AOP ID and Title:

SNAPSHOT

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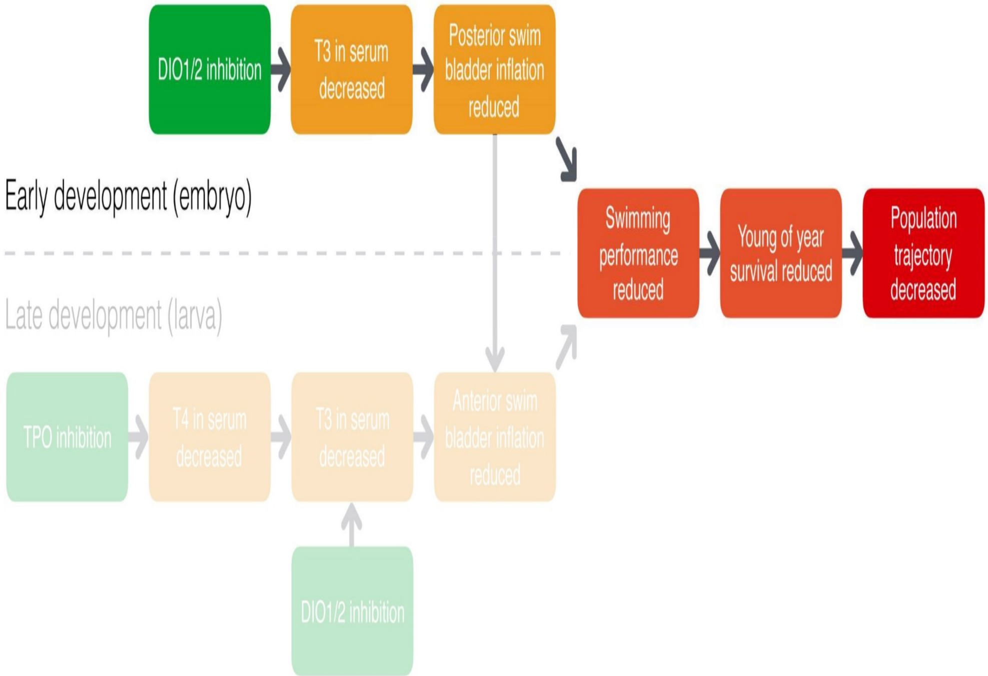
AOP 155: Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation

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 Do not cite	Under Development	1.35	Included in OECD Work Plan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. submitted; 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).

Disruption of the thyroid hormone (TH) system is increasingly being recognized as an important mode of action that can lead to adverse outcomes, especially during development. The description of AOPs may help to select or develop assays to identify TH disrupting compounds and add such assays to existing test guidelines using fish.

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; 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. submitted; 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. submitted; 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 clear.

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
5	KE	1006	Reduced, Young of year survival (https://aopwiki.org/events/1006)	Reduced, Young of year survival
6	KE	1007	Reduced, Anterior swim bladder inflation (https://aopwiki.org/events/1007)	Reduced, Anterior swim bladder inflation
8	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		
Decreased, Triiodothyronine (T3) in serum (https://aopwiki.org/relationships/1027)	adjacent	Reduced, Posterior swim bladder inflation		
Reduced, Posterior swim bladder inflation (https://aopwiki.org/relationships/1028)	adjacent	Reduced, Swimming performance		Low
Reduced, Swimming performance (https://aopwiki.org/relationships/1029)	adjacent	Reduced, Young of year survival		
Reduced, Young of year survival (https://aopwiki.org/relationships/1030)	adjacent	Decrease, Population trajectory		
Reduced, Posterior swim bladder inflation (https://aopwiki.org/relationships/1031)	adjacent	Reduced, Anterior swim bladder inflation		
Reduced, Anterior swim bladder inflation (https://aopwiki.org/relationships/1034)	adjacent	Reduced, Swimming performance		
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/1041)	non-adjacent	Reduced, Young of year survival	High	Low

Overall Assessment of the AOP

Overall, the weight of evidence for the sequence of key events laid out in the AOP is moderate to high. Nonetheless, the exact underlying mechanism of TH disruption leading to impaired swim bladder inflation is not understood. The current domain of applicability is larval life stages of zebrafish and fathead minnow pending future research in other fish species such as medaka.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	

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. submitted; 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.

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 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 confidence in the supporting data for essentiality of KEs within the AOP is high since there is direct evidence from specifically designed experimental studies (knockdown and knockout studies) illustrating that the impact on downstream KEs corresponds to what is predicted by the AOP.

Weight of Evidence Summary

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. Especially for some of the upstream KERs biological plausibility is high.

Overall, the empirical support for the KERs in the AOP is moderate since dependent changes in sets of KEs following exposure to a small number of specific stressors has been demonstrated, but there are still some data gaps.

Quantitative Consideration

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

Considerations for Potential Applications of the AOP (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. (submitted) provides 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 reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	MolecularInitiatingEvent
Aop:156 - Deiodinase 2 inhibition leading to reduced young of year survival via 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)

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

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).

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).

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 tot 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.

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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		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:155 - Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	KeyEvent
Aop:156 - Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/156)	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	KeyEvent
Aop:158 - Deiodinase 1 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/158)	KeyEvent

AOP ID and Name	Event Type
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 reduced young of year survival via 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		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
fathead minnow	Pimephales promelas		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

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.

Key Event Description

There are two biological active thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), and a few inactive 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. Least common is analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates, though methods employing HLPC and mass spectrometry (DeVito et al., 1999; Miller et al., 2009).

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 reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to reduced young of year survival via 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

The evidence for impaired posterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow.

Key Event Description

The swim bladder of bony fish is evolutionary homologous to the lung (Zheng et al., 2011). The teleost swim bladder is a gas-filled structure that consists of two chambers, the posterior and anterior chamber. In zebrafish, the posterior chamber inflates around 96 h post fertilization (hpf) which is 2 days post hatch, and the anterior chamber inflates around 21 dpf. In fathead minnow, the posterior and anterior chamber inflate around 6 and 14 dpf respectively.

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.

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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 reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	KeyEvent
Aop:156 - Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/156)	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	KeyEvent
Aop:158 - Deiodinase 1 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/158)	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to reduced young of year survival via 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		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
teleost fish	teleost fish		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=70862)

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Mixed	

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

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).

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

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Event: 1006: Reduced, Young of year survival (https://aopwiki.org/events/1006)

Short Name: Reduced, Young of year survival

Key Event Component

Process	Object	Action
survival		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:155 - Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	KeyEvent
Aop:156 - Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/156)	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	KeyEvent
Aop:158 - Deiodinase 1 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/158)	KeyEvent

AOP ID and Name	Event Type
Aop:159 - Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/159)	KeyEvent

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

Survival is important for all species.

Key Event Description

Young of year refers to young animals (usually fish) produced in one reproductive year, which have not yet reached one year of age. Small fish, hatched from eggs spawned in the current year, are considered young of year.

Young of year survival directly impacts population structure, growth and fitness. Maintenance of sustainable fish and wildlife populations is an accepted regulatory goal upon which risk assessments and risk management decisions are based.

How it is Measured or Detected

Young of year survival can be measured:

- in the lab by recording survival 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

Event: 1007: Reduced, Anterior swim bladder inflation (https://aopwiki.org/events/1007)

Short Name: Reduced, Anterior swim bladder inflation

Key Event Component

Process	Object	Action
swim bladder inflation	anterior chamber swim bladder	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:155 - Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	KeyEvent
Aop:156 - Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/156)	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	KeyEvent
Aop:158 - Deiodinase 1 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/158)	KeyEvent

AOP ID and Name	Event Type
Aop:159 - Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/159)	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		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
fathead minnow	Pimephales promelas		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow.

Key Event Description

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

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

How it is Measured or Detected

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

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List of Adverse Outcomes in this AOP

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

Short Name: Decrease, Population trajectory

Key Event Component

Process	Object	Action
population growth rate		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: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 reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	AdverseOutcome
Aop:156 - Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/156)	AdverseOutcome
Aop:157 - Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	AdverseOutcome
Aop:158 - Deiodinase 1 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/158)	AdverseOutcome
Aop:159 - Thyroperoxidase inhibition leading to reduced young of year survival via 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 - DNA methyltransferase inhibition leading to reduced fecundity associated population decline (https://aopwiki.org/aops/290)	AdverseOutcome

AOP ID and Name	Event Type
Aop:291 - DNA methyltransferase inhibition leading to transgenerational DNA methylation associated population decline (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:341 - DNA methyltransferase inhibition leading to transgenerational effects (#2) (https://aopwiki.org/aops/341)	AdverseOutcome

Biological Context

Level of Biological Organization
Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species		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.

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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 reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	**adjacent**		
Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/156)	**adjacent**		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

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.

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.

Key Event Relationship Description

Iodothyronine deiodinase or DIO is a peroxidase enzyme that is involved in the activation or deactivition of thyroid hormones. Currently, three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that locally activate or inactivate THs and are therefore important mediators of TH action. All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain the amino acid selenocysteine in their catalytic centre. DIO1 and DIO2 are capable of converting T4 into the more biologically active T3. DIO3 on the other hand converts T4 and T3 to the inactive forms of THs. The inhibition of DIO 1 and 2 enzymes results in decreased serum T3 levels and decreased T3 levels at the site of action.

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. (submitted) showed that IOP reduced T3 levels in zebrafish in 21 and 32 day old larvae that had been exposed starting from fertilization.

Uncertainties and Inconsistencies
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

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 thyroid 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.

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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 reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	**adjacent**		
Deiodinase 1 inhibition leading to reduced young of year survival via 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

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 thryoid hormone receptor alpha and beta failed to inflate the posterior chamber of the swim bladder by 120 hpf, suggesting that the action of T3 is needed for proper inflation of the posterior chamber.
- 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 (Wang et al., 2020). 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.

Evidence of dose-concordance:

- Rehberger et al. (2018) observed decreased T3 levels in the thyroid follicles (which is expected to result in decreased T3 levels in serum) of 120 hpf zebrafish embryos after exposure to PTU starting from 10 mg/L PTU. Stinckens et al.(2018) showed that the downstream KE, impaired posterior chamber inflation, occured at much higher concentrations (EC10: 184 mg/L)

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 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.

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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 reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	**adjacent**		**Low**
Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	**adjacent**		**Low**

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Importance of proper functioning of the swim bladder for supporting natural swimming behaviour is generally applicable to fish across sexes and lifestages.

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, by altering the volume of the swim bladder (Roberston et al., 2007). Fish rely on the lipid and gas content in their body to regulate their position within the water column, with the latter being more efficient at increasing body buoyancy. Therefore, fish with functional swim bladders have no problem supporting their body (Brix 2002), while it is highly likely that impaired inflation severely impacts swimming performance, 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.

Empirical Evidence

Buoyancy is one of the primary mechanisms of fish to regulate behaviour, swimming performance and energy expenditure.

Lindsey et al., 2010 reported that larvae that fail to inflate their swim bladder use additional energy to maintain buoyancy (Lindsey et al., 2010, Goodsell et al., 1996), possibly contributing to reduced swimming activity. 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 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).

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., unpublished). 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.

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: 1029: Reduced, Swimming performance leads to Reduced, Young of year survival (https://aopwiki.org/relationships/1029)

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Importance of swimming performance on young of year survival is generally applicable to fish.

Evidence Supporting this KER

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 young-of-year survival, especially in a non-laboratory environment where food is scarce and predators are abundant.

Empirical Evidence
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., unpublished). Since larvae from the same group that were able to inflate the posterior chamber survived, 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.

Quantitative Understanding of the Linkage

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. (unpublished) 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.

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., unpublished). Since larvae from the same group that were able to inflate the posterior chamber survived, 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.

Relationship: 1030: Reduced, Young of year survival leads to Decrease, Population trajectory (https://aopwiki.org/relationships/1030)

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Key Event Relationship Description

If young of year survival is reduced, ultimately the population trajectory will decrease.

Evidence Supporting this KER

Biological Plausibility
It is widely accepted that if young of year survival is reduced, the population trajectory will eventually decrease.

Relationship: 1031: Reduced, Posterior swim bladder inflation leads to Reduced, Anterior swim bladder inflation (https://aopwiki.org/relationships/1031)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	**adjacent**		
Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	**adjacent**		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

There is no evidence of sex-dependent processes involved in swim bladder chamber develoment and inflation. Additionally, zebrafish are undifferentiated gonochorists, and gonad differentiation starts only around 23-25 dpf (Uchida et al., 2002), after the time point of anterior chamber inflation (around 21 dpf).

Evidence Supporting this KER

Biological Plausibility
The anterior chamber is formed by evagination from the cranial end of the posterior chamber (Robertson et al., 2007, Winata et al., 2009). Therefore it is plausible to assume that the anterior chamber cannot inflate in cases where the posterior chamber is not inflated. Additionally, it is plausible to assume that when the posterior chamber is smaller due to incomplete inflation, the anterior chamber will also fail to completely inflate and therefore will also be smaller.

Empirical Evidence
Stinckens et al. (unpublished) showed that zebrafish larvae exposed to iopanoic acid that failed to inflate the posterior swim bladder chamber by 9 days post fertilization (while normal inflation should occur at 5 days post fertilization) died before the period of anterior chamber inflation.

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Relationship: 1034: Reduced, Anterior swim bladder inflation leads to Reduced, Swimming performance (https://aopwiki.org/relationships/1034)

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

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

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 anterior swim bladder inflation and reduced swimming performance, is weak.

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

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

Empirical Evidence

- After exposure to 2-mercaptobenzothiazole, a TPO inhibitor, from 0 to 32 days post fertilization (dpf) in zebrafish, the swimming activity of fish was impacted starting at 26 dpf if the inflation of the anterior chamber of the swim bladder was impaired or had no normal structure/size (Stinckens et al., 2016).
- Methimazole (MMI) and propylthiouracil (PTU), two thyroperoxidase inhibitors, and iopanoic acid (IOP), a deiodinase inhibitor, each reduced both anterior chamber (AC) inflation and swimming distance in zebrafish exposed from fertlization until the age of 32 days (Stinckens et al., submitted). The current dataset provides evidence for a specific, direct link between AC inflation and reduced swimming performance. First, after 21 d of exposure to 111 mg/L PTU around 30% of ACs were not inflated and swimming distance was reduced, while by 32 dpf all larvae had inflated ACs and the effect on swimming distance had disappeared. The most direct way to assess the role of AC inflation in swimming performance, however, is to compare larvae with and without inflated AC at the same time point and within the same experimental treatment. Both in the PTU exposure at 21 dpf and in the IOP exposure at 21 and 32 dpf, swimming distance was clearly reduced in larvae lacking an inflated AC, while the swimming distance of larvae with inflated AC was equal to that of controls.
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- It has also been reported that larvae that fail to inflate their swim bladder use additional energy to maintain buoyancy (Lindsey et al., 2010, Goodsell et al. 1996), possibly contributing to reduced swimming activity. Furthermore, Chatain (1994) associated 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 (Poppe et al. 1997). 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).

Uncertainties and Inconsistencies

After exposure to 100 mg/L MMI, 95% of the zebrafish larvae failed to inflate their AC at 32 dpf and swimming distance was reduced (Stinckens et al., submitted). On the other hand, there was no effect of impaired AC inflation on swimming distance in the MMI exposure of 50 mg/L. Also, inflated but smaller ACs did not result in a decreased swimming performance in the present study. A similar result, where non-inflated ACs did not consistently lead to reduced swimming performance, was previously found after exposure to MBT (Stinckens et al., 2016). In summary, the precise relationship between these two KEs is not easy to determine and may be different for different chemicals. Swimming capacity can be affected via other processes which may or may not depend on the HPT axis, such as decreased cardiorespiratory function, energy metabolism and growth.

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

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

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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 reduced young of year survival via 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

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.

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.

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).

Key Event Relationship Description

Iodothyronine deiodinase or DIO is a peroxidase enzyme that is involved in the activation or deactivition of thyroid hormones. Currently, three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that locally activate or inactivate THs and are therefore important mediators of TH action. All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain the amino acid selenocysteine in their catalytic centre. DIO1 and DIO2 are capable of converting T4 into the more biologically active T3. DIO3 on the other hand converts T4 and T3 to the inactive forms of THs. The inhibition of DIO 1 and 2 enzymes results in decreased serum T3 levels and decreased T3 levels at the site of action. 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 activity is widely accepted to reduce the T3 levels in serum and is expected to reduce local T3 levels in target tissues, since the conversion of T4 to T3 is inhibited. In fish, many different adverse effects during early development resulting from disruption of the TH endocrine system have been reported, including effects on swim bladder inflation. As in amphibians, the transition in fish between the different developmental phases, including maturation and inflation of the swim bladder, have been shown to be mediated by THs.

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.
- 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).

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: 1041: Reduced, Posterior swim bladder inflation leads to Reduced, Young of year survival (https://aopwiki.org/relationships/1041)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	**non-adjacent**	**High**	**Low**
Deiodinase 1 inhibition leading to reduced young of year survival via 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		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
fathead minnow	Pimephales promelas		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Life Stage Applicability

Life Stage	Evidence
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

The literature provides strong support for the relevance of this KER for physoclistous fish 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 that maintain an open pneumatic duct into adulthood is less apparent. This likely reflects greater potential to inflate 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.

This KER is probably not sex-dependent since both females and males rely on the posterior swim bladder chamber to regulate buyoancy.

Key Event Relationship Description

See biological plausibility below.

Evidence Supporting this KER

Biological Plausibility
The fish swim bladder, particularly the posterior chamber is species with a two-chambered organ, plays a critical role in buoyancy control in fish. Modulation of the volume of air in the chamber allows for maintenance of neutral buoyancy at different depths in the water column. Neutral 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 (particularly the posterior chamber thought to be involved in buoyancy control in most species) would create increased oxygen and energy demands leading to decreased growth, which in turn leads to decreased probability of young of year survival. In particular, these impacts would be expected in non-laboratory environments where fish much expend energy to capture food and avoid predators and where available food is limited.

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. Specifically, mean daily growth rate of fish with inflated swim bladders was 0.50 +/- 0.02 mm/d versus 0.32 +/- 0.01 mm/d for fish without inflated swim bladders.
- 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 experienced significantly increased mortality and lower time to mortality in a foodless environment compared to those with inflated swim bladders.
- Yellow perch with non-inflated swim bladders had significantly greater oxygen consumption than fish of the same size class with inflated swim bladders.
- In Lake Michigan, no yellow perch with a total length >20 mm were collected. Around 15 mm the number of fish collected with non-inflated swim bladders dropped off dramatically. These results reflect both the approximate size at which swim bladder inflation normally occurs within the species and inability to survive and grow to sizes exceeding 20 mm if the swim bladder fails to inflate.
- 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 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).

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