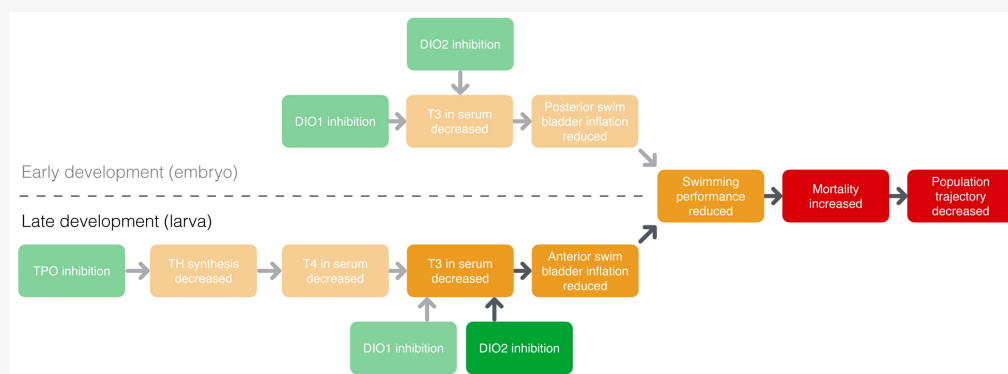


AOP ID and Title:

AOP 156: Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation

Short Title: DIO2i anterior swim bladder

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Open for adoption	Under Development	1.35	Included in OECD Work Plan

Abstract

Starting from reduced serum T3 levels, this AOP is identical to the AOP leading from thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (<https://aopwiki.org/aops/159>).

This AOP describes the sequence of events leading from deiodinase inhibition to increased mortality via reduced anterior swim bladder inflation. Disruption of the thyroid hormone system is increasingly being recognized as an important toxicity pathway that can cause many adverse outcomes, including disruption of developmental processes. Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of TH action. Type II deiodinase (DIO2) has thyroxine (T4) as a preferred substrate and is mostly important for converting T4 to the more biologically active triiodothyronine (T3). Inhibition of DIO2 therefore reduces T3 levels. Thyroid hormones are critical in regulating developmental processes and thyroid hormone disruption can interfere with normal development. Swim bladder inflation is known to be under TH control (Brown et al., 1988; Liu and Chan, 2002). Many fish species have a swim bladder which is a gas-filled organ that typically consists of two chambers (Robertson et al., 2007). The posterior chamber inflates during early development in the embryonic phase, while the anterior chamber inflates during late development in the larval phase. Both the posterior and the anterior chamber have an important role in regulating buoyancy, and the anterior chamber has an additional role in hearing (Robertson et al., 2017). This AOP describes how inhibition of DIO2 reduces levels of T3, thereby prohibiting proper inflation of the anterior chamber. Due to its role in regulating buoyancy, this results in reduced swimming performance. Since reduced swimming performance results in a decreased ability to forage and avoid predators, this reduces chances of survival. The final adverse outcome is a decrease of the population trajectory. Since many AOPs eventually lead to this more general adverse outcome at the population level, the more specific and informative adverse outcome at the organismal level, increased mortality, is used in the AOP title. Support for this AOP is mainly based on chemical exposures in zebrafish and fathead minnows (Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020). This AOP is part of a larger AOP network describing how decreased synthesis and/or decreased biological activation of THs leads to incomplete or improper inflation of the swim bladder, leading to reduced swimming performance, increased mortality and decreased population trajectory (Knapen et al., 2018; Knapen et al., 2020; Villeneuve et al., 2018).

Background

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

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

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name
1	MIE	1002	Inhibition, Deiodinase 2	Inhibition, Deiodinase 2
2	KE	1003	Decreased, Triiodothyronine (T3) in serum	Decreased, Triiodothyronine (T3) in serum
3	KE	1007	Reduced, Anterior swim bladder inflation	Reduced, Anterior swim bladder inflation
4	KE	1005	Reduced, Swimming performance	Reduced, Swimming performance
5	AO	351	Increased Mortality	Increased Mortality
6	AO	360	Decrease, Population trajectory	Decrease, Population trajectory

Key Event Relationships

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

Overall Assessment of the AOP

The attached document includes:

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

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

Domain of Applicability

Life Stage Applicability

Life Stage Evidence

larvae High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

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

Life stage: The current AOP is only applicable to larval development, which is the period where the anterior swim bladder chamber inflates. In all life stages, the conversion of T4 into more biologically active T3 is essential. Inhibition of deiodinase (DIO) therefore impacts swim bladder inflation in both early (<https://aopwiki.org/aops/155>) and late developmental life stages.

Sex: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization, sex differences are expected to play a minor role.

Essentiality of the Key Events

Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is evidence from deiodinase knockdowns showing the link with reduced posterior chamber inflation, but anterior chamber inflation was not studied. There is additional indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation.

Weight of Evidence Summary

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

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

Quantitative Consideration

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

Considerations for Potential Applications of the AOP (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. Villeneuve et al. (2014) discussed the relevance of swim bladder inflation as a potential key event and endpoint of interest in fish tests. Knapen et al. (2020) provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. 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. Specifically, for this AOP, deiodinase inhibition can be assessed using an in chemico assay, measurements of T3 levels could be added to the Fish Embryo Acute Toxicity (FET) test (OECD TG 236) and to the Fish Early Life Stage Toxicity (FELS) Test (OECD TG210), and assessments of anterior chamber inflation and swimming performance could be added to the FELS Test.

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Appendix 1

List of MIEs in this AOP

[Event: 1002: Inhibition, Deiodinase 2](#)

Short Name: Inhibition, Deiodinase 2

Key Event Component

Process	Object	Action
catalytic activity	type II iodothyronine deiodinase	decreased

AOPs Including This Key Event

AOP ID and Name		Event Type
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation		MolecularInitiatingEvent
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation		MolecularInitiatingEvent
Aop:190 - Type II iodothyronine deiodinase (DIO2) inhibition leading to altered amphibian metamorphosis		MolecularInitiatingEvent
Stressors		
Name		
iopanoic acid		
PERFLUOROOCTANOIC ACID		
Biological Context		
Level of Biological Organization		
Molecular		
Evidence for Perturbation by Stressor		
Overview for Molecular Initiating Event		
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
human	Homo sapiens	High NCBI
pigs	Sus scrofa	Moderate NCBI
Oreochromis niloticus	Oreochromis niloticus	Moderate NCBI
zebrafish	Danio rerio	Moderate NCBI
fathead minnow	Pimephales promelas	Moderate NCBI
African clawed frog	Xenopus laevis	NCBI
Life Stage Applicability		
Life Stage	Evidence	
All life stages	Moderate	
Sex Applicability		

Sex	Evidence
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Unspecific	High
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Taxonomic: Deiodination by DIO enzymes is known to exist in a wide range of vertebrates and invertebrates. Reports of inhibition of DIO2 activity are relatively scarce compared to DIO1. Studies reporting DIO2 inhibition have used human recombinant DIO2 enzyme (Olker et al., 2019), primary human astrocytes (Roberts et al., 2015), rat pituitary (Li et al., 2012), pig liver (Stinckens et al., 2018), Nile tilapia (*Oreochromis niloticus*) liver (Walpita et al., 2007). Evidence for zebrafish is indirect since DIO enzyme activity is usually not measured in chemical exposure experiments using zebrafish. Stinckens et al. (2018) showed that chemicals with DIO inhibitory potential in pig liver impaired swim bladder inflation in zebrafish, a thyroid hormone regulated process. Based on these results, DIO2 seemed to be more important than DIO1.

In mammals, DIO2 controls the intracellular concentration of T3. The cells that express DIO2 locally produce T3 that can more rapidly access the thyroid receptors in the nucleus than T3 from plasma (Bianco et al., 2002). For example, DIO2 is highly expressed in the mammalian brain. In teleosts, DIO2 has a markedly higher activity level compared to other vertebrates and it is expressed in liver (Orozco and Valverde, 2005). This could explain why DIO2 inhibition seems to be more important than DIO1 inhibition in determining the adverse outcome in zebrafish (Stinckens et al., 2018).

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

Sex: Deiodinases are important for TH homeostasis in both sexes. Sex-specific differences in this KE have not been described in fish.

Key Event Description

Disruption of the thyroid hormone system is increasingly being recognized as an important toxicity pathway, as it can cause many adverse outcomes. Thyroid hormones do not only play an important role in the adult individual, but they are also critical during embryonic development. Thyroid hormones (THs) play an important role in a wide range of biological processes in vertebrates including growth, development, reproduction, cardiac function, thermoregulation, response to injury, tissue repair and homeostasis. Numerous chemicals are known to disturb thyroid function, for example by inhibiting thyroperoxidase (TPO) or deiodinase (DIO), upregulating excretion pathways or modifying gene expression. The two major thyroid hormones are triiodothyronine (T3) and thyroxine (T4), both iodinated derivatives of tyrosine. The synthesis of the thyroid hormones is a process that involves several steps. Thyroglobulin, the thyroid hormone precursor, is produced by the thyroid epithelial cells and transported to the lumen via exocytosis. Then thyroperoxidase (TPO) plays an essential role in the production of mainly T4. The prohormone T4 is then released in the circulation under the influence of thyroid stimulating hormone (TSH), in order to be transported to the various tissues, including the liver, the kidneys and the heart. Most TH actions depend on the binding of T3 to its nuclear receptors. Active and inactive THs are tightly regulated by enzymes called iodothyronine deiodinases (DIO). The activation occurs via outer ring deiodination (ORD), i.e. removing iodine from the outer, phenolic ring of T4 to form T3, while inactivation occurs via inner ring deiodination (IRD), i.e. removing iodine from the inner tyrosol ring of T4 or T3.

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

How it is Measured or Detected

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

Several methods for deiodinase activity measurements are available. A first *in vitro* assay measures deiodinase activities by quantifying the radioactive iodine release from iodine-labelled substrates, depending on the preferred substrates of the isoforms of deiodinases (Forhead et al., 2006; Pavelka, 2010; Houbrechts et al., 2016; Stinckens et al., 2018). Each of these assays requires a source of deiodinase which can be obtained for example using unexposed pig liver tissue (available from slaughterhouses) or rat liver tissue. Olker et al. (2019) on the other hand used an adenovirus expression system to produce the DIO2 enzyme and developed an assay for nonradioactive measurement of iodide released using the Sandell-Kolthoff method in a 96-well plate format. This assay was then used to screen the ToxCast Phase 1 chemical library. The specific synthesis of DIO2 through the adenovirus expression system provides an important advantage over other methods where activity of the different deiodinase isoforms needs to be distinguished in other ways, such as based on differences in enzyme kinetics.

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List of Key Events in the AOP

[Event: 1003: Decreased, Triiodothyronine \(T3\) in serum](#)

Short Name: Decreased, Triiodothyronine (T3) in serum

Key Event Component

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

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	KeyEvent
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	KeyEvent
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent

Aop:189 - Type I iodothyronine deiodinase (DIO1) inhibition leading to altered amphibian metamorphosis	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered eye structure	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Organ term

Organ term

serum

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage Evidence

All life stages High

Sex Applicability

Sex Evidence

Unspecific High

Taxonomic: The overall evidence supporting taxonomic applicability is strong. With few exceptions vertebrate species have circulating T3 and T4 that are bound to transport proteins in blood. Clear species differences exist in transport proteins (Yamauchi and Ishihara, 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, whereas the half-life in humans is 5-9 days (Capen, 1997). While these species differences impact hormone half-life, possibly regulatory feedback mechanisms, and quantitative dose-response relationships, measurement of 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 different animal and plant kingdoms (Eales, 1997; Heyland and Moroz, 2005), while their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of TH in the different species may differ depending on the expression or function of specific proteins (e.g receptors or enzymes) that are related to TH function, and therefore extrapolation between species should be done with cautious.

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

Sex: Thyroid hormones are essential in both sexes.

Key Event Description

There are two biologically active thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), and a few less active iodothyronines (rT3, 3,5-T2), which are all derived from the modification of tyrosine molecules (Hulbert, 2000). However, the

plasma concentrations of the other iodothyronines are significantly lower than those of T3 and T4. The different iodothyronines are formed by the sequential outer or inner ring monodeiodination of T4 by the deiodinating enzymes, Dio1, Dio2, and Dio3 (Gereben et al., 2008). Deiodinase structure is considered to be unique, as THs are the only molecules in the body that incorporate iodide.

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

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

It is notable that the changes measured in the TH concentration reflect mainly the changes in the serum transport proteins rather than changes in the thyroid status. These thyroid-binding proteins serve as hormonal store which ensure their even and constant distribution in the different tissues, while they protect the most sensitive ones in the case of severe changes in thyroid availability, like in thyroidectomies (Obregon et al., 1981). Until recently, it was believed that all of the effects of TH were mediated by the binding of T3 to the thyroid nuclear receptors (TR α and TR β), 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 succeeded by the efficient regulatory mechanism of the thyroid hormone axis which consists of the following: (1) the hypothalamic secretion of the thyrotropin-releasing hormone (TRH), (2) the thyroid-stimulating hormone (TSH) secretion from the anterior pituitary, (3) hormonal transport by the plasma binding proteins, (4) cellular uptake mechanisms in the cell level, (5) intracellular control of TH concentration by the deiodinating mechanism (6) transcriptional function of the nuclear thyroid hormone receptor and (7) in the fetus, the transplacental passage of T4 and T3 (Cheng et al., 2010).

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

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

How it is Measured or Detected

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

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

mass spectrometry have become more common (DeVito et al., 1999; Miller et al., 2009; Hornung et al., 2015; Nelson et al., 2016; Stinckens et al., 2016).

Any of these measurements should be evaluated for fit-for-purpose, relationship to the actual endpoint of interest, repeatability, and reproducibility. All three of the methods summarized above would be fit-for-purpose, depending on the number of samples to be evaluated and the associated costs of each method. Both RIA and ELISA measure THs by 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: 1007: Reduced, Anterior swim bladder inflation](#)

Short Name: Reduced, Anterior swim bladder inflation

Key Event Component

Process	Object	Action
swim bladder inflation	anterior chamber swim bladder	decreased

AOPs Including This Key Event

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

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

swim bladder

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage Evidence

larvae High

Sex Applicability

Sex Evidence

Unspecific High

Taxonomic: Teleost fish can be divided in two groups according to swim bladder morphology: physoclistous (e.g., yellow perch) and physostomus (e.g., zebrafish and fathead minnow). Physostomus fish retain a duct between the digestive tract and the swim bladder during adulthood allowing them to gulp air at the surface to fill the swim bladder. In contrast, in physoclistous fish, once

initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Woolley and Qin, 2010). The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2016; Nelson et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020). While zebrafish and fathead minnows are physostomous fish with a two-chambered swim bladder, the Japanese rice fish (*Oryzias latipes*) is a physoclistous fish with a single chambered swim bladder that inflates during early development. The key event 'reduced anterior chamber inflation' is not applicable to such fish species.

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

Sex: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization, sex differences are expected to play a minor role.

Key Event Description

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

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

How it is Measured or Detected

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

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Event: 1005: Reduced, Swimming performance

Short Name: Reduced, Swimming performance

Key Event Component

Process	Object	Action
aquatic locomotion		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	KeyEvent
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	KeyEvent
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	KeyEvent
Aop:242 - Inhibition of lysyl oxidase leading to enhanced chronic fish toxicity	KeyEvent
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	KeyEvent

Biological Context

Level of Biological Organization

Individual

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage Evidence

All life stages High

Sex Applicability

Sex Evidence

Unspecific High

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

Life stage: Importance of swimming performance for natural behaviour is generally applicable across all life stages.

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

Key Event Description

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

How it is Measured or Detected

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

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

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List of Adverse Outcomes in this AOP

Event: 351: Increased Mortality

Short Name: Increased Mortality

Key Event Component

Process	Object	Action
mortality		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	AdverseOutcome
Aop:96 - Axonal sodium channel modulation leading to acute mortality	AdverseOutcome
Aop:104 - Altered ion channel activity leading impaired heart function	AdverseOutcome
Aop:113 - Glutamate-gated chloride channel activation leading to acute mortality	AdverseOutcome
Aop:160 - Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality	AdverseOutcome
Aop:161 - Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality	AdverseOutcome
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:186 - unknown MIE leading to renal failure and mortality	AdverseOutcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	AdverseOutcome
Aop:320 - Binding of viral S-glycoprotein to ACE2 receptor leading to acute respiratory distress associated	

mortality	AOP ID and Name	Adverse Outcome Event Type
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation		Adverse Outcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation		Adverse Outcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation		Adverse Outcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation		Adverse Outcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation		Adverse Outcome
Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered eye structure		Adverse Outcome

Biological Context

Level of Biological Organization

Population

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage **Evidence**

All life stages High

Sex Applicability

Sex **Evidence**

Unspecific High

All living things are susceptible to mortality.

Key Event Description

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

How it is Measured or Detected

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

Mortality can be measured:

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

Regulatory Significance of the AO

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

Event: 360: Decrease, Population trajectory

Short Name: Decrease, Population trajectory

Key Event Component

Process	Object	Action
population growth rate	population of organisms	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)	AdverseOutcome
Aop:25 - Aromatase inhibition leading to reproductive dysfunction	AdverseOutcome
Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction	AdverseOutcome
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	AdverseOutcome
Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior	AdverseOutcome
Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	AdverseOutcome
Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	AdverseOutcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release	AdverseOutcome
Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I/metaphase I transition	AdverseOutcome
Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction	AdverseOutcome
Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint	AdverseOutcome
Aop:292 - Inhibition of tyrosinase leads to decreased population in fish	AdverseOutcome
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	AdverseOutcome
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	AdverseOutcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	AdverseOutcome
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	AdverseOutcome
Aop:336 - DNA methyltransferase inhibition leading to population decline (1)	AdverseOutcome
Aop:337 - DNA methyltransferase inhibition leading to population decline (2)	AdverseOutcome
Aop:338 - DNA methyltransferase inhibition leading to population decline (3)	AdverseOutcome
Aop:339 - DNA methyltransferase inhibition leading to population decline (4)	AdverseOutcome
Aop:340 - DNA methyltransferase inhibition leading to transgenerational effects (1)	AdverseOutcome
Aop:341 - DNA methyltransferase inhibition leading to transgenerational effects (2)	AdverseOutcome
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	AdverseOutcome
Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline	AdverseOutcome
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	AdverseOutcome
Aop:299 - Excessive reactive oxygen species production leading to population decline via reduced fatty acid beta-oxidation	AdverseOutcome
Aop:311 - Excessive reactive oxygen species production leading to population decline via mitochondrial	AdverseOutcome

dysfunction	AOP ID and Name	Event Type
	Aop:216 - Excessive reactive oxygen species production leading to population decline via follicular atresia	AdverseOutcome
	Aop:238 - Excessive reactive oxygen species production leading to population decline via lipid peroxidation	AdverseOutcome
	Aop:326 - Thermal stress leading to population decline (3)	AdverseOutcome
	Aop:325 - Thermal stress leading to population decline (2)	AdverseOutcome
	Aop:324 - Thermal stress leading to population decline (1)	AdverseOutcome
	Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered eye structure	AdverseOutcome

Biological Context

Level of Biological Organization

Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

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

Key Event Description

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
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All life stages High

Sex Applicability

Sex	Evidence
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Unspecific High

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

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

Key Event Relationship Description

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

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

Evidence Supporting this KER

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

Biological Plausibility

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

Empirical Evidence

- Houbrechts et al. (2016) developed a Dio2 knockout and confirmed both the absence of the full length Dio2 protein in the liver and the dramatical decrease of T4 activating enzyme activity in liver, brain and eyes. Finally, they found decreased levels of T3 in liver, brain and eyes.
- Winata et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in DIO1+2 and DIO2 knockdown fish. These effects were rescued after T3 supplementation but not by T4 supplementation, confirming that decreased T3 levels were at the basis of the observed effects.
- In the study of Cavallin et al. (2017) fathead minnow larvae were exposed to IOP, a model iodothyronine deiodinase inhibitor that is assumed to inhibit all three deiodinase enzymes (DIO1,2,3). Transcriptional analysis showed that especially DIO2, but also DIO3 mRNA levels (in some treatments), were increased in 10 to 21 day old larvae exposed to IOP as of the age of 6 days. This suggests that IOP effectively inhibited DIO2 and DIO3 in the larvae and that mRNA levels increased as a compensatory response. The authors also observed pronounced decreases of whole body T3 concentrations and increases of whole body T4 concentrations.
- Stinckens et al. (2020) showed that IOP reduced T3 levels in zebrafish in 21 and 32 day old larvae that had been exposed starting from fertilization.
- While DIO1 has a high Km and rT3 is its preferred substrate, DIO2 has a low Km and T4 is its preferred substrate, indicating that DIO2 is more important than DIO1 in converting T4 to T3 in a physiological situation (Darras and Van Herck, 2012).

Uncertainties and Inconsistencies

Since in fish early life stages THs are typically measured on a whole body level, it is currently uncertain whether T3 level changes occur at the serum and/or tissue level. Pending more dedicated studies, whole body TH levels are considered a proxy for serum TH levels.

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

In DIO2 knockout mice it seemed that the negative feedback system was blocked resulting in increased levels of T4 and TSH and in normal rather than decreased T3 levels compared to WT.

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

Quantitative Understanding of the Linkage

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

Known Feedforward/Feedback loops influencing this KER

Thyroid hormone levels are regulated via negative feedback, influencing this KER. Additionally, deiodinases regulate the activity of thyroid 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: 1035: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Anterior swim bladder inflation

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	<i>Danio rerio</i>	High	NCBI
fathead minnow	<i>Pimephales promelas</i>	High	NCBI

Life Stage Applicability

Life Stage Evidence

larvae High

Sex Applicability**Sex Evidence**

Unspecific High

Taxonomic: Teleost fish can be divided in two groups according to swim bladder morphology: physoclistous (e.g., yellow perch) and physostomus (e.g., zebrafish and fathead minnow). Physostomus fish retain a duct between the digestive tract and the swim bladder during adulthood allowing them to gulp air at the surface to fill the swim bladder. In contrast, in physoclistous fish, once initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Woolley and Qin, 2010). The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2016; Nelson et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020).

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

Sex: This KER is probably not sex-dependent since both females and males rely on THs for regulation of vital processes. 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 in zebrafish).

Key Event Relationship Description

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

Evidence Supporting this KER**Biological Plausibility**

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

Empirical Evidence

- In a study in which embryo-larval fathead minnows (*Pimephales promelas*) were exposed to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole (MBT), T3 concentrations measured at 14dpf were reduced at the same concentration (1 mg/L) that significantly reduced anterior swim bladder inflation at the same time-point (Nelson et al. 2016).
- 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 the study of Cavallin et al. (2017) fathead minnow larvae were exposed to IOP, a model iodothyronine deiodinase inhibitor that is assumed to inhibit all three deiodinase enzymes (DIO1,2,3). The authors observed pronounced decreases of whole body T3 concentrations and increases of whole body T4 concentrations, together with impaired inflation of the anterior swim bladder chamber. More specifically, inflation was delayed and the size of the swim bladder chamber was reduced until the end of the exposure experiment. Since exposure was started after inflation of the posterior chamber, this study shows that DIO inhibition can directly affect anterior chamber inflation.
- In the study of Stinckens et al. (2020) a strong correlation between reduced T3 levels and reduced anterior chamber inflation was observed in zebrafish exposed to iopanoic acid, a deiodinase inhibitor, as well as methimazole and propylthiouracil, both thyroperoxidase inhibitors, from fertilization until the age of 32 days. Anterior chamber inflation was delayed and a number of larvae did not manage to inflate the anterior chamber by the end of the 32 day exposure period. Additionally, exposed fish that had inflated the swim bladder had reduced anterior chamber sizes.

Uncertainties and Inconsistencies

- Since in fish early life stages THs are typically measured on a whole body level, it is currently uncertain whether TH levels changes occur at the serum and/or tissue level. Pending more dedicated studies, whole body TH levels are considered a proxy for serum TH levels.
- Reduced anterior chamber inflation upon disruption of the thyroid hormone system is in most cases, but not always,

accompanied by reduced whole body T3 levels. Stinckens et al. (2016) found a consistent relationship between reduced whole body T4 levels, but not T3 levels, and reduced anterior chamber inflation. Possibly, local T4 levels in the swim bladder tissue were too low to allow for enough local activation to T3. This relates to the general uncertainty on serum versus tissue TH levels.

- The mechanism underlying the link between reduced T3 and reduced anterior chamber inflation remains unclear, but several hypotheses exist (Stinckens et al., 2020). For example, altered gas distribution between chambers could be the result of impaired development of smooth muscle fibers, delayed and/or impaired evagination of the anterior chamber, impaired anterior budding through altered Wnt and hedgehog signalling, etc.
- Increased T3 levels also seem to result in reduced swim bladder inflation. For example, Li et al. (2011) reported impairment of swim bladder inflation in Chinese rare minnows (*Gobiocypris rarus*) exposed to exogenous T3.

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI

fathead minnow	Pimephales promelas	Low	NCRI
Term	Scientific Term	Evidence	Links

Life Stage Applicability

Life Stage Evidence

larvae High

Sex Applicability

Sex Evidence

Unspecific High

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

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

Sex: Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization, sex differences are expected to play a minor role.

Key Event Relationship Description

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

Evidence Supporting this KER

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

Biological Plausibility

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

Several studies in zebrafish and fathead minnow showed that a smaller AC was associated with a larger posterior chamber (Nelson et al., 2016; Stinckens et al., 2016; Cavallin et al., 2017, Stinckens et al., 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

- Lindsey et al. (2010) showed that zebrafish started swimming deeper down in the water column upon inflation of the anterior chamber, confirming a role of the anterior chamber in supporting swimming performance.
- After exposure to 2-mercaptobenzothiazole, a TPO inhibitor, from 0 to 32 days post fertilization (dpf) in zebrafish, the swimming activity of fish was impacted starting at 26 dpf if the inflation of the anterior chamber of the swim bladder was impaired or had no normal structure/size (Stinckens et al., 2016).
- Methimazole (MMI) and propylthiouracil (PTU), two thyroperoxidase inhibitors, and iopanoic acid (IOP), a deiodinase inhibitor, each reduced both anterior chamber inflation and swimming distance in zebrafish exposed from fertilization until the age of 32 days (Stinckens et al., 2020). Stinckens et al. (2020) showed a specific, direct link between reduced anterior chamber inflation and reduced swimming performance. First, after 21 d of exposure to 111 mg/L propylthiouracil around 30% of anterior chambers were not inflated and swimming distance was reduced, while by 32 days post fertilization all larvae had inflated their anterior chamber (although chamber surface was still smaller) and the effect on swimming distance had disappeared. The most direct way to assess the role of anterior chamber inflation in swimming performance, however, is to compare larvae with and without inflated anterior chamber at the same time point and within the same experimental treatment. Both in the propylthiouracil exposure at 21 days post fertilization and in the iopanoic acid exposure at 21 and 32 days post fertilization, swimming distance was clearly reduced in larvae lacking an inflated anterior chamber, while the

swimming distance of larvae with inflated anterior chamber was equal to that of controls.

- 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 methimazole, 95% of the zebrafish larvae failed to inflate their anterior chamber at 32 dpf and swimming distance was reduced (Stinckens et al., 2020). On the other hand, there was no effect of impaired anterior chamber inflation on swimming distance in the methimazole exposure of 50 mg/L. Also, inflated but smaller anterior chambers did not result in a decreased swimming performance in this study. A similar result, where non-inflated anterior chambers did not consistently lead to reduced swimming performance, was previously found after exposure to 2-mercaptobenzothiazole (Stinckens et al., 2016). In summary, the precise relationship between these two KEs is not easy to determine and may be different for different chemicals. 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 increased mortality as hearing loss would affect their ability to respond to their surrounding environment, thus impacting ecological relevant endpoints such as predator avoidance or prey seeking (Wisenden et al., 2008; Fay, 2009).

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	<i>Danio rerio</i>	Moderate	NCBI
fathead minnow	<i>Pimephales promelas</i>	Moderate	NCBI

Life Stage Applicability

Life Stage Evidence

Adult	Moderate
Juvenile	Moderate
larvae	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Importance of swimming performance on survival is generally applicable to all hatched fish across life stages and sexes.

Key Event Relationship Description

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

Evidence Supporting this KER

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

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

Biological Plausibility

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

Empirical Evidence

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

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

Uncertainties and Inconsistencies

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

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage is currently limited.

Time-scale

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

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

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Relationship: 2013: Increased Mortality leads to Decrease, Population trajectory

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	adjacent		
Acetylcholinesterase inhibition leading to acute mortality	adjacent	Moderate	Moderate
Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	adjacent	High	Moderate
Deiodinase 2 inhibition leading to increased mortality via reduced anterior	adjacent	High	Moderate

swim bladder inflation Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AOP Name	Adjacency	Weight of Evidence	Quantitative Data
Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation		adjacent	High	Moderate
Thyropoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation		adjacent	High	Moderate
Thyropoxidase inhibition leading to increased mortality via altered eye structure		adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage Evidence

All life stages High

Sex Applicability

Sex Evidence

Unspecific High

Taxonomic: All organisms must survive to reproductive age in order to reproduce and sustain populations. The additional considerations related to survival made above are applicable to other fish species in addition to zebrafish and fathead minnows with the same reproductive strategy (r-strategist as described in the theory of MaxArthur and Wilson (1967)). The impact of reduced survival on population size is even greater for k-strategists that invest more energy in a lower number of offspring.

Life stage: Density dependent effects start to play a role in the larval stage of fish when free-feeding starts (Hazlerigg et al., 2014).

Sex: This linkage is independent of sex.

Key Event Relationship Description

- Increased mortality in the reproductive population may lead to a declining population.
- Increased mortality may lead to a declining population. This depends on the excess mortality due to the applied stressor and the environmental parameters such as food availability and predation rate. Most fish species are r-strategist, meaning they produce a lot of offspring instead of investing in parental care. This results in natural high larval mortality causing only a small percentage of the larvae to survive to maturity. If the excess larval mortality due to a stressor is small, the population dynamics might result in constant population size. Should the larval excess be more significant, or last on the long-term, this will affect the population. To calculate the long-term persistence of the population, population dynamic models should be used.

Evidence Supporting this KER

Survival rate is an obvious determinant of population size and is therefore included in population modeling (e.g., Miller et al., 2020).

Biological Plausibility

- Survival to reproductive maturity is a parameter of demographic significance. Assuming resource availability (i.e., food, habitat, etc.) is not limiting to the extant population, sufficient mortality in the reproductive population may ultimately lead to declining population trajectories.
- Under some conditions, reduced larval survival may be compensated by reduced predation and increased food availability, and therefore not result in population decline (Stige et al., 2019).

Empirical Evidence

- According to empirical data, combined with population dynamic models, feeding larvae are the crucial life stage in zebrafish (and other r-strategists) for the regulation of the population. (Schäfers et al., 1993)

- In fathead minnow, natural survival of early life stages has been found to be highly variable and influential on population growth (Miller and Ankley, 2004)

Uncertainties and Inconsistencies

- The extent to which larval mortality affects population size could depend on the fraction of surplus mortality compared to a natural situation.
- There are scenarios in which individual mortality may not lead to declining population size. These include instances where populations are limited by the availability of habitat and food resources, which can be replenished through immigration. Effects of mortality in the larvae can be compensated by reduced competition for resources (Stige et al., 2019).
- The direct impact of pesticides on migration behavior can be difficult to track in the field, and documentation of mortality during migration is likely underestimated (Eng 2017).
- In general, there is not enough empirical data on the relationships between survival and population level effects in fish (Rearick et al., 2018) to optimize population models.

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