014).

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 and ultimately to reduced survival. (Knapen et al., 2018; Knapen et al., 2020; Villeneuve et al., 2018). Specific parts of the AOP network are relevant to different life stages. The swim bladder is an internal gas-filled organ found in many bony fish species and typically consists of two gas-filled chambers. The posterior chamber inflates during early development and contributes to the ability of fish to control their buoyancy, while the anterior chamber inflates during late development and has an additional role as a resonating chamber to produce or receive sound (Robertson et al., 2007). 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, early developmental processes that are dependent on THs, such as posterior swim bladder chamber inflation, appear to be less sensitive to inhibition of TH synthesis. On the other hand, when maternally derived THs are depleted during late development (larval stage), endogenous TH synthesis becomes more important and inhibition of TPO interferes with proper inflation of the anterior swim bladder chamber (Stinckens et al. 2020; Nelson et al., 2016; Stinckens et al., 2016; Godfrey et al., 2017). In all life stages however, the conversion of T4 into T3 is essential. Inhibition of deiodinase (DIO) therefore impacts swim bladder inflation in both early and late developmental life stages (Stinckens et al. 2020; Jomaa et al., 2014; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2018).

In addition to evidence from chemical exposure summarized above, data from knockdowns, knockouts and TH supplementation has been instrumental in supporting the AOP network (Walpita et al., 2009, 2010; Heijlen et al., 2013, 2014; Bagci et al., 2015; Houbrechts et al., 2016; Chopra et al., 2019). Although there is strong evidence for the link between TH and swim bladder inflation, the exact underlying mechanism (e.g., impairment of development and/or inflation process) is not understood. Another uncertainty relates to serum versus tissue TH levels. Since collecting blood from early life stages of fish is not feasible, whole body TH measurements are typically used as a proxy for serum TH levels. Finally, the role of DIO1 versus DIO2 in TH activation in serum or locally and the overall importance of DIO1 versus DIO2 and in fish is not exactly clear. Available evidence suggests that DIO2 is more important for proper swim bladder inflation in fish.

*Text used from Knapen et al. (2020)*

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

# 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 (https://aopwiki.org/events/1002) | Inhibition, Deiodinase 2 |
| | | | | |
| 2 | KE | 1003 | Decreased, Triiodothyronine (T3) in serum (https://aopwiki.org/events/1003) | Decreased, Triiodothyronine (T3) in serum |
| 3 | KE | 1004 | Reduced, Posterior swim bladder inflation (https://aopwiki.org/events/1004) | Reduced, Posterior swim bladder inflation |
| 4 | KE | 1005 | Reduced, Swimming performance (https://aopwiki.org/events/1005) | Reduced, Swimming performance |
| | | | | |

| Sequence | Type | Event ID | Title | Short name |
|---|---|---|---|---|
| 5 | AO | 351 | Increased Mortality (https://aopwiki.org/events/351) | Increased Mortality |
| 6 | AO | 360 | Decrease, Population trajectory (https://aopwiki.org/events/360) | Decrease, Population trajectory |

## Key Event Relationships

| Upstream Event | Relationship Type | Downstream Event | Evidence | Quantitative Understanding |
|---|---|---|---|---|
| Inhibition, Deiodinase 2 (https://aopwiki.org/relationships/1026) | adjacent | Decreased, Triiodothyronine (T3) in serum | Moderate | Low |
| Decreased, Triiodothyronine (T3) in serum (https://aopwiki.org/relationships/1027) | adjacent | Reduced, Posterior swim bladder inflation | Moderate | Low |
| Reduced, Posterior swim bladder inflation (https://aopwiki.org/relationships/1028) | adjacent | Reduced, Swimming performance | Moderate | Low |
| Reduced, Swimming performance (https://aopwiki.org/relationships/2212) | adjacent | Increased Mortality | Moderate | Low |
| Increased Mortality (https://aopwiki.org/relationships/2013) | adjacent | Decrease, Population trajectory | High | Moderate |
| | | | | |
| Inhibition, Deiodinase 2 (https://aopwiki.org/relationships/1042) | non-adjacent | Reduced, Posterior swim bladder inflation | Moderate | Low |
| Reduced, Posterior swim bladder inflation (https://aopwiki.org/relationships/2213) | non-adjacent | Increased Mortality | High | Low |

## Stressors

| Name | Evidence |
|---|---|
| iopanoic acid | High |

### iopanoic acid

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

# Overall Assessment of the AOP

The attached document includes:

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

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

## Domain of Applicability

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| Embryo | High |

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Life stage**: The current AOP is only applicable to early embryonic development, which is the period where the posterior swim bladder chamber inflates. 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, early developmental processes that are dependent on THs, such as posterior swim bladder chamber inflation, appear to be less sensitive to inhibition of TH synthesis. When maternally derived THs are depleted during late development (larval stage), endogenous TH synthesis becomes more important and inhibition of TPO interferes with proper inflation of the anterior swim bladder chamber (Stinckens et al. 2020; Nelson et al., 2016; Stinckens et al., 2016; Godfrey et al., 2017). In all life stages however, the conversion of T4 into T3 is essential. Inhibition of deiodinase (DIO) therefore impacts swim bladder inflation in both early and late developmental life stages.

**Taxonomic**: The AOP is currently mainly based on experimental evidence from studies on zebrafish and fathead minnow. A first logical step in expanding the applicability of the AOP network is to assess its relevance to other species that are frequently used in existing fish test guidelines, such as the Japanese rice fish (medaka), three-spined stickleback and rainbow trout.

**Sex**: Sex differences are typically not investigated in tests using early life stages of fish and it is currently unclear whether sex-related differences are important in this AOP. Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization, when sex differentiation has not started yet, sex differences are expected to play a minor role in the current AOP.

## Essentiality of the Key Events

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

## Weight of Evidence Summary

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

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

## Quantitative Consideration

Quantitative understanding of this AOP is currently limited.

# Considerations for Potential Applications of the AOP (optional)

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. 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. 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, using the levels of assessment of the OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals as a guide.

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# Appendix 1

## List of MIEs in this AOP

Event: 1002: Inhibition, Deiodinase 2 (https://aopwiki.org/events/1002)

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 (https://aopwiki.org/aops/155) | MolecularInitiatingEvent |
| Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156) | MolecularInitiatingEvent |
| Aop:190 - Type II iodothyronine deiodinase (DIO2) inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/190) | MolecularInitiatingEvent |

### Stressors

| Name |
|---|
| iopanoic acid |
| PERFLUOROOCTANOIC ACID |

### Biological Context

| Level of Biological Organization |
|---|
| Molecular |

## Evidence for Perturbation by Stressor

### Overview for Molecular Initiating Event

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

Stinckens et al. (2018)

PERFLUOROOCTANOIC ACID

Stinckens et al. (2018)

## Domain of Applicability

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| rat | Rattus norvegicus | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116) |
| human | Homo sapiens | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606) |
| pigs | Sus scrofa | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9823) |
| Oreochromis niloticus | Oreochromis niloticus | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8128) |
| zebrafish | Danio rerio | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | Moderate |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: Deiodination by DIO enzymes is known to exist in a wide range of vertebrates and invertebrates. Reports of inibition 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 zebrafish is indirect since DIO enzyme activity is usually not measured in chemical exposure experiments using zebrafish. 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. Based on these results, DIO2 seemed to be more important than DIO1.

In mammals, DIO2 controls the intracellular concentration of T3. 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. In teleosts, DIO2 has a markedly higher activity level compared to other vertebrates and it is expressed in liver (Orozco and Valverde, 2005). 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**: Deiodinases are important for TH homeostasis in both sexes. Sex-specific differences in this KE have not been described in fish.

## 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. The synthesis of the thyroid hormones is a process that involves several steps. Thyroglobulin, the thyroid hormone precursor, is produced by the thyroid epithelial cells and transported to the lumen via exocytosis. Then thyroperoxidase (TPO) plays an essential role in the production of mainly T4. The prohormone T4 is then released in the circulation under the influence of thyroid stimulating hormone (TSH), in order to be transported to the various tissues, including the liver, the kidneys and the heart. 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 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 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.

## 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 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.

## References

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## List of Key Events in the AOP

### Event: 1003: Decreased, Triiodothyronine (T3) in serum (https://aopwiki.org/events/1003)

Short Name: Decreased, Triiodothyronine (T3) in serum

#### Key Event Component

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

#### AOPs Including This Key Event

| AOP ID and Name | Event Type |
|---|---|
| Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155) | KeyEvent |
| Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156) | KeyEvent |
| Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157) | KeyEvent |
| Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/158) | KeyEvent |
| Aop:189 - Type I iodothyronine deiodinase (DIO1) inhibition leading to altered amphibian metamorphosis (https://aopwiki.org/aops/189) | KeyEvent |
| Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159) | KeyEvent |

#### Biological Context

| Level of Biological Organization |
|---|
| Tissue |

#### Organ term

| Organ term |
|---|
| serum |

#### Domain of Applicability

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |
| African clawed frog | Xenopus laevis | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8355) |

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| | | | |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: The overall evidence supporting taxonomic applicability is strong. With few exceptions vertebrate species have circulating T3 and T4 that are bound to transport proteins in blood. Clear species differences exist in transport proteins (Yamauchi and Isihara, 2009). Specifically, the majority of supporting data for TH decreases in serum come from rat studies, and the predominant iodothyronine binding protein in rat serum is transthyretin (TT4). TT4 demonstrates a reduced binding affinity for T4 when compared with thyroxine binding globulin (TBG), the predominant serum binding protein for T4 in humans. This difference in serum binding protein affinity for THs is thought to modulate serum half-life for T4; the half-life of T4 in rats is 12-24 hr, wherease the half-life in humans is 5-9 days (Capen, 1997). While these species differences impact hormone half-life, possibly regulatory feedback mechanisms, and quantitative dose-response relationships, measurement of serum THs is still regarded as a measurable key event causatively linked to downstream adverse outcomes.

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

**Life stage**: Thyroid hormones are essential in all life stages, but elevations of circulating levels are associated with specific developmental events.

**Sex**: Thyroid hormones are essential in both sexes.

## Key Event Description

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

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

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

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

The production of THs in the thyroid gland and the circulation levels in the bloodstream are self-controlled by an efficiently regulated feedback mechanism across the Hypothalamus-Pituitary-Thyroid (HPT) axis. One of the most unique characteristics of TH is their ability to regulate their own concentration, not only in the plasma level, but also in the individual cell level, to maintain their homeostasis. This is succeed by the efficient regulatory mechanism of the thyroid hormone axis which consists of the following: (1) the hypothalamic secretion of the thyrotropin-releasing

hormone (TRH), (2) the thyroid-stimulating hormone (TSH) secretion from the anterior pituitary, (3) hormonal transport by the plasma binding proteins, (4) cellular uptake mechanisms in the cell level, (5) intracellular control of TH concentration by the deiodinating mechanism (6) transcriptional function of the nuclear thyroid hormone receptor and (7) in the fetus, the transplacental passage of T4 and T3 (Cheng et al., 2010).

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

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

## How it is Measured or Detected

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

Recently, analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates through methods employing HLPC 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.

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## Event: 1004: Reduced, Posterior swim bladder inflation (https://aopwiki.org/events/1004)

Short Name: Reduced, Posterior swim bladder inflation

### Key Event Component

| Process | Object | Action |
|---|---|---|
| swim bladder inflation | posterior chamber swim bladder | decreased |

### AOPs Including This Key Event

| AOP ID and Name | Event Type |
|---|---|
| Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155) | KeyEvent |
| Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157) | KeyEvent |

### Biological Context

| Level of Biological Organization |
|---|
| Organ |

## Organ term

| Organ term |
|---|
| swim bladder |

## Domain of Applicability

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| Embryo | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: Teleost fish can be divided in two groups according to swim bladder morphology: physoclistous (e.g., yellow perch) and physostomus (e.g., zebrafish and fathead minnow). Physostomus fish retain a duct between the digestive tract and the swim bladder during adulthood allowing them to gulp air at the surface to fill the swim bladder. In contrast, in physoclistous fish, once initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Wooley and Qin, 2010). Much of the evidence for impaired posterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2018; Cavallin et al., 2017; Wang et al., 2020).

**Life stage**: The posterior chamber inflates during a specific developmental time frame. In zebrafish, the posterior chamber inflates around 96 h post fertilization (hpf) which is 2 days post hatch. In the fathead minnow, the posterior chamber inflates around 6 dpf. Therefore this KE is only applicable to the embryonic life stage.

**Sex**: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization, when sex differentiation has not started yet, sex differences are expected to play a minor role.

## Key Event Description

The teleost swim bladder is a gas-filled structure that consists of two chambers, the posterior and anterior chamber. In zebrafish, the posterior chamber inflates around 96 h post fertilization (hpf) which is 2 days post hatch, and the anterior chamber inflates around 21 dpf (days post fertilization). In fathead minnow, the posterior and anterior chamber inflate around 6 and 14 dpf respectively.

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

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

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

## How it is Measured or Detected

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

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

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## Event: 1005: Reduced, Swimming performance (https://aopwiki.org/events/1005)

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 (https://aopwiki.org/aops/155) | KeyEvent |
| Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156) | KeyEvent |
| Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157) | KeyEvent |
| Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/158) | KeyEvent |
| Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159) | KeyEvent |
| Aop:242 - Inhibition of lysyl oxidase leading to enhanced chronic fish toxicity (https://aopwiki.org/aops/242) | KeyEvent |
| Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration (https://aopwiki.org/aops/334) | KeyEvent |

### Biological Context

| Level of Biological Organization |
|---|
| Individual |

## Domain of Applicability

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| teleost fish | teleost fish | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=70862) |
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: Importance of swimming performance for natural behaviour is generally applicable to fish.

**Life stage**: Importance of swimming performance for natural behaviour is generally applicable across all 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, a swim tunnel has been used to measure various data (Fu et al., 2013).

## References

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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 (https://aopwiki.org/events/351)

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 (https://aopwiki.org/aops/16) | AdverseOutcome |
| Aop:96 - Axonal sodium channel modulation leading to acute mortality (https://aopwiki.org/aops/96) | AdverseOutcome |
| Aop:104 - Altered ion channel activity leading impaired heart function (https://aopwiki.org/aops/104) | AdverseOutcome |
| Aop:113 - Glutamate-gated chloride channel activation leading to acute mortality (https://aopwiki.org/aops/113) | AdverseOutcome |
| Aop:160 - Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality (https://aopwiki.org/aops/160) | AdverseOutcome |
| Aop:161 - Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality (https://aopwiki.org/aops/161) | AdverseOutcome |
| Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality (https://aopwiki.org/aops/138) | AdverseOutcome |
| Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality (https://aopwiki.org/aops/177) | AdverseOutcome |
| Aop:186 - unknown MIE leading to renal failure and mortality (https://aopwiki.org/aops/186) | AdverseOutcome |
| Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement (https://aopwiki.org/aops/312) | AdverseOutcome |
| Aop:320 - Binding of viral S-glycoprotein to ACE2 receptor leading to acute respiratory distress associated mortality (https://aopwiki.org/aops/320) | AdverseOutcome |
| Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155) | AdverseOutcome |
| Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156) | AdverseOutcome |
| Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157) | AdverseOutcome |
| Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/158) | AdverseOutcome |
| Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159) | AdverseOutcome |

Biological Context

| Level of Biological Organization |
|---|
| Population |

Domain of Applicability

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| Gallus gallus | Gallus gallus | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9031) |
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

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 is typically measured by observation. Lack of any heart beat, gill movement, and body movement are typical signs of death used in the evaluation of mortality of animals.

Mortality can be measured:

- in the lab by recording mortality during prolonged exposure experiments
- in dedicated mesocosms, or in drainable ponds
- in the field, for example by determining age structure after one capture, or by capture-tag-recapture efforts

### Regulatory Significance of the AO

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

### Event: 360: Decrease, Population trajectory (https://aopwiki.org/events/360)

Short Name: Decrease, Population trajectory

### Key Event Component

| Process | Object | Action |
|---|---|---|
| population growth rate | population of organisms | decreased |

### AOPs Including This Key Event

| AOP ID and Name | Event Type |
|---|---|
| Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish) (https://aopwiki.org/aops/23) | AdverseOutcome |
| Aop:25 - Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25) | AdverseOutcome |
| Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/29) | AdverseOutcome |

| AOP ID and Name | Event Type |
|---|---|
| Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30) | AdverseOutcome |
| Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior (https://aopwiki.org/aops/100) | AdverseOutcome |
| Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122) | AdverseOutcome |
| Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123) | AdverseOutcome |
| Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155) | AdverseOutcome |
| Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156) | AdverseOutcome |
| Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157) | AdverseOutcome |
| Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/158) | AdverseOutcome |
| Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159) | AdverseOutcome |
| Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release (https://aopwiki.org/aops/101) | AdverseOutcome |
| Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I /metaphase I transition (https://aopwiki.org/aops/102) | AdverseOutcome |
| Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/63) | AdverseOutcome |
| Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint (https://aopwiki.org/aops/103) | AdverseOutcome |
| Aop:290 - Mitochondrial ATP synthase inhibition leading to growth arrest (1) (https://aopwiki.org/aops/290) | AdverseOutcome |
| Aop:291 - Mitochondrial ATP synthase inhibition leading to growth arrest (2) (https://aopwiki.org/aops/291) | AdverseOutcome |
| Aop:292 - Inhibition of tyrosinase leads to decreased population in fish (https://aopwiki.org/aops/292) | AdverseOutcome |
| Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR (https://aopwiki.org/aops/310) | AdverseOutcome |
| Aop:16 - Acetylcholinesterase inhibition leading to acute mortality (https://aopwiki.org/aops/16) | AdverseOutcome |
| Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement (https://aopwiki.org/aops/312) | AdverseOutcome |
| Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration (https://aopwiki.org/aops/334) | AdverseOutcome |
| Aop:336 - DNA methyltransferase inhibition leading to population decline (1) (https://aopwiki.org/aops/336) | AdverseOutcome |
| Aop:337 - DNA methyltransferase inhibition leading to population decline (2) (https://aopwiki.org/aops/337) | AdverseOutcome |
| Aop:338 - DNA methyltransferase inhibition leading to population decline (3) (https://aopwiki.org/aops/338) | AdverseOutcome |
| Aop:339 - DNA methyltransferase inhibition leading to population decline (4) (https://aopwiki.org/aops/339) | AdverseOutcome |
| Aop:340 - DNA methyltransferase inhibition leading to transgenerational effects (1) (https://aopwiki.org/aops/340) | AdverseOutcome |

| AOP ID and Name | Event Type |
|---|---|
| Aop:341 - DNA methyltransferase inhibition leading to transgenerational effects (2) (https://aopwiki.org/aops/341) | AdverseOutcome |
| Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish (https://aopwiki.org/aops/289) | AdverseOutcome |
| Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline (https://aopwiki.org/aops/297) | AdverseOutcome |
| Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation (https://aopwiki.org/aops/346) | AdverseOutcome |
| Aop:299 - Excessive reactive oxygen species production leading to population decline via reduced fatty acid beta-oxidation (https://aopwiki.org/aops/299) | AdverseOutcome |
| Aop:311 - Excessive reactive oxygen species production leading to population decline via mitochondrial dysfunction (https://aopwiki.org/aops/311) | AdverseOutcome |
| Aop:216 - Excessive reactive oxygen species production leading to population decline via follicular atresia (https://aopwiki.org/aops/216) | AdverseOutcome |
| Aop:238 - Excessive reactive oxygen species production leading to population decline via lipid peroxidation (https://aopwiki.org/aops/238) | AdverseOutcome |

## Biological Context

| Level of Biological Organization |
|---|
| Population |

## Domain of Applicability

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| all species | all species | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0) |

**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

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) in serum (https://aopwiki.org/relationships/1026)

AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155)** | **adjacent** | **Moderate** | **Low** |
| **Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156)** | **adjacent** | **Moderate** | **Low** |

Evidence Supporting Applicability of this Relationship

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: Deiodinases are important for the activation of T4 to T3 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 and sex**: Deiodinases are important for the activation of T4 to T3 across all life stages and sexes.

## 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)

## 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 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 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 fish. 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 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 (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. 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. 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).

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. Inibition 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, influencing this KER. Additionally, deiodinases regulate the activity of thryoid hormones, not only in serum and target organs, but also in the thryoid gland. Deiodinases themselves are known to be involved in 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.

### References

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## Relationship: 1027: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Posterior swim bladder inflation (https://aopwiki.org/relationships/1027)

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155)** | **adjacent** | **Moderate** | **Low** |
| **Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157)** | **adjacent** | **Moderate** | **Low** |

### Evidence Supporting Applicability of this Relationship

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| Embryo | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: The indirect relationship between deiodinase inhibition, expected to lead to reduced T3 levels, and reduced posterior chamber inflation has been confirmed in early zebrafish larvae around the time of posterior chamber inflation, i.e. around 5 days post fertilization in zebrafish (Stinckens et al., 2018) and around 6 days post fertilization in fathead minnows (Cavallin et al., 2017).

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

**Sex**: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization, when sex differentiation has not started yet, sex differences are expected to play a minor role.

## Key Event Relationship Description

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

## Evidence Supporting this KER

There is convincing evidence that decreased T3 levels result in impaired posterior chamber inflation, but the underlying mechanisms are not completely understood. The quantitative understanding is currently very limited because T3 levels and posterior inflation are seldom measured in the same study. Therefore the evidence supporting this KER can be considered moderate.

**Biological Plausibility**

Thyroid hormones are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition (Liu and Chan, 2002) and larval-to-juvenile transition (Brown et al., 1997) in fish. Inflation of the posterior chamber is part of the embryonic-to-larval transition in fish, together with structural and functional maturation of the mouth and gastrointestinal tract, and resorption of the yolk sac (Liu and Chan, 2002). Marelli et al. (2016) showed that thyroid hormone receptor alpha and beta are both expressed in swim bladder tissue of zebrafish at 5 days post fertilization, corresponding to the timing of posterior inflation. this time point has additionally been shown to coincide with increased T3 and T4 levels (Chang et al., 2012), suggesting that posterior inflation is under thyroid hormone regulation.

**Empirical Evidence**

- Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass (Morone saxatilis) lead to significant increases in both swim bladder inflation and survival (Brown et al., 1988).
- Dong et al. (2013) and Thisse et al. (2003) showed localized expression of DIO1 and DIO2 in the swim bladder tissue of 96 and 120 hpf zebrafish larvae, suggesting that local activation of thryoid hormones (i.e. conversion of T4 to T3) is requried in swim bladder tissue around that time period.
- Marelli et al. (2016) used morpholinos to block translation of thryoid hormone receptor alpha or beta in zebrafish. They found that thyroid hormone receptor alpha and beta knockdowns failed to inflate the posterior chamber of the swim bladder by 120 hpf, indicating that the action of T3 is needed for proper inflation of the posterior chamber. High T3 doses partially rescued the negative impact in partially resistant mutants, further confirming the importance of T3 in this process.
- Stinckens et al. (2018) showed that effects on posterior chamber inflation in zebrafish could be predicted based on in chemico DIO2 inhibition potential with only few false positives and false negatives. While T3 levels were not determined in this study, DIO2 inhibition is expected to result in decreased T3 levels.

- Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of DIO1+2 in zebrafish resulted in impairment of the inflation of the posterior chamber of the swim bladder. DIO1 and 2 knockdown is expected to result in reduced T3 levels. Indeed, Walpita et al. (2009, 2010) showed that T3 supplementation effectively rescued the effects of DIO1 and 2 knockdown, while T4 supplementation did not.
- de Vrieze et al. (2014) found that knockdown of monocarboxylate transporter 8 (mct8) in zebrafish resulted in a dose-dependent impairment of posterior chamber inflation. Since this transporter is known to transport thyroid hormones across cell membranes, this supports the importance of thyroid hormones in regulating posterior chamber inflation.
- Shi et al. (2019) found that exposure of adult zebrafish to 6:2 chlorinated polyfluorinated ether sulfonate (F-53B), an alternative to perfluorooctanesulfonate (PFOS), decreased T3 levels in both male and female zebrafish. Additionally, F-53B was maternally transferred to the offspring. Decreased T3 levels together with impaired posterior chamber inflation was observed in the F1 offspring.
- Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids from fertilization until the age of 5 days. Exogeneous T3 or T4 supplementation partly rescued PFECA-induced posterior swim bladder malformation, confirming the causal relationship between reduced T3 levels and reduced posterior chamber inflation.
- Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. This confirms that T3 plays an important role in posterior swim bladder inflation.

**Uncertainties and Inconsistencies**

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

Another uncertainty lies in the relative importance of the different T4 actvating iodothyronine deiodinases (DIO1, DIO2) in regulating swim bladder inflation. Stinckens et al. (2018) showed that exposure of zebrafish embryos to seven strong DIO1 inhibitors (measured using in chemico enzyme inhibition assays), six out of seven compounds impaired posterior chamber inflation. Exposure to strong DIO2 inhibitors on the other hand affected posterior chamber inflation and/or surface area in all cases. These results suggest that DIO2 enzymes may play a more important role in swim bladder inflation compared to DIO1 enzymes. it has been previously suggested that DIO2 is the major contributor to TH activation in developing zebrafish embryos (Darras et al., 2015; Walpita et al., 2010). It has been shown that a morpholino knockdown targeting DIO1 mRNA alone did not affect embryonic development in zebrafish, while knockdown of DIO2 delayed progression of otic vesicle length, head-trunk angle and pigmentation index (Houbrechts et al., 2016; Walpita et al., 2010, 2009). DIO1 inhibition may only become essential in hypothyroidal circumstances, for example when DIO2 is inhibited or in case of iodine deficiency, in zebrafish (Walpita et al., 2010) and mice (Galton et al., 2009; Schneider et al., 2006).

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

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

The earliest life stages of teleost fish rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). As a result, posterior swim bladder chamber inflation, which occurs early during development, appears to be less sensitive to inhibition of TH synthesis than to inhibition of the conversion of T4 to T3 (Stinckens et al., 2016, 2018; Nelson et al., 2016). There have however been a few reports of reduced posterior inflation upon inhibition of TH synthesis (Liu and Chan, 2002). It must however be noted that these observations could reflect delayed inflation due to a general delay in development rather than a direct effect on the swim bladder. Longer observations would have to clarify this.

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## Relationship: 1028: Reduced, Posterior swim bladder inflation leads to Reduced, Swimming performance (https://aopwiki.org/relationships/1028)

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155)** | **adjacent** | **Moderate** | **Low** |
| **Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157)** | **adjacent** | **Moderate** | **Low** |

### Evidence Supporting Applicability of this Relationship

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |
| bluefin tuna | Thunnus thynnus | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8237) |
| Dicentrarchus labrax | Dicentrarchus labrax | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=13489) |
| Perca flavescens | Perca flavescens | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8167) |
| Salmo salar | Salmo salar | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8030) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| Embryo | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: Importance of proper functioning of the swim bladder for supporting natural swimming behaviour can be plausibly assumed to be generally applicable to fish possessing a posterior chamber. Evidence exists for a wide variety of freshwater and marine fish species.

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

**Sex**: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while

male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization, when sex differentiation has not started yet, sex differences are expected to play a minor role.

## Key Event Relationship Description

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 lower young-of-year survival.

## Evidence Supporting this KER

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

**Biological Plausibility**
The posterior chamber of the swim bladder has a function in regulating the buoyancy of fish (Roberston et al., 2007). Fish rely on the lipid and gas content in their body to regulate their position within the water column, with the latter being more efficient at increasing body buoyancy. Therefore, fish with functional swim bladders have no problem supporting their body (Brix 2002), while it is highly likely that impaired inflation severely impacts swimming performance, as has been suggested previously (Bagci et al., 2015; Hagenaars et al., 2014). Fish without a functional swim bladder are severely disadvantaged, making the likelihood of surviving smaller. Stoyek et al. (2011) showed that the posterior chamber volume is maintained at a stable level at varying pressures corresponding to varying depths through gas exchange with the anteror chamber.

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

- Stewart and Gee (1981) showed that fathead minnows swimming from still water to a current resorbed gas to fill the swim bladder and tailor buoyancy precisely to the level were swimming is most efficient.
- Lindsey et al., 2010 reported that zebrafish larvae that fail to inflate their swim bladder use additional energy to maintain buoyancy (Lindsey et al., 2010, Goodsell et al., 1996), possibly contributing to reduced swimming activity. Furthermore, they reported that the range of swimming depth varies with stages of swim bladder development.
- Czesny et al., 2005 reported that yellow perch larvae without inflated swim bladders capture free-swimming prey poorly and expend more energy on feeding and maintaining their position within the water column, due to impacted swimming behaviour.
- Kurata et al., 2014 observed that Bluefin tuna larvae present at the bottom of a tank, incapable of swimming upwards, had significantly lower swim bladder inflation.
- Chatain (1994) associated sea bass larvae with non-inflated swim bladders with numerous complications, such as spinal deformities and lordosis and reduced growth rates, adding to the impact on swimming behaviour.
- An increasing incidence of swim bladder non-inflation has also been reported in Atlantic salmon. Affected fish had severely altered balance and buoyancy, observed through a specific swimming behaviour, as the affected fish were swimming upside down in an almost vertical position (Poppe et al., 1997).
- Permanent DIO 2 deficiency in zebrafish was shown to result in reduced posterior chamber inflation and disturbed locomotor activity (Houbrechts et al., 2016).
- Michiels et al. (2017) showed that both for controls and zebrafish embryos exposed to an environmental sample, the swimming distance was significantly lower in larvae that failed to inflate the posterior chamber compared to larvae from the same treatment that had inflated posterior chambers.
- Exposure of zebrafish embryos to thyroid disrupting compounds resulted in an effect on posterior chamber inflation as well as on the swimming distance in the larval stage (Stinckens et al., unpublished).
- All zebrafish larvae that failed to inflate the posterior chamber after exposure to 2 mg/L iopanoic acid (IOP), died by the age of 9 dpf (Stinckens et al., 2020). Since larvae from the same group that were able to inflate the posterior chamber survived, it is plausible to assume that uninflated posterior chambers limited the ability to swim and find food.
- Hagenaars et al. (2014) showed that zebrafish embryos exposed to 4.28 mg/L PFOS had lower swimming speeds when the posterior chamber was not inflated. It should be noted that almost all larvae with a non-inflated swimbladder had a spinal curvature and it could therefore not statistically be determined whether the reduced swimming speed was due to a spinal curvature, a non-inflated swim bladder or the interaction of both.
- Knockdown of deiodinase 3 (expected to lead to hyperthyroidism) in zebrafish was shown to result in both impaired inflation of the posterior chamber and reduced swimming activity and escape response (Heijlen et al., 2014; Bagci et al., 2015).
- Massei et al. (in preparation) showed that impaired swim bladder inflation and reduced swimming activity of 5 day old zebrafish larvae were correlated after exposure to narcotics.

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

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

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

## Quantitative Understanding of the Linkage

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

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

**Time-scale**
The data of Michiels et al. (2017) and Stinckens et al. (unpublished) on swim bladder inflation and swimming activity have been collected on the same day. The process of posterior chamber inflation normally occurs during a specific developmental time frame, resulting in limited flexibility to explore temporal concordance. Based on the biologically plausible direct importance of swim bladder functionality to swimming performance, no lag is expected.

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## Relationship: 2212: Reduced, Swimming performance leads to Increased Mortality (https://aopwiki.org/relationships/2212)

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155)** | **adjacent** | **Moderate** | **Low** |
| **Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156)** | **adjacent** | **Moderate** | **Low** |
| **Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157)** | **adjacent** | **Moderate** | **Low** |
| **Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/158)** | **adjacent** | **Moderate** | **Low** |
| **Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159)** | **adjacent** | **Moderate** | **Low** |

### Evidence Supporting Applicability of this Relationship

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**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.

### Key Event Relationship Description

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.

### 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 (https://aopwiki.org/relationships/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.

**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 day 5 when the yolk is almost completely depleted, there was a lag period of around 4 days after which reduced feeding resulted in mortality. Obviously, in a laboratory setup there is no increased risk of being caught by a predator.

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

## Relationship: 2013: Increased Mortality leads to Decrease, Population trajectory (https://aopwiki.org/relationships/2013)

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement (https://aopwiki.org/aops/312)** | **adjacent** | | |
| **Acetylcholinesterase inhibition leading to acute mortality (https://aopwiki.org/aops/16)** | **adjacent** | **Moderate** | **Moderate** |
| **Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155)** | **adjacent** | **High** | **Moderate** |
| **Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/156)** | **adjacent** | **High** | **Moderate** |

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157)** | **adjacent** | **High** | **Moderate** |
| **Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/158)** | **adjacent** | **High** | **Moderate** |
| **Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159)** | **adjacent** | **High** | **Moderate** |

## Evidence Supporting Applicability of this Relationship

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| All life stages | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: All organisms must survive to reproductive age in order to reproduce and sustain populations. The additional considerations related to young-of-year 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 young of year 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.
- Increased mortality in the young-of-year cohort 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 young-of-year has been found to be highly variable and influential on population growth (Miller and Ankley, 2004)

**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).
- In general, there is not enough empirical data on the relationships between survival and population level effects in fish (Rearick et al., 2018) to optimize population models.

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## List of Non Adjacent Key Event Relationships

### Relationship: 1042: Inhibition, Deiodinase 2 leads to Reduced, Posterior swim bladder inflation (https://aopwiki.org/relationships/1042)

AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155)** | **non-adjacent** | **Moderate** | **Low** |

Evidence Supporting Applicability of this Relationship

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| Embryo | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

**Taxonomic**: The evidence for a relationship between DIO2 inhibition and inflation of the posterior chamber of the swim bladder is currently based on work in zebrafish and fathead minnow but is expected to be broadly applicable to fish.

**Sex**: This KER is probably not sex-dependent since both females and males rely on activation of THs by deiodinase for regulation of vital processes. Additionally, zebrafish are undifferentiated gonochorists, and gonad differentiation starts only around 23-25 dpf (Uchida et al., 2002), well after the time point of posterior chamber inflation (around 5 dpf).

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

## Key Event Relationship Description

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

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

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

## Evidence Supporting this KER

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

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

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

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

- Chang et al., (2012) established a base-line for TH levels during zebrafish development and observed peaks in whole-body T3 content at 5

dpf when the posterior chamber of the swim bladder inflates.
- Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of DIO1+2 in zebrafish resulted in impairment of the inflation of the posterior chamber of the swim bladder.
- Permanent DIO 2 deficiency in zebrafish was shown to result in reduced posterior chamber inflation (Houbrechts et al., 2016).
- DIO1 and DIO2 mRNA has also been shown to be present in zebrafish swim bladder tissue at 96 hpf using whole mount in situ hybridization (Heijlen et al., 2013; Dong et al., 2013), suggesting a tissue-specific role of T3 in the inflation process of the posterior chamber.
- Exposure to PTU, a very potent DIO1 inhibitor, caused thyroid hypertrophy in X. leavis because of the inhibition of the peripheral conversion of T4 to T3 (Degitz et al., 2005). PTU also decreased serum T3 levels in the rat (Frumess and Larsen, 1975) and resulted in effects on posterior chamber inflation in zebrafish (Jomaa et al., 2014; Stinckens et al., 2018). It should be noted that there are some uncertainties related to the species-specific susceptibility of DIO1 to inhibition by PTU, as teleostean DIO1 seems to be less sensitive to inhibition by PTU (Orozco and Valverde, 2005; Kuiper et al., 2006; Orozco et al., 2012).
- Stinckens et al. (2018) showed that effects on posterior chamber inflation in zebrafish could be predicted based on in chemico DIO2 inhibition potential with only few false positives and false negatives.
- After exposure of fathead minnows (Pimephales promelas) to the non-specific deiodinase inhibitor IOP from 1-6 dpf, Incidence and length of inflated posterior swim bladders were significantly reduced (Cavallin et al., 2017).
- While DIO1 has a high Km and rT3 is its preferred substrate, DIO2 has a low Km and T4 is its preferred substrate, indicating that DIO2 is more important than DIO1 in converting T4 to T3 in a physiological situation (Darras and Van Herck, 2012). It follows that DIO2 inhibition is likely more important than DIO1 inhibition in reducing posterior chamber inflation.

**Uncertainties and Inconsistencies**

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

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

Another uncertainty lies in the relative importance of the different T4 activating iodothyronine deiodinases (DIO1, DIO2) in regulating swim bladder inflation. Stinckens et al. (2018) showed that when exposing zebrafish embryos to seven strong DIO1 inhibitors (measured using in chemico enzyme inhibition assays), six out of seven compounds impaired posterior chamber inflation. Exposure to strong DIO2 inhibitors on the other hand affected posterior chamber inflation and/or surface area in all cases. These results suggest that DIO2 enzymes may play a more important role in swim bladder inflation compared to DIO1 enzymes. it has been previously suggested that DIO2 is the major contributor to TH activation in developing zebrafish embryos (Darras et al., 2015; Walpita et al., 2010). It has been shown that a morpholino knockdown targeting DIO1 mRNA alone did not affect embryonic development in zebrafish, while knockdown of DIO2 delayed progression of otic vesicle length, head-trunk angle and pigmentation index (Houbrechts et al., 2016; Walpita et al., 2010, 2009). DIO1 inhibition may only become essential in hypothyroidal circumstances, for example when DIO2 is inhibited or in case of iodine deficiency, in zebrafish (Walpita et al., 2010) and mice (Galton et al., 2009; Schneider et al., 2006).

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

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## Relationship: 2213: Reduced, Posterior swim bladder inflation leads to Increased Mortality (https://aopwiki.org/relationships/2213)

### AOPs Referencing Relationship

| AOP Name | Adjacency | Weight of Evidence | Quantitative Understanding |
|---|---|---|---|
| **Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/155)** | **non-adjacent** | **High** | **Low** |
| **Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (https://aopwiki.org/aops/157)** | **non-adjacent** | **High** | **Low** |

## Evidence Supporting Applicability of this Relationship

**Taxonomic Applicability**

| Term | Scientific Term | Evidence | Links |
|---|---|---|---|
| zebrafish | Danio rerio | High | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955) |
| fathead minnow | Pimephales promelas | Moderate | NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988) |

**Life Stage Applicability**

| Life Stage | Evidence |
|---|---|
| Embryo | High |
| larvae | High |

**Sex Applicability**

| Sex | Evidence |
|---|---|
| Unspecific | High |

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

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

**Sex**: This KER is probably not sex-dependent since both females and males rely on the posterior swim bladder chamber to regulate buyoancy. Furthermore, zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization, when sex differentiation has not started yet, sex differences are expected to play a minor role.

## Key Event Relationship Description

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

## Evidence Supporting this KER

**Biological Plausibility**

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

**Empirical Evidence**

- Czesny et al. (2005) demonstrated that swim bladder non-inflation was associated with multiple phenotypic and behavioral outcomes that would be expected to adversely impact young of year survival.

    - Yellow perch with non-inflated swim bladders grew more slowly than those with inflated swim bladders, both in the laboratory and in the field.
    - Yellow perch with non-inflated swim bladders always captured prey less efficiently than those with inflated swim bladders of the same size class.
    - Yellow perch with non-inflated swim bladders suffered from increased predation risk.
    - Yellow perch with non-inflated swim bladders experienced significantly increased mortality and lower time to mortality in a foodless environment compared to those with inflated swim bladders, indicating greater energy expenditure.
    - Yellow perch with non-inflated swim bladders had significantly greater oxygen consumption than fish of the same size class with inflated swim bladders, again indicating greater energy expenditure.
    - The authors hypothesized that failed swim bladder inflation occurs frequently in natural systems, but these individuals rarely survive in a natural environment where food resources are limited.
    - Note: yellow perch are a physoclistous species in which initial inflation can only occur during a narrow window of development in which the pneumatic duct is still connected to the gut, allowing the fish to gulp air and inflate its swim bladder. Once the pneumatic duct closes, normal inflation is no longer possible.
- In aquaculture systems, failure to inflate the swim bladder has been shown to reduce growth rates and cause high mortalities in a wide range of species (reviewed by Woolley and Qin, 2010).
- Pond-cultured walleye with non-inflated swim bladders were found to be smaller (weight and length) than fish with inflated swim bladders. There was also association with deformities (e.g., lordosis) that were expected to impair survival (Kindschi and Barrows, 1993).
- Review of failed swim bladder inflation in wild perch and 26 other physoclistous species showed that fish whose swim bladders failed to inflate had higher mortality, reduced growth, and increased incidence of spinal malformations stereotypical of persistent upward swimming (Egloff, 1996).
- Chatain (1994) reported that sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*) with non-inflated swim bladders were 20-30% less in weight than those with inflated swim bladders and more susceptible to stress-induced mortality (e.g., associated with handling, hypoxia, etc.). It was suggested this was due to both increased energetic demands and decreased feeding efficiency.
- Marty et al. 1995 measured increased oxygen consumption in Japanese medaka (*Oryzias latipes*) with non-inflated swim bladders compared to those whose swim bladders had inflated.
- In zebrafish (*Danio rerio*) whose smim bladder inflation was prevented by holding in a closed chamber (preventing air gulping to inflate the swim bladder), larval survival was significantly less than that of fish held in open chambers whose swim bladders could inflate. There was also increased incidence of spinal curvature in the closed chamber fish whose swim bladders were prevented from inflating (Goolish and Oukutake, 1999).
- Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass (Morone saxatilis) lead to significant increases in both swim bladder inflation and survival (Brown et al., 1988).
- In striped bass, (Morone saxatilis) failure to inflate the swimbladder was reported to results in dysfunctional buoyancy control, deformities, and poor larval survival and growth (Martin-Robichaud and Peterson, 2008).
- All zebrafish larvae that failed to inflate the posterior chamber after exposure to 2 mg/L iopanoic acid (IOP), died by the age of 9 dpf (Stinckens et al., 2020). Since larvae from the same group that were able to inflate the posterior chamber survived, it is plausible to assume that uninflated posterior chambers limited the ability to swim and find food.

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