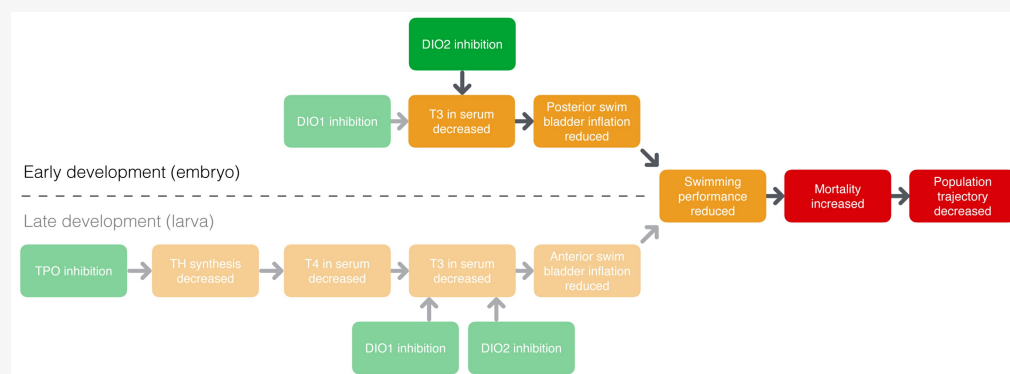


## AOP ID and Title:

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

**Short Title: DIO2i posterior swim bladder**

## Graphical Representation



## Authors

Lucia Vergauwen [1], [lucia.vergauwen(at)uantwerpen.be]

Evelyn Stinckens [1], [evelyn.stinckens(at)uantwerpen.be]

Dan Villeneuve [2], [villeneuve.dan(at)epa.gov]

Dries Knapen [1], [dries.knapen (at)uantwerpen.be]

[1] Zebrafishlab, Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

[2] United States Environmental Protection Agency, Mid-Continent Ecology Division, 6201 Congdon Blvd, Duluth, MN, USA.

## Status

| Author status     | OECD status         | OECD project | SAAOP status               |
|-------------------|---------------------|--------------|----------------------------|
| Open for adoption | EAGMST Under Review | 1.35         | Included in OECD Work Plan |

## Abstract

Other than the difference in deiodinase (DIO) isoform, the current AOP is identical to the corresponding AOP leading from DIO1 inhibition to increased mortality via posterior swim bladder inflation (<https://aopwiki.org/aops/157>). The overall importance of DIO1 versus DIO2 in fish is not exactly clear. The current state of the art suggests that DIO2 is more important than DIO1 in regulating swim bladder inflation. Therefore the current AOP is may be of higher biological relevance compared to AOP 155. 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 posterior swim bladder inflation. Disruption of the thyroid hormone system is increasingly being recognized as an important toxicity pathway that can cause many adverse outcomes, including developmental abnormalities. Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of thyroid hormone (TH) action. Type II deiodinase (DIO2) has thyroxine (T4) as a preferred substrate and is mostly important for converting T4 to the more biologically active triiodothyronine (T3). Inhibition of DIO2 therefore reduces T3 levels. As in amphibians, the transition between the different developmental phases in fish, including maturation and inflation of the swim bladder, is mediated by THs (Brown et al., 1988; Liu and Chan, 2002). The swim bladder is a gas-filled organ that typically consists of two chambers (Robertson et al., 2007). The posterior chamber inflates during early development in the embryonic phase, while the anterior chamber inflates during late development in the larval phase. This AOP describes how DIO2 inhibition results in reduced T3 levels, which prohibit normal inflation of the posterior chamber of the swim bladder in the embryonic phase. The posterior chamber is important for regulating buoyancy and thus for swimming performance (Robertson et al., 2007). Reduced swimming performance reduces chances of survival due to a decreased ability to forage and avoid predators. The final adverse outcome is a decrease of the population 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 (Jomaa et al., 2014; Cavallin et al., 2017; Stinckens et al., 2018) and on knockdown/knockout and TH supplementation studies in zebrafish embryos where the DIO2 gene is inactivated (Walpita et al., 2009, 2010; Heijlen et al., 2014; Bagci et al., 2015; Houbrechts et al., 2016). This AOP is part of a larger AOP network describing how decreased synthesis and/or decreased biological activation of THs leads to incomplete or improper inflation of the swim bladder, leading to reduced swimming performance, increased mortality and decreased population trajectory (Knapen et al., 2018; Knapen et al., 2020; Villeneuve et al., 2018).

## Background

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

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

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

## 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     | <a href="#">Inhibition, Deiodinase 2</a>                  | Inhibition, Deiodinase 2                  |
| 2        | KE   | 1003     | <a href="#">Decreased, Triiodothyronine (T3) in serum</a> | Decreased, Triiodothyronine (T3) in serum |
| 3        | KE   | 1004     | <a href="#">Reduced, Posterior swim bladder inflation</a> | Reduced, Posterior swim bladder inflation |
| 4        | KE   | 1005     | <a href="#">Reduced, Swimming performance</a>             | Reduced, Swimming performance             |
| 5        | AO   | 351      | <a href="#">Increased Mortality</a>                       | Increased Mortality                       |
| 6        | AO   | 360      | <a href="#">Decrease, Population trajectory</a>           | Decrease, Population trajectory           |

### Key Event Relationships

| Upstream Event                                            | Relationship Type | Downstream Event                          | Evidence | Quantitative Understanding |
|-----------------------------------------------------------|-------------------|-------------------------------------------|----------|----------------------------|
| <a href="#">Inhibition, Deiodinase 2</a>                  | adjacent          | Decreased, Triiodothyronine (T3) in serum | Moderate | Low                        |
| <a href="#">Decreased, Triiodothyronine (T3) in serum</a> | adjacent          | Reduced, Posterior swim bladder inflation | Moderate | Low                        |
| <a href="#">Reduced, Posterior swim bladder inflation</a> | adjacent          | Reduced, Swimming performance             | Moderate | Low                        |
| <a href="#">Reduced, Swimming performance</a>             | adjacent          | Increased Mortality                       | Moderate | Low                        |
| <a href="#">Increased Mortality</a>                       | adjacent          | Decrease, Population trajectory           | Moderate | Moderate                   |
| <a href="#">Inhibition, Deiodinase 2</a>                  | non-adjacent      | Reduced, Posterior swim bladder inflation | Moderate | Low                        |
| <a href="#">Reduced, Posterior swim bladder inflation</a> | non-adjacent      | Increased Mortality                       | High     | Low                        |

## Stressors

### Name Evidence

iopanoic acid High

## iopanoic acid

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

## Overall Assessment of the AOP

The attached document includes:

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

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

## Domain of Applicability

### Life Stage Applicability

#### Life Stage Evidence

Embryo High

### Taxonomic Applicability

| Term           | Scientific Term            | Evidence | Links                |
|----------------|----------------------------|----------|----------------------|
| fathead minnow | <i>Pimephales promelas</i> | High     | <a href="#">NCBI</a> |
| zebrafish      | <i>Danio rerio</i>         | High     | <a href="#">NCBI</a> |

### Sex Applicability

#### Sex Evidence

Unspecific Moderate

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

**Taxonomic:** Organogenesis of the swim bladder begins with an evagination from the gut. In physostomous fish, a connection between the swim bladder and the gut is retained. In 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. Knowledge could be expanded to physoclistous fish, such as the Japanese rice fish (*Oryzias latipes*) that has a single chambered swim bladder that inflates during early development.

**Sex:** All key events in this AOP are plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In Medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes of the sequence of events along this AOP. Sex differences are typically not investigated in tests using early life stages of fish and it is currently unclear whether sex-related differences are important in this AOP. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, sex differences are expected to play a minor role in the current AOP. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be

used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

## Essentiality of the Key Events

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

## Weight of Evidence Summary

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

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

## Quantitative Consideration

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

## Considerations for Potential Applications of the AOP (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), the Fish Early Life Stage Toxicity (FELS) Test (OECD TG210) and the Fish Sexual Development Test (FSDT) (OECD TG 234), and assessments of posterior chamber inflation and swimming performance could be added to the FELS Test and FSDT.

Thyroid hormone system disruption causes multiple unspecific effects. Addition of TH measurements could aid in increasing the diagnostic capacity of a battery of endpoints since they are specific to the TH system. A battery of endpoints would ideally include the MIE, the AO and TH levels as the causal link. It is also in this philosophy that TH measurements are currently being considered as one of the endpoints in project 2.64 of the OECD TG work plan, "Inclusion of thyroid endpoints in OECD fish Test Guidelines". While T3 measurements showed low levels of variation and were highly predictive of downstream effects in dedicated experiments to support this AOP, more variability may be present in other studies. Because of the rapid development in fish, it is important to compare T3 levels within specific developmental stages. For example, clear changes in T3 levels have been observed in zebrafish at 14, 21 and 32 dpf (Stinckens et al., 2020) and in fathead minnows at 4, 6, 10, 14, 18 and 21 dpf (Nelson et al., 2016; Cavallin et al., 2017) using liquid chromatography tandem mass spectrometry (LC–MS/MS).

## References

- Bagci, E., Heijlen, M., Vergauwen, L., Hagenaars, A., Houbrechts, A.M., Esguerra, C.V., Blust, R., Darras, V.M., Knapen, D., 2015. Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. *PLOS One* 10, e0123285.
- Brown, C.L., Doroshov, S.I., Nunez, J.M., Hadley, C., Vaneenennaam, J., Nishioka, R.S., Bern, H.A., 1988. MATERNAL TRIIODOTHYRONINE INJECTIONS CAUSE INCREASES IN SWIMBLADDER INFLATION AND SURVIVAL RATES IN LARVAL STRIPED BASS, MORONE-SAXATILIS. *Journal of Experimental Zoology* 248, 168-176.
- Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. *Environmental Toxicology and Chemistry* 36, 2942-2952.

- Heijlen, M., Houbrechts, A., Bagci, E., Van Herck, S., Kersseboom, S., Esguerra, C., Blust, R., Visser, T., Knapen, D., Darras, V., 2014. Knockdown of type 3 iodothyronine deiodinase severely perturbs both embryonic and early larval development in zebrafish. *Endocrinology* 155, 1547-1559.
- Houbrechts, A.M., Delarue, J., Gabriels, I.J., Sourbron, J., Darras, V.M., 2016. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. *Endocrinology* 157, 3668-3681.
- Jomaa, B., Hermesen, S.A.B., Kessels, M.Y., van den Berg, J.H.J., Peijnenburg, A.A.C.M., Aarts, J.M.M.J.G., Piersma, A.H., Rietjens, I.M.C.M., 2014. Developmental Toxicity of Thyroid-Active Compounds in a Zebrafish Embryotoxicity Test. *Altex-Alternatives to Animal Experimentation* 31, 303-317.
- Knapen, D., Angrish, M.M., Fortin, M.C., Katsiadaki, I., Leonard, M., Margiotta-Casaluci, L., Munn, S., O'Brien, J.M., Pollesch, N., Smith, L.C., Zhang, X.W., Villeneuve, D.L., 2018. Adverse outcome pathway networks I: Development and applications. *Environmental Toxicology and Chemistry* 37, 1723-1733.
- Knapen, D., Stinckens, E., Cavallin, J.E., Ankley, G.T., Holbech, H., Villeneuve, D.L., Vergauwen, L., 2020. Toward an AOP Network-Based Tiered Testing Strategy for the Assessment of Thyroid Hormone Disruption. *Environmental Science & Technology* 54, 8491-8499.
- Liu, Y.W., Chan, W.K., 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. *Differentiation* 70, 36-45.
- Maack, G., Segner, H., 2003. Morphological development of the gonads in zebrafish. *Journal of Fish Biology* 62, 895-906.
- Robertson, G.N., McGee, C.A.S., Dumbarton, T.C., Croll, R.P., Smith, F.M., 2007. Development of the swimbladder and its innervation in the zebrafish, *Danio rerio*. *Journal of Morphology* 268, 967-985.
- Stinckens, E., Vergauwen, L., Ankley, G.T., Blust, R., Darras, V.M., Villeneuve, D.L., Witters, H., Volz, D.C., Knapen, D., 2018. An AOP-based alternative testing strategy to predict the impact of thyroid hormone disruption on swim bladder inflation in zebrafish. *Aquatic Toxicology* 200, 1-12.
- Villeneuve, D., Angrish, M., Fortin, M., Katsiadaki, I., Leonard, M., Margiotta-Casaluci, L., Munn, S., O'Brien, J., Pollesch, N., Smith, L., Zhang, X., Knapen, D., 2018. Adverse Outcome Pathway Networks II: Network Analytics. *Environ Toxicol Chem* doi: 10.1002/etc.4124.
- Villeneuve, D., Volz, D.C., Embry, M.R., Ankley, G.T., Belanger, S.E., Leonard, M., Schirmer, K., Tanguay, R., Truong, L., Wehmas, L., 2014. Investigating alternatives to the fish early-life stage test: a strategy for discovering and annotating adverse outcome pathways for early fish development. *Environmental Toxicology and Chemistry* 33, 158-169.
- Walpita, C.N., Crawford, A.D., Janssens, E.D., Van der Geyten, S., Darras, V.M., 2009. Type 2 iodothyronine deiodinase is essential for thyroid hormone-dependent embryonic development and pigmentation in zebrafish. *Endocrinology* 150, 530-539.
- Walpita, C.N., Crawford, A.D., Darras, V.M., 2010. Combined antisense knockdown of type 1 and type 2 iodothyronine deiodinases disrupts embryonic development in zebrafish (*Danio rerio*). *Gen Comp Endocrinol* 166, 134-141.
- Woolley, L.D., Qin, J.G., 2010. Swimbladder inflation and its implication to the culture of marine finfish larvae. *Reviews in Aquaculture* 2, 181-190.

## Appendix 1

### 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               |
|-------------------------------------------------------------------------------------------------------------------------------|--------------------------|
| <a href="#">Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | MolecularInitiatingEvent |

| AOP ID and Name                                                                                                                                                                                                                                                                                                                                                                                                                 |                       | Event Type |                      |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|------------|----------------------|
| Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation                                                                                                                                                                                                                                                                                                                    |                       | Molecular  | InitiatingEvent      |
| Aop:190 - Type II iodothyronine deiodinase (DIO2) inhibition leading to altered amphibian metamorphosis                                                                                                                                                                                                                                                                                                                         |                       | Molecular  | InitiatingEvent      |
| Stressors                                                                                                                                                                                                                                                                                                                                                                                                                       |                       |            |                      |
| Name                                                                                                                                                                                                                                                                                                                                                                                                                            |                       |            |                      |
| iopanoic acid                                                                                                                                                                                                                                                                                                                                                                                                                   |                       |            |                      |
| PERFLUOROOCTANOIC ACID                                                                                                                                                                                                                                                                                                                                                                                                          |                       |            |                      |
| Biological Context                                                                                                                                                                                                                                                                                                                                                                                                              |                       |            |                      |
| Level of Biological Organization                                                                                                                                                                                                                                                                                                                                                                                                |                       |            |                      |
| Molecular                                                                                                                                                                                                                                                                                                                                                                                                                       |                       |            |                      |
| Evidence for Perturbation by Stressor                                                                                                                                                                                                                                                                                                                                                                                           |                       |            |                      |
| Overview for Molecular Initiating Event                                                                                                                                                                                                                                                                                                                                                                                         |                       |            |                      |
| <p>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.</p> |                       |            |                      |
| 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   | <a href="#">NCBI</a> |
| human                                                                                                                                                                                                                                                                                                                                                                                                                           | Homo sapiens          | High       | <a href="#">NCBI</a> |
| pigs                                                                                                                                                                                                                                                                                                                                                                                                                            | Sus scrofa            | Moderate   | <a href="#">NCBI</a> |
| Oreochromis niloticus                                                                                                                                                                                                                                                                                                                                                                                                           | Oreochromis niloticus | Moderate   | <a href="#">NCBI</a> |
| zebrafish                                                                                                                                                                                                                                                                                                                                                                                                                       | Danio rerio           | Moderate   | <a href="#">NCBI</a> |
| fathead minnow                                                                                                                                                                                                                                                                                                                                                                                                                  | Pimephales promelas   | Moderate   | <a href="#">NCBI</a> |
| African clawed frog                                                                                                                                                                                                                                                                                                                                                                                                             | Xenopus laevis        |            | <a href="#">NCBI</a> |
| Life Stage Applicability                                                                                                                                                                                                                                                                                                                                                                                                        |                       |            |                      |
| Life Stage                                                                                                                                                                                                                                                                                                                                                                                                                      | Evidence              |            |                      |
| All life stages                                                                                                                                                                                                                                                                                                                                                                                                                 | Moderate              |            |                      |
| Sex Applicability                                                                                                                                                                                                                                                                                                                                                                                                               |                       |            |                      |
| Sex                                                                                                                                                                                                                                                                                                                                                                                                                             | Evidence              |            |                      |
| Unspecific                                                                                                                                                                                                                                                                                                                                                                                                                      | Moderate              |            |                      |
| Taxonomic: Deiodination by DIO enzymes is known to exist in a wide range of vertebrates and invertebrates. This KE is plausibly                                                                                                                                                                                                                                                                                                 |                       |            |                      |

applicable across vertebrates. Reports of inhibition of DIO2 activity are relatively scarce compared to DIO1. Studies reporting DIO2 inhibition have used human recombinant DIO2 enzyme (Olker et al., 2019), primary human astrocytes (Roberts et al., 2015), rat pituitary (Li et al., 2012), pig liver (Stinckens et al., 2018), Nile tilapia (*Oreochromis niloticus*) liver (Walpita et al., 2007). Evidence for fish (e.g., zebrafish and fathead minnow) is mostly indirect since DIO enzyme activity is usually not measured in chemical exposure experiments. 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. Houbrechts et al. (2016) did however confirm decreased DIO2 activity in a DIO1-DIO2 knockdown zebrafish at the ages of 3 and 7 days post fertilization and Noyes confirmed decreased outer ring deiodination activity in fathead minnows exposed to BDE-209. Walpita et al. (2007) showed decreased DIO2 activity in the liver of Nile tilapia injected with dexamethasone.

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

## Key Event Description

Disruption of the thyroid hormone system is increasingly being recognized as an important toxicity pathway, as it can cause many adverse outcomes. Thyroid hormones do not only play an important role in the adult individual, but they are also critical during embryonic development. Thyroid hormones (THs) play an important role in a wide range of biological processes in vertebrates including growth, development, reproduction, cardiac function, thermoregulation, response to injury, tissue repair and homeostasis. Numerous chemicals are known to disturb thyroid function, for example by inhibiting thyroperoxidase (TPO) or deiodinase (DIO), upregulating excretion pathways or modifying gene expression. The two major thyroid hormones are triiodothyronine (T3) and thyroxine (T4), both iodinated derivatives of tyrosine. Most TH actions depend on the binding of T3 to its nuclear receptors. Active and inactive THs are tightly regulated by enzymes called iodothyronine deiodinases (DIO). The activation occurs via outer ring deiodination (ORD), i.e. removing iodine from the outer, phenolic ring of T4 to form T3, while inactivation occurs via inner ring deiodination (IRD), i.e. removing iodine from the inner tyrosol ring of T4 or T3.

Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of TH action. All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain selenocysteine in their catalytic centre. Type I deiodinase is capable to convert T4 into T3, as well as to convert 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 ( $\mu\text{M}$  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.

Measurements of *in vivo* deiodinase activity in tissues collected from animal experiments are scarce. Noyes et al. (2011) showed decreased rate of outer ring deiodination (mediated by DIO1 and DIO2) in whole fish microsomes after exposure to BDE-209. After incubation with the substrate, thyroid hormone levels were measured using LC-MS/MS. Houbrechts et al. (2016) confirmed DIO2 deiodination activity in a DIO1-DIO2 knockdown zebrafish at the ages of 3 and 7 days post fertilization. Decreased T3 levels are often used as evidence of DIO inhibition, for example after exposure to iopanoic acid, in fish species such as zebrafish (Stinckens et al., 2020) and fathead minnow (Cavallin et al., 2017). It should be noted that it is difficult to make the distinction between decreased

T3 levels caused by outer ring deiodination mediated by DIO2 inhibition or DIO1 inhibition.

## References

- Bianco, A.C., Salvatore, D., Gereben, B., Berry, M.J., Larsen, P.R., 2002. Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocrine Reviews* 23, 38-89.
- Cavallin JE, Ankley GT, Blackwell BR, Blanksma CA, Fay KA, Jensen KM, Kahl MD, Knapen D, Kosian PA, Poole ST et al. 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. *Environmental Toxicology and Chemistry*. 36(11):2942-2952.
- Darras, V.M., Van Herck, S.L.J., 2012. Iodothyronine deiodinase structure and function: from ascidians to humans. *Journal of Endocrinology* 215, 189-206.
- Forhead, A.J., Curtis, K., Kaptein, E., Visser, T.J., Fowden, A.L., 2006. Developmental control of iodothyronine deiodinases by cortisol in the ovine fetus and placenta near term. *Endocrinology* 147, 5988-5994.
- Houbrechts, A.M., Delarue, J., Gabriels, I.J., Sourbron, J., Darras, V.M., 2016. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. *Endocrinology* 157, 3668-3681.
- Li, N.N., Jiang, Y.Q., Shan, Z.Y., Teng, W.P., 2012. Prolonged high iodine intake is associated with inhibition of type 2 deiodinase activity in pituitary and elevation of serum thyrotropin levels. *British Journal of Nutrition* 107, 674-682.
- Noyes PD, Hinton DE, Stapleton HM. 2011. Accumulation and debromination of decabromodiphenyl ether (bde-209) in juvenile fathead minnows (pimephales promelas) induces thyroid disruption and liver alterations. *Toxicological Sciences*. 122(2):265-274.
- Olker, J.H., Korte, J.J., Denny, J.S., Hartig, P.C., Cardon, M.C., Knutsen, C.N., Kent, P.M., Christensen, J.P., Degitz, S.J., Hornung, M.W., 2019. Screening the ToxCast Phase 1, Phase 2, and e1k Chemical Libraries for Inhibitors of Iodothyronine Deiodinases. *Toxicological Sciences* 168, 430-442.
- Orozco, A., Valverde, R.C., 2005. Thyroid hormone deiodination in fish. *Thyroid* 15, 799-813.
- Pavelka, S., 2010. Radiometric enzyme assays: development of methods for extremely sensitive determination of types 1, 2 and 3 iodothyronine deiodinase enzyme activities. *Journal of Radioanalytical and Nuclear Chemistry* 286, 861-865.
- Renko, K., Schache, S., Hoefig, C.S., Welsink, T., Schwiebert, C., Braun, D., Becker, N.P., Kohrle, J., Schomburg, L., 2015. An Improved Nonradioactive Screening Method Identifies Genistein and Xanthohumol as Potent Inhibitors of Iodothyronine Deiodinases. *Thyroid* 25, 962-968.
- Roberts, S.C., Bianco, A.C., Stapleton, H.M., 2015. Disruption of Type 2 Iodothyronine Deiodinase Activity in Cultured Human Glial Cells by Polybrominated Diphenyl Ethers. *Chemical Research in Toxicology* 28, 1265-1274.
- Stinckens, E., Vergauwen, L., Ankley, G.T., Blust, R., Darras, V.M., Villeneuve, D.L., Witters, H., Volz, D.C., Knapen, D., 2018. An AOP-based alternative testing strategy to predict the impact of thyroid hormone disruption on swim bladder inflation in zebrafish. *Aquatic Toxicology* 200, 1-12.
- Stinckens E, Vergauwen L, Blackwell BR, Ankley GT, Villeneuve DL, Knapen D. 2020. Effect of thyroperoxidase and deiodinase inhibition on anterior swim bladder inflation in the zebrafish. *Environmental Science & Technology*. 54(10):6213-6223.
- Vergauwen, L., Cavallin, J.E., Ankley, G.T., Bars, C., Gabriels, I.J., Michiels, E.D.G., Fitzpatrick, K.R., Periz-Stanacev, J., Randolph, E.C., Robinson, S.L., Saari, T.W., Schroeder, A.L., Stinckens, E., Swintek, J., Van Cruchten, S.J., Verbueken, E., Villeneuve, D.L., Knapen, D., 2018. Gene transcription ontogeny of hypothalamic-pituitary-thyroid axis development in early-life stage fathead minnow and zebrafish. *General and Comparative Endocrinology* 266, 87-100.
- Walpita, C.N., Grommen, S.V., Darras, V.M., Van der Geyten, S., 2007. The influence of stress on thyroid hormone production and peripheral deiodination in the Nile tilapia (*Oreochromis niloticus*). *Gen Comp Endocrinol* 150, 18-25.

## List of Key Events in the AOP

**Event: 1003: Decreased, Triiodothyronine (T3) 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 |
|---------------------------------------------------------------------------------------------------------------------------------|------------|
| <a href="#">Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>   | KeyEvent   |
| <a href="#">Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>    | KeyEvent   |
| <a href="#">Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>   | KeyEvent   |
| <a href="#">Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>    | KeyEvent   |
| <a href="#">Aop:189 - Type I iodothyronine deiodinase (DIO1) inhibition leading to altered amphibian metamorphosis</a>          | KeyEvent   |
| <a href="#">Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation</a> | KeyEvent   |
| <a href="#">Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure</a>         | KeyEvent   |
| <a href="#">Aop:364 - Thyroperoxidase inhibition leading to increased mortality via decreased eye size</a>                      | KeyEvent   |
| <a href="#">Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning</a>        | 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     | <a href="#">NCBI</a> |
| fathead minnow      | Pimephales promelas | High     | <a href="#">NCBI</a> |
| African clawed frog | Xenopus laevis      | High     | <a href="#">NCBI</a> |

## Life Stage Applicability

## Life Stage Evidence

All life stages High

## Sex Applicability

## Sex Evidence

Unspecific Moderate

**Taxonomic:** The overall evidence supporting taxonomic applicability is strong. With few exceptions vertebrate species have circulating T3 and T4 that are bound to transport proteins in blood. Therefore, the current key event is plausibly applicable to vertebrates in general. 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 (TTR). TTR demonstrates a reduced binding affinity for T4 when compared with thyroxine binding globulin (TBG), the predominant serum binding protein for T4 in humans. This difference in serum binding protein affinity for THs is thought to modulate serum half-life for T4; the half-life of T4 in rats is 12-24 hr, whereas the half-life in humans is 5-9 days (Capen, 1997). While these species differences impact hormone half-life, possibly regulatory feedback mechanisms, and quantitative dose-response relationships, measurement of 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 (Manzon and Youson, 1997; Yaoita and Brown, 1990) as well as fish development, embryo-to-larval transition and larval-to-juvenile transition (Thienpont et al., 2011; Liu and Chan, 2002) is well established. Their 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 caution.

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

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

## Key Event Description

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

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

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

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

The production of THs in the thyroid gland and the circulation levels in the bloodstream are self-controlled by an efficiently regulated feedback mechanism across the Hypothalamus-Pituitary-Thyroid (HPT) axis. TH levels are regulated, not only in the plasma level, but also in the individual cell level, to maintain homeostasis. This is succeeded by the efficient regulatory mechanism of the thyroid hormone axis which consists of the following: (1) the hypothalamic secretion of the thyrotropin-releasing hormone (TRH), (2) the thyroid-stimulating hormone (TSH) secretion from the anterior pituitary, (3) hormonal transport by the plasma binding proteins, (4) cellular uptake mechanisms in the cell level, (5) intracellular control of TH concentration by the deiodinating mechanism (6) transcriptional function of the nuclear thyroid hormone receptor and (7) in the fetus, the transplacental passage of T4 and T3 (Cheng et al., 2010).

In regards to the brain, the TH concentration involves also an additional level of regulation, namely the hormonal transport through the Blood Brain Barrier (BBB) (Williams, 2008). The TRH and the TSH 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 (Furrow 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 (Glinioer, 2001), emphasizing the role of TH in neurodevelopment in all above cases. In adults, the thyroid-related effects are mainly linked to metabolic activities, such as deficiencies in oxygen consumption, and in the metabolism of the vitamin, proteins, lipids and carbohydrates, but these defects are subtle and reversible (Oetting and Yen, 2007). Blood tests to detect the amount of thyroid hormone (T4) and thyroid stimulating hormone (TSH) are routinely done for newborn babies for the diagnosis of congenital hypothyroidism at the earliest stage possible.

Although the components of the thyroid hormone system as well as thyroid hormone synthesis and action are highly conserved across vertebrates, there are some taxon-specific considerations.

Although the HPT axis is highly conserved, there are some differences between fish and mammals (Blanton and Specker, 2007; Deal and Volkoff, 2020). For example, in **fish**, corticotropin releasing hormone (CRH) often plays a more important role in regulating thyrotropin (TSH) secretion by the pituitary and thus thyroid hormone synthesis compared to TSH-releasing hormone (TRH). TTRs from fish have low sequence identity with human TTR, for example seabream TTR has 54% sequence identity with human TTR but the only amino acid difference within the thyroxine-binding site is the conservative substitution of Ser117 in human TTR to Thr117 in seabream TTR (Santos and Power, 1999; Yamauchi et al., 1999; Eneqvist et al., 2004). In vitro binding experiments showed that TH disrupting chemicals bind with equal or weaker affinity to seabream TTR than to the human TTR with polar TH disrupting chemicals, in particular, showing a more than 500-fold lower affinity for seabream TTR compared to human TTR (Zhang et al., 2018).

Zebrafish and fathead minnows are oviparous fish species in which maternal thyroid hormones are transferred to the eggs and regulate early embryonic developmental processes during external (versus intra-uterine in mammals) development (Power et al., 2001; Campinho et al., 2014; Ruuskanen and Hsu, 2018) until embryonic thyroid hormone synthesis is initiated. Maternal transfer of thyroid hormones, both T4 and T3, to the eggs has been demonstrated in zebrafish (Walpita et al., 2007; Chang et al., 2012) and fathead minnows (Crane et al., 2004; Nelson et al., 2016).

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

### How it is Measured or Detected

T3 and T4 can be measured as free (unbound) or total (bound + unbound). 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.

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

### References

- Bartalena L, Robbins J. Thyroid hormone transport proteins. Clin Lab Med. 1993 Sep;13(3):583-98.
- Bassett JH, Harvey CB, Williams GR. (2003). Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol Cell Endocrinol. 213:1-11.

- Bianco AC, Kim BW. (2006). Deiodinases: implications of the local control of thyroid hormone action. *J Clin Invest.* 116: 2571–2579.
- Blanton ML, Specker JL. 2007. The hypothalamic-pituitary-thyroid (hpt) axis in fish and its role in fish development and reproduction. *Crit Rev Toxicol.* 37(1-2):97-115.
- Brent GA. (2012). Mechanisms of thyroid hormone action. *J Clin Invest.* 122: 3035-3043.
- Campinho MA, Saraiva J, Florindo C, Power DM. 2014. Maternal thyroid hormones are essential for neural development in zebrafish. *Molecular Endocrinology.* 28(7):1136-1149.
- Cavallin JE, Ankley GT, Blackwell BR, Blanksma CA, Fay KA, Jensen KM, Kahl MD, Knapen D, Kosian PA, Poole ST et al. 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. *Environmental Toxicology and Chemistry.* 36(11):2942-2952.
- Chang J, Wang M, Gui W, Zhao Y, Yu L, Zhu G. 2012. Changes in thyroid hormone levels during zebrafish development. *Zoological Science.* 29(3):181-184.
- Cheng SY, Leonard JL, Davis PJ. (2010). Molecular aspects of thyroid hormone actions. *Endocr Rev.* 31:139–170.
- Crane HM, Pickford DB, Hutchinson TH, Brown JA. 2004. Developmental changes of thyroid hormones in the fathead minnow, *pimephales promelas*. *General and Comparative Endocrinology.* 139(1):55-60.
- Davis PJ, Zhou M, Davis FB, Lansing L, Mousa SA, Lin HY. (2010). Mini-review: Cell surface receptor for thyroid hormone and nongenomic regulation of ion fluxes in excitable cells. *Physiol Behav.* 99:237–239.
- Deal CK, Volkoff H. 2020. The role of the thyroid axis in fish. *Frontiers in Endocrinology.* 11.
- DeVito M, Biegel L, Brouwer A, Brown S, Brucker-Davis F, Cheek AO, Christensen R, Colborn T, Cooke P, Crissman J, Crofton K, Doerge D, Gray E, Hauser P, Hurley P, Kohn M, Lazar J, McMaster S, McClain M, McConnell E, \*Meier C, Miller R, Tietge J, Tyl R. (1999). Screening methods for thyroid hormone disruptors. *Environ Health Perspect.* 107:407-415.
- Eales JG. (1997). Iodine metabolism and thyroid related functions in organisms lacking thyroid follicles: Are thyroid hormones also vitamins? *Proc Soc Exp Biol Med.* 214:302-317.
- Eneqvist T, Lundberg E, Karlsson A, Huang SH, Santos CRA, Power DM, Sauer-Eriksson AE. 2004. High resolution crystal structures of piscine transthyretin reveal different binding modes for triiodothyronine and thyroxine. *Journal of Biological Chemistry.* 279(25):26411-26416.
- Friesema EC, Jansen J, Milici C, Visser TJ. (2005). Thyroid hormone transporters. *Vitam Horm.* 70: 137–167.
- Furlow JD, Neff ES. (2006). A developmental switch induced by thyroid hormone: *Xenopus laevis* metamorphosis. *Trends Endocrinol Metab.* 17:40–47.
- Gereben B, Zavacki AM, Ribich S, Kim BW, Huang SA, Simonides WS, Zeöld A, Bianco AC. (2008). Cellular and molecular basis of deiodinase-regulated thyroid hormone signalling. *Endocr Rev.* 29:898–938.
- Gilbert ME, Rovet J, Chen Z, Koibuchi N. (2012). Developmental thyroid hormone disruption: prevalence, environmental contaminants and neurodevelopmental consequences. *Neurotoxicology.* 33: 842-852.
- Glinioer D. (2001). Potential consequences of maternal hypothyroidism on the offspring: evidence and implications. *Horm Res.* 55:109-114.
- Hennemann G, Docter R, Friesema EC, de Jong M, Krenning EP, Visser TJ. (2001). Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocr Rev.* 22:451-476.
- Hernandez-Mariano JA, Torres-Sanchez L, Bassol-Mayagoitia S, Escamilla-Nunez M, Cebrian ME, Villeda-Gutierrez EA, Lopez-Rodriguez G, Felix-Arellano EE, Blanco-Munoz J. 2017. Effect of exposure to p,p'-dde during the first half of pregnancy in the maternal thyroid profile of female residents in a mexican floriculture area. *Environmental Research.* 156:597-604.
- Heyland A, Hodin J. (2004). Heterochronic developmental shift caused by thyroid hormone in larval sand dollars and its implications for phenotypic plasticity and the evolution of non-feeding development. *Evolution.* 58: 524-538.
- Heyland A, Moroz LL. (2005). Cross-kingdom hormonal signaling: an insight from thyroid hormone functions in marine larvae. *J Exp Biol.* 208:4355-4361.
- Hornung, M.W., Kosian, P.A., Haselman, J.T., Korte, J.J., Challis, K., Macherla, C., Nevalainen, E., Degitz, S.J., 2015. In Vitro, Ex Vivo, and In Vivo Determination of Thyroid Hormone Modulating Activity of Benzothiazoles. *Toxicological Sciences* 146, 254-264.
- Hulbert A J. (2000). Thyroid hormones and their effects: A new perspective. *Biol Rev.* 75: 519-631.
- Jansen J, Friesema EC, Milici C, Visser TJ. (2005). Thyroid hormone transporters in health and disease. *Thyroid.* 15: 757-768.
- Larsen PR. (2009). Type 2 iodothyronine deiodinase in human skeletal muscle: new insights into its physiological role and regulation. *J Clin Endocrinol Metab.* 94:1893-1895.

- Liu XS, Cai Y, Wang Y, Xu SH, Ji K, Choi K. 2019. Effects of tris(1,3-dichloro-2-propyl) phosphate (tdcpp) and triphenyl phosphate (tpp) on sex-dependent alterations of thyroid hormones in adult zebrafish. *Ecotoxicology and Environmental Safety*. 170:25-32.
- Liu YW, Chan WK. 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. *Differentiation*. 70(1):36-45.
- Manzon RG, Youson JH. (1997). The effects of exogenous thyroxine (T4) or triiodothyronine (T3), in the presence and absence of potassium perchlorate, on the incidence of metamorphosis and on serum T4 and T3 concentrations in larval sea lampreys (*Petromyzon marinus* L.). *Gen Comp Endocrinol*. 106:211-220.
- Miller MD, Crofton KM, Rice DC, Zoeller RT. (2009). Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. *Environ Health Perspect*. 117:1033-1041.
- Moeller LC, Dumitrescu AM, Seo H, Refetoff S. (2006). Thyroid hormone mediated changes in gene expression can be initiated by cytosolic action of the thyroid hormone receptor  $\beta$  through the phosphatidylinositol 3-kinase pathway. *NRS*. 4:1-4. Nelson, K., Schroeder, A., Ankley, G., Blackwell, B., Blanksma, C., Degitz, S., Flynn, K., Jensen, K., Johnson, R., Kahl, M., Knapen, D., Kosian, P., Milsk, R., Randolph, E., Saari, T., Stinckens, E., Vergauwen, L., Villeneuve, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part I: Fathead minnow. *Aquatic Toxicology* 173, 192-203. Obregon MJ, Mallol J, Escobar del Rey F, Morreale de Escobar G. (1981). Presence of l-thyroxine and 3,5,3-triiodo-l-thyronine in tissues from thyroidectomised rats. *Endocrinology* 109:908-913.
- Nelson K, Schroeder A, Ankley G, Blackwell B, Blanksma C, Degitz S, Flynn K, Jensen K, Johnson R, Kahl M et al. 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part i: Fathead minnow. *Aquatic Toxicology*. 173:192-203.
- Oetting A, Yen PM. (2007). New insights into thyroid hormone action. *Best Pract Res Clin Endocrinol Metab*. 21:193–208.
- Power DM, Llewellyn L, Faustino M, Nowell MA, Bjornsson BT, Einarsdottir IE, Canario AV, Sweeney GE. 2001. Thyroid hormones in growth and development of fish. *Comp Biochem Physiol C Toxicol Pharmacol*. 130(4):447-459.
- Ruuskanen S, Hsu BY. 2018. Maternal thyroid hormones: An unexplored mechanism underlying maternal effects in an ecological framework. *Physiological and Biochemical Zoology*. 91(3):904-916.
- Santos CRA, Power DM. 1999. Identification of transthyretin in fish (*Sparus aurata*): Cdna cloning and characterisation. *Endocrinology*. 140(5):2430-2433.
- Schussler, G.C. (2000). The thyroxine-binding proteins. *Thyroid* 10:141–149.
- Stinckens E, Vergauwen L, Blackwell BR, Anlley GT, Villeneuve DL, Knapen D. 2020. Effect of thyroperoxidase and deiodinase inhibition on anterior swim bladder inflation in the zebrafish. *Environmental Science & Technology*. 54(10):6213-6223.
- Stinckens, E., Vergauwen, L., Schroeder, A., Maho, W., Blackwell, B., Witters, H., Blust, R., Ankley, G., Covaci, A., Villeneuve, D., Knapen, D., 2016. Impaired anterior swim bladder inflation following exposure to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole part II: Zebrafish. *Aquatic Toxicology* 173, 204-217.
- Thienpont B, Tingaud-Sequeira A, Prats E, Barata C, Babin PJ, Raldua D. 2011. Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. *Environmental Science & Technology*. 45(17):7525-7532
- Visser WE, Friesema EC, Jansen J, Visser TJ. (2008). Thyroid hormone transport in and out of cells. *Trends Endocrinol Metab*. 19:50-56.
- Walpita CN, Van der Geyten S, Rurangwa E, Darras VM. 2007. The effect of 3,5,3'-triiodothyronine supplementation on zebrafish (*Danio rerio*) embryonic development and expression of iodothyronine deiodinases and thyroid hormone receptors. *Gen Comp Endocrinol*. 152(2-3):206-214.
- Wang JX, Shi GH, Yao JZ, Sheng N, Cui RN, Su ZB, Guo Y, Dai JY. 2020. Perfluoropolyether carboxylic acids (novel alternatives to pfoa) impair zebrafish posterior swim bladder development via thyroid hormone disruption. *Environment International*. 134.
- Webster GM, Venners SA, Mattman A, Martin JW. 2014. Associations between perfluoroalkyl acids (pfass) and maternal thyroid hormones in early pregnancy: A population-based cohort study. *Environmental Research*. 133:338-347.
- Williams GR. (2008). Neurodevelopmental and neurophysiological actions of thyroid hormone. *J Neuroendocrinol*. 20:784–794.
- Yamauchi K, Nakajima J, Hayashi H, Hara A. 1999. Purification and characterization of thyroid-hormone-binding protein from masu salmon serum - a homolog of higher-vertebrate transthyretin. *European Journal of Biochemistry*. 265(3):944-949.
- Yamauchi K1, Ishihara A. Evolutionary changes to transthyretin: developmentally regulated and tissue-specific gene expression. *FEBS J*. 2009 Oct;276(19):5357-66.
- Yaoita Y, Brown DD. (1990). A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. *Genes Dev*. 4:1917-1924.

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

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

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

### [Event: 1004: Reduced, Posterior swim bladder inflation](#)

**Short Name:** Reduced, Posterior swim bladder inflation

#### Key Event Component

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

#### AOPs Including This Key Event

| AOP ID and Name                                                                                                               | Event Type |
|-------------------------------------------------------------------------------------------------------------------------------|------------|
| <a href="#">Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | KeyEvent   |
| <a href="#">Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | 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     | <a href="#">NCBI</a> |
| fathead minnow | Pimephales promelas | High     | <a href="#">NCBI</a> |

##### Life Stage Applicability

##### Life Stage Evidence

Embryo High

##### Sex Applicability

##### Sex Evidence

Unspecific Moderate

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

swim bladder inflation in Medaka (*Oryzias latipes*) (Gonzalez-doncel et al., 2003; Dong et al., 2016; Kupsco et al., 2016; Mu et al., 2018; Pandelides et al., 2021). This KE is plausibly applicable across fish species with swim bladders, both physostomous and physoclistous.

**Life stage:** The posterior chamber inflates during a specific developmental time frame. In zebrafish, the posterior chamber inflates around 96-120 hpf which is 2-3 dph. In the fathead minnow, the posterior chamber inflates around 6 dpf. Therefore this KE is only applicable to the embryonic life stage.

**Sex:** This KE/KER is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In Medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE/KER. Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

## Key Event Description

The teleost swim bladder is a gas-filled structure that consists of two chambers, the posterior and anterior chamber. In zebrafish, the posterior chamber inflates around 96-120 h post fertilization (hpf) which is 2-3 days post hatch, and the anterior chamber inflates around 21 dpf (days post fertilization). In fathead minnow, the posterior and anterior chamber inflate around 6 and 14 dpf respectively.

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

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

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

## How it is Measured or Detected

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

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

## References

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

Dong W, Liu J, Wei LX, Yang JF, Chernick M, Hinton DE. 2016. Developmental toxicity from exposure to various forms of mercury compounds in medaka fish (*oryzias latipes*) embryos. *Peerj*. 4.

Godfrey A, Hooser B, Abdelmoneim A, Sepulveda MS. 2019. Sex-specific endocrine-disrupting effects of three halogenated chemicals in japanese medaka. *Journal of Applied Toxicology*. 39(8):1215-1223.

Gonzalez-Doncel M, de la Pena E, Barrueco C, Hinton DE. 2003. Stage sensitivity of medaka (*oryzias latipes*) eggs and embryos to

permethrin. *Aquatic Toxicology*. 62(3):255-268.

Kupsco A, Schlenk D. 2016. Stage susceptibility of japanese medaka (*oryzias latipes*) to selenomethionine and hypersaline developmental toxicity. *Environmental Toxicology and Chemistry*. 35(5):1247-1256.

Liu, Y.W., Chan, W.K., 2002. Thyroid hormones are important for embryonic tolarval transitory phase in zebrafish. *Differentiation* 70, 36–45, <http://dx.doi.org/10.1046/j.1432-0436.2002.700104.x>.

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

Mu JL, Chernick M, Dong W, Di Giulio RT, Hinton DE. 2017. Early life co-exposures to a real-world pah mixture and hypoxia result in later life and next generation consequences in medaka (*oryzias latipes*). *Aquatic Toxicology*. 190:162-173.

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

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

Pandelides Z, Ussery EJ, Overturf MD, Guchardi J, Holdway DA. 2021. Inhibition of swim bladder inflation in japanese medaka (*oryzias latipes*) embryos following exposure to select pharmaceuticals alone and in combination. *Aquatic Toxicology*. 234.

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

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

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

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

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

### 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 |
|---------------------------------------------------------------------------------------------------------------------------------|------------|
| <a href="#">Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>   | KeyEvent   |
| <a href="#">Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>    | KeyEvent   |
| <a href="#">Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>   | KeyEvent   |
| <a href="#">Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>    | KeyEvent   |
| <a href="#">Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation</a> | KeyEvent   |
| <a href="#">Aop:242 - Inhibition of lysyl oxidase leading to enhanced chronic fish toxicity</a>                                 | KeyEvent   |
| <a href="#">Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration</a>                                  | KeyEvent   |

#### **Biological Context**

**Level of Biological Organization**

Individual

**Domain of Applicability****Taxonomic Applicability**

| Term           | Scientific Term     | Evidence | Links                |
|----------------|---------------------|----------|----------------------|
| zebrafish      | Danio rerio         | High     | <a href="#">NCBI</a> |
| teleost fish   | teleost fish        | High     | <a href="#">NCBI</a> |
| fathead minnow | Pimephales promelas | High     | <a href="#">NCBI</a> |

**Life Stage Applicability****Life Stage Evidence**

|          |          |
|----------|----------|
| Larvae   | Moderate |
| Juvenile | Moderate |
| Adult    | Moderate |

**Sex Applicability****Sex Evidence**

|            |          |
|------------|----------|
| Unspecific | Moderate |
|------------|----------|

**Taxonomic:** Importance of swimming performance for natural behaviour is generally applicable to fish and the other taxa that rely on swimming to support vital behaviours.

**Life stage:** Importance of swimming performance for natural behaviour is generally applicable across all free-swimming life stages, i.e., post-embryonic life stages.

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

**Key Event Description**

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

**How it is Measured or Detected**

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

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

Little and Finger (1990) discussed swimming behavior as an indicator of sublethal toxicity in fish.

**References**

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

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

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

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

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

9-17.

**List of Adverse Outcomes in this AOP****Event: 351 : Increased Mortality****Short Name: Increased Mortality****Key Event Component**

| Process   | Object | Action    |
|-----------|--------|-----------|
| mortality |        | increased |

**AOPs Including This Key Event**

| AOP ID and Name                                                                                                                                                                    | Event Type     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <a href="#">Aop:16 - Acetylcholinesterase inhibition leading to acute mortality</a>                                                                                                | AdverseOutcome |
| <a href="#">Aop:96 - Axonal sodium channel modulation leading to acute mortality</a>                                                                                               | AdverseOutcome |
| <a href="#">Aop:104 - Altered ion channel activity leading impaired heart function</a>                                                                                             | AdverseOutcome |
| <a href="#">Aop:113 - Glutamate-gated chloride channel activation leading to acute mortality</a>                                                                                   | AdverseOutcome |
| <a href="#">Aop:160 - Ionotropic gamma-aminobutyric acid receptor activation mediated neurotransmission inhibition leading to mortality</a>                                        | AdverseOutcome |
| <a href="#">Aop:161 - Glutamate-gated chloride channel activation leading to neurotransmission inhibition associated mortality</a>                                                 | AdverseOutcome |
| <a href="#">Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality</a>                                                                       | AdverseOutcome |
| <a href="#">Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality</a>                                                                                | AdverseOutcome |
| <a href="#">Aop:186 - unknown MIE leading to renal failure and mortality</a>                                                                                                       | AdverseOutcome |
| <a href="#">Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination &amp; Movement</a>                                                      | AdverseOutcome |
| <a href="#">Aop:320 - Binding of viral S-glycoprotein to ACE2 receptor leading to acute respiratory distress associated mortality</a>                                              | AdverseOutcome |
| <a href="#">Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>                                                      | AdverseOutcome |
| <a href="#">Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>                                                       | AdverseOutcome |
| <a href="#">Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>                                                      | AdverseOutcome |
| <a href="#">Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>                                                       | AdverseOutcome |
| <a href="#">Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>                                                    | AdverseOutcome |
| <a href="#">Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure</a>                                                            | AdverseOutcome |
| <a href="#">Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Acute Respiratory Distress Syndrome (ARDS) and Multiple Organ Dysfunction (MOD)</a> | AdverseOutcome |
| <a href="#">Aop:364 - Thyroperoxidase inhibition leading to increased mortality via decreased eye size</a>                                                                         | AdverseOutcome |
| <a href="#">Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning</a>                                                           | AdverseOutcome |
| <a href="#">Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)</a>                                                                | AdverseOutcome |
| <a href="#">Aop:413 - Oxidation and antagonism of reduced glutathione leading to mortality via acute renal failure</a>                                                             | AdverseOutcome |
| <a href="#">Aop:410 - Repression of Gbx2 expression leads to defects in developing inner ear and consequently to increased mortality</a>                                           | KeyEvent       |

**Biological Context****Level of Biological Organization**

## Population Level of Biological Organization

### Domain of Applicability

#### Taxonomic Applicability

| Term        | Scientific Term | Evidence | Links                |
|-------------|-----------------|----------|----------------------|
| all species | all species     | High     | <a href="#">NCBI</a> |

#### Life Stage Applicability

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

#### Sex Applicability

| Sex        | Evidence |
|------------|----------|
| Unspecific | Moderate |

All living things are susceptible to mortality.

### Key Event Description

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

### How it is Measured or Detected

Mortality of animals is generally observed as cessation of the heart beat, breathing (gill or lung movement) and locomotory movements. Mortality is typically measured by observation. Depending on the size of the organism, instruments such as microscopes may be used. The reported metric is mostly the mortality rate: the number of deaths in a given area or period, or from a particular cause.

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

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

### Regulatory Significance of the AO

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

#### [Event: 360: Decrease, Population 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     |
|------------------------------------------------------------------------------------------------------------------|----------------|
| <a href="#">Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)</a> | AdverseOutcome |
| <a href="#">Aop:25 - Aromatase inhibition leading to reproductive dysfunction</a>                                | AdverseOutcome |
| <a href="#">Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction</a>                           | AdverseOutcome |
| <a href="#">Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction</a>                        | AdverseOutcome |

# AOP155

| AOP ID and Name                                                                                                                                         | Event Type     |
|---------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <a href="#">Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior</a>                      | AdverseOutcome |
| <a href="#">Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation</a>                    | AdverseOutcome |
| <a href="#">Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription</a>                                        | AdverseOutcome |
| <a href="#">Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>                           | AdverseOutcome |
| <a href="#">Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>                            | AdverseOutcome |
| <a href="#">Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>                           | AdverseOutcome |
| <a href="#">Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>                            | AdverseOutcome |
| <a href="#">Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>                         | AdverseOutcome |
| <a href="#">Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release</a>                             | AdverseOutcome |
| <a href="#">Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I/metaphase I transition</a> | AdverseOutcome |
| <a href="#">Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction</a>                                                                  | AdverseOutcome |
| <a href="#">Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint</a>               | AdverseOutcome |
| <a href="#">Aop:292 - Inhibition of tyrosinase leads to decreased population in fish</a>                                                                | AdverseOutcome |
| <a href="#">Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR</a>                      | AdverseOutcome |
| <a href="#">Aop:16 - Acetylcholinesterase inhibition leading to acute mortality</a>                                                                     | AdverseOutcome |
| <a href="#">Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination &amp; Movement</a>                           | AdverseOutcome |
| <a href="#">Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration</a>                                                          | AdverseOutcome |
| <a href="#">Aop:336 - DNA methyltransferase inhibition leading to population decline (1)</a>                                                            | AdverseOutcome |
| <a href="#">Aop:337 - DNA methyltransferase inhibition leading to population decline (2)</a>                                                            | AdverseOutcome |
| <a href="#">Aop:338 - DNA methyltransferase inhibition leading to population decline (3)</a>                                                            | AdverseOutcome |
| <a href="#">Aop:339 - DNA methyltransferase inhibition leading to population decline (4)</a>                                                            | AdverseOutcome |
| <a href="#">Aop:340 - DNA methyltransferase inhibition leading to transgenerational effects (1)</a>                                                     | AdverseOutcome |
| <a href="#">Aop:341 - DNA methyltransferase inhibition leading to transgenerational effects (2)</a>                                                     | AdverseOutcome |
| <a href="#">Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish</a>                                                       | AdverseOutcome |
| <a href="#">Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline</a>                                                         | AdverseOutcome |
| <a href="#">Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation</a>                                      | AdverseOutcome |
| <a href="#">Aop:299 - Excessive reactive oxygen species production leading to population decline via reduced fatty acid beta-oxidation</a>              | AdverseOutcome |
| <a href="#">Aop:311 - Excessive reactive oxygen species production leading to population decline via mitochondrial dysfunction</a>                      | AdverseOutcome |
| <a href="#">Aop:216 - Excessive reactive oxygen species production leading to population decline via follicular atresia</a>                             | AdverseOutcome |
| <a href="#">Aop:238 - Excessive reactive oxygen species production leading to population decline via lipid peroxidation</a>                             | AdverseOutcome |
| <a href="#">Aop:326 - Thermal stress leading to population decline (3)</a>                                                                              | AdverseOutcome |
| <a href="#">Aop:325 - Thermal stress leading to population decline (2)</a>                                                                              | AdverseOutcome |
| <a href="#">Aop:324 - Thermal stress leading to population decline (1)</a>                                                                              | AdverseOutcome |
| <a href="#">Aop:363 - Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure</a>                                 | AdverseOutcome |
| <a href="#">Aop:349 - Inhibition of 11β-hydroxylase leading to decreased population trajectory</a>                                                      | AdverseOutcome |
| <a href="#">Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory</a>                                     | AdverseOutcome |
| <a href="#">Aop:376 - Androgen receptor agonism leading to male-biased sex ratio</a>                                                                    | AdverseOutcome |
| <a href="#">Aop:386 - Increased reactive oxygen species production leading to population decline via inhibition of photosynthesis</a>                   | AdverseOutcome |

| AOP ID and Name                                                                                                                    | Event Type     |
|------------------------------------------------------------------------------------------------------------------------------------|----------------|
| <a href="#">Aop:387 - Increased reactive oxygen species production leading to population decline via mitochondrial dysfunction</a> | AdverseOutcome |
| <a href="#">Aop:388 - DNA damage leading to population decline via programmed cell death</a>                                       | AdverseOutcome |
| <a href="#">Aop:389 - Oxygen-evolving complex damage leading to population decline via inhibition of photosynthesis</a>            | AdverseOutcome |
| <a href="#">Aop:364 - Thyroperoxidase inhibition leading to increased mortality via decreased eye size</a>                         | AdverseOutcome |
| <a href="#">Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning</a>           | AdverseOutcome |
| <a href="#">Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)</a>                | AdverseOutcome |

## Biological Context

### Level of Biological Organization

Population

### Domain of Applicability

#### Taxonomic Applicability

| Term        | Scientific Term | Evidence | Links                |
|-------------|-----------------|----------|----------------------|
| all species | all species     | High     | <a href="#">NCBI</a> |

#### Life Stage Applicability

| Life Stage      | Evidence      |
|-----------------|---------------|
| All life stages | Not Specified |

#### Sex Applicability

| Sex        | Evidence      |
|------------|---------------|
| Unspecific | Not Specified |

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

### Key Event Description

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

### How it is Measured or Detected

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

### Regulatory Significance of the AO

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

### References

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

## Appendix 2

## List of Key Event Relationships in the AOP

### List of Adjacent Key Event Relationships

[Relationship: 1026: Inhibition, Deiodinase 2 leads to Decreased, Triiodothyronine \(T3\) in serum](#)

#### AOPs Referencing Relationship

| AOP Name                                                                                                            | Adjacency | Weight of Evidence | Quantitative Understanding |
|---------------------------------------------------------------------------------------------------------------------|-----------|--------------------|----------------------------|
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | adjacent  | Moderate           | Low                        |
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>  | adjacent  | Moderate           | Low                        |

#### Evidence Supporting Applicability of this Relationship

##### Taxonomic Applicability

| Term           | Scientific Term     | Evidence | Links                |
|----------------|---------------------|----------|----------------------|
| zebrafish      | Danio rerio         | High     | <a href="#">NCBI</a> |
| fathead minnow | Pimephales promelas | High     | <a href="#">NCBI</a> |

##### Life Stage Applicability

| Life Stage | Evidence |
|------------|----------|
|------------|----------|

All life stages High

##### Sex Applicability

| Sex | Evidence |
|-----|----------|
|-----|----------|

Unspecific Moderate

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

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

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

#### Key Event Relationship Description

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

Three types of iodothyronine deiodinases (DIO1-3) have been described in vertebrates that activate or inactivate THs and are therefore important mediators of TH action. All deiodinases are integral membrane proteins of the thioredoxin superfamily that contain selenocysteine in their catalytic centre. Type I deiodinase is capable of converting T4 into T3, as well as to convert rT3 to the inactive thyroid hormone 3,3'-T2, through outer ring deiodination. rT3, rather than T4, is the preferred substrate for DIO1. Furthermore, DIO1 has a very high  $K_m$  ( $\mu\text{M}$  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 zebrafish Dio2 knockout and confirmed both the absence of the full length Dio2 protein in the liver and the dramatical decrease of T4 activating enzyme activity in liver, brain and eyes. Finally, they found decreased levels of T3 in liver, brain and eyes.
- Winata et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in DIO1+2 and DIO2 knockdown zebrafish. These effects were rescued after T3 supplementation but not by T4 supplementation, confirming that decreased T3 levels were at the basis of the observed effects.
- In the study of Cavallin et al. (2017) fathead minnow larvae were exposed to IOP, a model iodothyronine deiodinase inhibitor that is assumed to inhibit all three deiodinase enzymes (DIO1,2,3). Transcriptional analysis showed that especially DIO2, but also DIO3 mRNA levels (in some treatments), were increased in 10 to 21 day old larvae exposed to IOP as of the age of 6 days. This suggests that IOP effectively inhibited DIO2 and DIO3 in the larvae and that mRNA levels increased as a compensatory response. The authors also observed pronounced decreases of whole body T3 concentrations and increases of whole body T4 concentrations.
- Stinckens et al. (2020) showed that IOP reduced T3 levels in zebrafish in 21 and 32 day old larvae that had been exposed starting from fertilization.
- While DIO1 has a high  $K_m$  and rT3 is its preferred substrate, DIO2 has a low  $K_m$  and T4 is its preferred substrate, indicating that DIO2 is more important than DIO1 in converting T4 to T3 in a physiological situation across species (Darras and Van Herck, 2012).

### Uncertainties and Inconsistencies

Since in fish early life stages THs are typically measured on a whole body level, it is currently uncertain whether T3 level changes occur at the serum and/or tissue level. 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).

Deiodinase 2 inhibition may not always directly lead to decreased T3 levels as there may be age-specific, exposure window-specific, and exposure duration-specific effects that may deviate from that dynamic. Differences in feedback mechanisms may be an important contributor. In DIO2 knockout mice it seemed that the negative feedback system was blocked resulting in increased levels of T4 and TSH and in normal rather than decreased T3 levels compared to WT.

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

## Quantitative Understanding of the Linkage

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

### Known Feedforward/Feedback loops influencing this KER

Thyroid hormone levels are regulated via negative feedback, 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.

### References

- Bianco, A.C., Kim, B.W., 2006. Deiodinases: implications of the local control of thyroid hormone action. *Journal of Clinical Investigation* 116, 2571-2579.
- Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. *Environmental Toxicology and Chemistry* 36, 2942-2952.
- Darras, V.M., Houbrechts, A.M., Van Herck, S.L.J., 2015. Intracellular thyroid hormone metabolism as a local regulator of nuclear thyroid hormone receptor-mediated impact on vertebrate development.
- Darras, V.M., Van Herck, S.L.J., 2012. Iodothyronine deiodinase structure and function: from ascidians to humans. *Journal of Endocrinology* 215, 189-206.
- Hernandez-Mariano JA, Torres-Sanchez L, Bassol-Mayagoitia S, Escamilla-Nunez M, Cebrian ME, Villeda-Gutierrez EA, Lopez-Rodriguez G, Felix-Arellano EE, Blanco-Munoz J. 2017. Effect of exposure to p,p'-dDE during the first half of pregnancy in the maternal thyroid profile of female residents in a Mexican floriculture area. *Environmental Research*. 156:597-604.
- Houbrechts, A.M., Delarue, J., Gabriels, I.J., Sourbron, J., Darras, V.M., 2016. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. *Endocrinology* 157, 3668-3681.
- Liu XS, Cai Y, Wang Y, Xu SH, Ji K, Choi K. 2019. Effects of tris(1,3-dichloro-2-propyl) phosphate (tdcpp) and triphenyl phosphate (tpp) on sex-dependent alterations of thyroid hormones in adult zebrafish. *Ecotoxicology and Environmental Safety*. 170:25-32.
- Maia, A.L., Kim, B.W., Huang, S.A., Harney, J.W., Larsen, P.R., 2005. Type 2 iodothyronine deiodinase is the major source of plasma T-3 in euthyroid humans. *Journal of Clinical Investigation* 115, 2524-2533.
- Mol, K.A., Van der Geyten, S., Burel, C., Kuhn, E.R., Boujard, T., Darras, V.M., 1998. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. *Fish Physiology and Biochemistry* 18, 253-266.
- Orozco, A., Valverde, R.C., 2005. Thyroid hormone deiodination in fish. *Thyroid* 15, 799-813.
- Power, D.M., Llewellyn, L., Faustino, M., Nowell, M.A., Bjornsson, B.T., Einarsdottir, I.E., Canario, A.V., Sweeney, G.E., 2001. Thyroid hormones in growth and development of fish. *Comp Biochem Physiol C Toxicol Pharmacol* 130, 447-459.
- Schneider, M.J., Fiering, S.N., Pallud, S.E., Parlow, A.F., St Germain, D.L., Galton, V.A., 2001. Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T-4. *Molecular Endocrinology* 15, 2137-2148.
- Stinckens, E., Vergauwen, L., Blackwell, B.R., Anley, G.T., Villeneuve, D.L., Knapen, D., 2020. Effect of Thyroperoxidase and Deiodinase Inhibition on Anterior Swim Bladder Inflation in the Zebrafish. *Environmental Science & Technology* 54, 6213-6223.
- Walpita, C.N., Van der Geyten, S., Rurangwa, E., Darras, V.M., 2007. The effect of 3,5,3'-triiodothyronine supplementation on zebrafish (*Danio rerio*) embryonic development and expression of iodothyronine deiodinases and thyroid hormone receptors. *Gen Comp Endocrinol* 152, 206-214.
- Webster GM, Venners SA, Mattman A, Martin JW. 2014. Associations between perfluoroalkyl acids (pfass) and maternal thyroid hormones in early pregnancy: A population-based cohort study. *Environmental Research*. 133:338-347.
- Winata, C.L., Korzh, S., Kondrychyn, I., Korzh, V., Gong, Z. 2010. The role of vasculature and blood circulation in zebrafish swim bladder development. *Dev. Biol.* 10:3.
- Winata, C.L., Korzh, S., Kondrychyn, I., Zheng, W., Korzh, V., Gong, Z. 2009. Development of zebrafish swimbladder: the requirement of Hedgehog signaling in specification and organization of the three tissue layers. *Dev. Biol.* 331, 222-236, <http://dx.doi.org/10.1016/j.ydbio.2009.04.035>.

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

**AOPs Referencing Relationship**

| AOP Name                                                                                                            | Adjacency | Weight of Evidence | Quantitative Understanding |
|---------------------------------------------------------------------------------------------------------------------|-----------|--------------------|----------------------------|
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | adjacent  | Moderate           | Low                        |
| <a href="#">Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | adjacent  | Moderate           | Low                        |

**Evidence Supporting Applicability of this Relationship**

**Taxonomic Applicability**

| Term           | Scientific Term     | Evidence | Links                |
|----------------|---------------------|----------|----------------------|
| zebrafish      | Danio rerio         | High     | <a href="#">NCBI</a> |
| fathead minnow | Pimephales promelas | Moderate | <a href="#">NCBI</a> |

**Life Stage Applicability**

**Life Stage Evidence**

Embryo High

**Sex Applicability**

**Sex Evidence**

Unspecific Moderate

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

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

**Sex:** This KE/KER is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In Medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE/KER. Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

## Key Event Relationship Description

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 thyroid hormones (i.e. conversion of T4 to T3) is required in swim bladder tissue around that time period.
- Marelli et al. (2016) used morpholinos to block translation of thyroid hormone receptor alpha or beta in zebrafish. They found that thyroid hormone receptor alpha and beta knockdowns failed to inflate the posterior chamber of the swim bladder by 120 hpf, indicating that the action of T3 is needed for proper inflation of the posterior chamber. High T3 doses partially rescued the negative impact in partially resistant mutants, further confirming the importance of T3 in this process.
- Stinckens et al. (2018) showed that effects on posterior chamber inflation in zebrafish could be predicted based on in chemico DIO2 inhibition potential with only few false positives and false negatives. While T3 levels were not determined in this study, DIO2 inhibition is expected to result in decreased T3 levels.
- Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of DIO1+2 in zebrafish resulted in impairment of the inflation of the posterior chamber of the swim bladder. DIO1 and 2 knockdown is expected to result in reduced T3 levels. Indeed, Walpita et al. (2009, 2010) showed that T3 supplementation effectively rescued the effects of DIO1 and 2 knockdown, while T4 supplementation did not.
- de Vrieze et al. (2014) found that knockdown of monocarboxylate transporter 8 (mct8) in zebrafish resulted in a dose-dependent impairment of posterior chamber inflation. Since this transporter is known to transport thyroid hormones across cell membranes, this supports the importance of thyroid hormones in regulating posterior chamber inflation.
- Shi et al. (2019) found that exposure of adult zebrafish to 6:2 chlorinated polyfluorinated ether sulfonate (F-53B), an alternative to perfluorooctanesulfonate (PFOS), decreased T3 levels in both male and female zebrafish. Additionally, F-53B was maternally transferred to the offspring. Decreased T3 levels together with impaired posterior chamber inflation was observed in the F1 offspring.
- Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids from fertilization until the age of 5 days. Exogenous T3 or T4 supplementation partly rescued PFECA-induced posterior swim bladder malformation, confirming the causal relationship between reduced T3 levels and reduced posterior chamber inflation.
- Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. This confirms that T3 plays an important role in posterior swim bladder inflation.

## Uncertainties and Inconsistencies

The mechanism through which altered TH levels result in impaired posterior chamber inflation still needs to be elucidated. It is currently unclear which aspect of swim bladder development and inflation is affected by TH disruption. Based on the developmental stages of the posterior chamber, several hypotheses could explain effects on posterior chamber inflation due to disrupted TH levels. A first hypothesis includes effects on the budding of the posterior chamber inflation. Secondly, the effect on posterior chamber inflation could also be caused by disturbing the formation and growth of the three tissue layers of this organ. It has been reported that the Hedgehog signalling pathway plays an essential role in swim bladder development and is required for growth and differentiation of cells of the swim bladder. The Wnt/ $\beta$ -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; Stelow and Shi, 1995). Molla et al. (2019) showed that insulin-like growth factor (IGF-1) plays a role in swim bladder inflation/maturation in zebrafish. Reinwald et al. (2021) showed that T3 and propylthiouracil treatment of zebrafish embryos altered expression of genes involved in muscle contraction and functioning in an opposing fashion. The authors suggested impaired muscle function as an additional key event between decreased T3 levels and reduced swim bladder inflation. Several other hypotheses include effects on the successful initial inflation of the posterior

chamber, effects on lactic acid production that is required for the maintenance of the swim bladder volume, or effects on the production of surfactant that is crucial to maintain the surface tension necessary for swim bladder inflation.

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

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

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

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

## References

- Bagci, E., Heijlen, M., Vergauwen, L., Hagenaaars, A., Houbrechts, A.M., Esguerra, C.V., Blust, R., Darras, V.M., Knapen, D., 2015. Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. *PLOS One* 10, e0123285.
- Brown, C.L., Doroshov, S.I., Nunez, J.M., Hadley, C., Vaneennaam, J., Nishioka, R.S., Bern, H.A., 1988. MATERNAL TRIIODOTHYRONINE INJECTIONS CAUSE INCREASES IN SWIMBLADDER INFLATION AND SURVIVAL RATES IN LARVAL STRIPED BASS, MORONE-SAXATILIS. *Journal of Experimental Zoology* 248, 168-176.
- Brown, D.D., 1997. The role of thyroid hormone in zebrafish and axolotl development. *Proceedings of the National Academy of Sciences of the United States of America* 94, 13011-13016.
- Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. *Environmental Toxicology and Chemistry* 36, 2942-2952.
- Chang, J., Wang, M., Gui, W., Zhao, Y., Yu, L., Zhu, G., 2012. Changes in Thyroid Hormone Levels during Zebrafish Development. *Zoological Science* 29, 181-184.
- Darras, V.M., Houbrechts, A.M., Van Herck, S.L.J., 2015. Intracellular thyroid hormone metabolism as a local regulator of nuclear thyroid hormone receptor-mediated impact on vertebrate development. *Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms* 1849, 130-141.
- de Vrieze, E., van de Wiel, S.M.W., Zethof, J., Flik, G., Klaren, P.H.M., Arjona, F.J., 2014. Knockdown of Monocarboxylate Transporter 8 (mct8) Disturbs Brain Development and Locomotion in Zebrafish. *Endocrinology* 155, 2320-2330.
- Dong, W., Macaulay, L.J., Kwok, K.W., Hinton, D.E., Stapleton, H.M., 2013. Using whole mount in situ hybridization to examine thyroid hormone deiodinase expression in embryonic and larval zebrafish: a tool for examining OH-BDE toxicity to early life stages. *Aquat Toxicol* 132-133, 190-199.
- Galton, V.A., Schneider, M.J., Clark, A.S., St Germain, D.L., 2009. Life without thyroxine to 3,5,3'-triiodothyronine conversion: studies in mice devoid of the 5'-deiodinases. *Endocrinology* 150, 2957-2963.
- Godfrey A, Hooser B, Abdelmoneim A, Sepulveda MS. 2019. Sex-specific endocrine-disrupting effects of three halogenated

chemicals in japanese medaka. *Journal of Applied Toxicology*. 39(8):1215-1223.

Heijlen, M., Houbrechts, A., Bagci, E., Van Herck, S., Kersseboom, S., Esguerra, C., Blust, R., Visser, T., Knapen, D., Darras, V., 2014. Knockdown of type 3 iodothyronine deiodinase severely perturbs both embryonic and early larval development in zebrafish. *Endocrinology* 155, 1547-1559.

Heijlen, M., Houbrechts, A.M., Darras, V.M., 2013. Zebrafish as a model to study peripheral thyroid hormone metabolism in vertebrate development. *Gen.Comp. Endocrinol.* 188, 289–296, <http://dx.doi.org/10.1016/j.ygcen.2013.04.004>.

Kress, E., Rezza, A., Nadjar, J., Samarut, J., Plateroti, M., 2009. The frizzled-related sFRP2 gene is a target of thyroid hormone receptor alpha1 and activates beta-catenin signaling in mouse intestine. *J Biol Chem* 284, 1234-1241.

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

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

Marelli, F., Carra, S., Agostini, M., Cotelli, F., Peeters, R., Chatterjee, K., Persani, L., 2016. Patterns of thyroid hormone receptor expression in zebrafish and generation of a novel model of resistance to thyroid hormone action. *Molecular and Cellular Endocrinology* 424, 102-117.

Molla, M.H.R., Hasan, M.T., Jang, W.J., Diaz, C.D.S., Appenteng, P., Marufchoni, H., Jahan, B., Brown, C.L., 2019. Thyroid hormone-induced swim bladder and eye maturation are transduced by IGF-1 in zebrafish embryos. *Aquaculture Research* 50, 3462-3470.

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

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

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

Plateroti, M., Kress, E., Mori, J.I., Samarut, J., 2006. Thyroid hormone receptor alpha1 directly controls transcription of the beta-catenin gene in intestinal epithelial cells. *Mol Cell Biol* 26, 3204-3214.

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

Schneider, M.J., Fiering, S.N., Thai, B., Wu, S.Y., St Germain, E., Parlow, A.F., St Germain, D.L., Galton, V.A., 2006. Targeted disruption of the type 1 selenodeiodinase gene (Dio1) results in marked changes in thyroid hormone economy in mice. *Endocrinology* 147, 580-589.

Shi, G., Wang, J., Guoa, H., Shenga, N., Cui, Q., Pan, Y., Guob, Y., Sun, Y., Dai, J., 2019. Parental exposure to 6:2 chlorinated polyfluorinated ether sulfonate (F-53B) induced transgenerational thyroid hormone disruption in zebrafish. *Science of the Total Environment* 665, 855-863.

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

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

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

Stolow, M.A., Shi, Y.B., 1995. XENOPUS SONIC HEDGEHOG AS A POTENTIAL MORPHOGEN DURING EMBRYOGENESIS AND THYROID HORMONE-DEPENDENT METAMORPHOSIS. *Nucleic Acids Research* 23, 2555-2562.

Thisse, C., Degraeve, A., Kryukov, G.V., Gladyshev, V.N., Obrecht-Pflumio, S., Krol, A., Thisse, B., Lescure, A., 2003. Spatial and temporal expression patterns of selenoprotein genes during embryogenesis in zebrafish. *Gene Expr Patterns* 3, 525-532.

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

Walpita, C.N., Crawford, A.D., Darras, V.M., 2010. Combined antisense knockdown of type 1 and type 2 iodothyronine deiodinases

disrupts embryonic development in zebrafish (*Danio rerio*). *General and Comparative Endocrinology* 166, 134-141.

Walpita, C.N., Crawford, A.D., Janssens, E.D., Van der Geyten, S., Darras, V.M., 2009. Type 2 iodothyronine deiodinase is essential for thyroid hormone-dependent embryonic development and pigmentation in zebrafish. *Endocrinology* 150, 530-539.

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

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

Winata, C.L., Korzh, S., Kondrychyn, I., Zheng, W., Korzh, V., Gong, Z., 2009. Development of zebrafish swimbladder: The requirement of Hedgehog signaling in specification and organization of the three tissue layers. *Developmental Biology* 331, 222-236.

Woolley LD, Qin JG. 2010. Swimbladder inflation and its implication to the culture of marine finfish larvae. *Reviews in Aquaculture*. 2(4):181-190.

Yin, A., Korzh, S., Winata, C.L., Korzh, V., Gong, Z., 2011. Wnt signaling is required for early development of zebrafish swimbladder. *PLoS One* 6, e18431.

Yin, A., Korzh, V., Gong, Z., 2012. Perturbation of zebrafish swimbladder development by enhancing Wnt signaling in *Wif1* morphants. *Biochim Biophys Acta* 1823, 236-244.

### **Relationship: 1028: Reduced, Posterior swim bladder inflation leads to Reduced, Swimming performance**

#### **AOPs Referencing Relationship**

| AOP Name                                                                                                            | Adjacency | Weight of Evidence | Quantitative Understanding |
|---------------------------------------------------------------------------------------------------------------------|-----------|--------------------|----------------------------|
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | adjacent  | Moderate           | Low                        |
| <a href="#">Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | adjacent  | Moderate           | Low                        |

#### **Evidence Supporting Applicability of this Relationship**

##### **Taxonomic Applicability**

| Term                        | Scientific Term             | Evidence | Links                |
|-----------------------------|-----------------------------|----------|----------------------|
| zebrafish                   | <i>Danio rerio</i>          | High     | <a href="#">NCBI</a> |
| fathead minnow              | <i>Pimephales promelas</i>  | Moderate | <a href="#">NCBI</a> |
| bluefin tuna                | <i>Thunnus thynnus</i>      | Moderate | <a href="#">NCBI</a> |
| <i>Dicentrarchus labrax</i> | <i>Dicentrarchus labrax</i> | Moderate | <a href="#">NCBI</a> |
| <i>Perca flavescens</i>     | <i>Perca flavescens</i>     | Moderate | <a href="#">NCBI</a> |
| <i>Salmo salar</i>          | <i>Salmo salar</i>          | Moderate | <a href="#">NCBI</a> |

##### **Life Stage Applicability**

###### **Life Stage Evidence**

Embryo High

##### **Sex Applicability**

###### **Sex Evidence**

Unspecific Moderate

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

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

**Sex:** This KE/KER is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In Medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE/KER. Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

### Key Event Relationship Description

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

### Evidence Supporting this KER

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

### Biological Plausibility

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

### Empirical Evidence

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

- Stewart and Gee (1981) showed that fathead minnows swimming from still water to a current resorbed gas to fill the swim bladder and tailor buoyancy precisely to the level where swimming is most efficient.
- Lindsey et al., 2010 reported that zebrafish larvae that fail to inflate their swim bladder use additional energy to maintain buoyancy (Lindsey et al., 2010, Goodsell et al., 1996), possibly contributing to reduced swimming activity. Furthermore, they reported that the range of swimming depth varies with stages of swim bladder development.
- Czesny et al., 2005 reported that yellow perch larvae without inflated swim bladders capture free-swimming prey poorly and expend more energy on feeding and maintaining their position within the water column, due to impacted swimming behaviour.
- Kurata et al., 2014 observed that Bluefin tuna larvae present at the bottom of a tank, incapable of swimming upwards, had significantly lower swim bladder inflation.
- Chatain (1994) associated sea bass larvae with non-inflated swim bladders with numerous complications, such as spinal deformities and lordosis and reduced growth rates, adding to the impact on swimming behaviour.
- An increasing incidence of swim bladder non-inflation has also been reported in Atlantic salmon. Affected fish had severely altered balance and buoyancy, observed through a specific swimming behaviour, as the affected fish were swimming upside down in an almost vertical position (Poppe et al., 1977).
- Permanent DIO 2 deficiency in zebrafish was shown to result in reduced posterior chamber inflation and disturbed locomotor activity (Houbrechts et al., 2016).
- Michiels et al. (2017) showed that both for controls and zebrafish embryos exposed to an environmental sample, the swimming distance was significantly lower in larvae that failed to inflate the posterior chamber compared to larvae from the same treatment that had inflated posterior chambers.
- Exposure of zebrafish embryos to thyroid disrupting compounds resulted in an effect on posterior chamber inflation as well as on the swimming distance in the larval stage (Stinckens et al., unpublished).
- All zebrafish larvae that failed to inflate the posterior chamber after exposure to 2 mg/L iopanoic acid (IOP), died by the age of 9 dpf (Stinckens et al., 2020). Since larvae from the same group that were able to inflate the posterior chamber survived, it is plausible to assume that uninflated posterior chambers limited the ability to swim and find food.
- Hagenaars et al. (2014) showed that zebrafish embryos exposed to 4.28 mg/L PFOS had lower swimming speeds when the posterior chamber was not inflated. It should be noted that almost all larvae with a non-inflated swimbladder had a spinal

curvature and it could therefore not statistically be determined whether the reduced swimming speed was due to a spinal curvature, a non-inflated swim bladder or the interaction of both.

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

### Uncertainties and Inconsistencies

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

Exposure of zebrafish embryos to 6-propylthiouracil (PTU) resulted in an effect on posterior chamber inflation, but did not result in a direct effect on the swimming distance in the larval stage (Stinckens et al., unpublished). Vergauwen et al. (2015) reported decreased swimming activity as well as impaired posterior chamber inflation after exposure to phenanthrene, a non-polar narcotic, but there was no significant difference between swimming activity of larvae with or 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 swimming 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.

### References

- Bagci, E., Heijlen, M., Vergauwen, L., Hagenaaars, A., Houbrechts, A.M., Esguerra, C.V., Blust, R., Darras, V.M., Knapen, D., 2015. Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. *PLoS One* 10, e0123285, <http://dx.doi.org/10.1371/journal.pone.0123285>.
- Brix O (2002) The physiology of living in water. In: Hart PJ, Reynolds J (eds) *Handbook of Fish Biology and Fisheries*, Vol. 1, pp. 70–96. Blackwell Publishing, Malden, USA.
- Chatain, B., 1994. Abnormal swimbladder development and lordosis in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*). *Aquaculture* 119:371–379.
- Czesny, S.J., Graeb, B.D.S., Dettmersn, J.M., 2005. Ecological consequences of swimbladder noninflation for larval yellow perch. *Trans. Am. Fish. Soc.* 134, 1011–1020, <http://dx.doi.org/10.1577/T04-016.1>.
- Godfrey A, Hooser B, Abdelmoneim A, Sepulveda MS. 2019. Sex-specific endocrine-disrupting effects of three halogenated chemicals in Japanese medaka. *Journal of Applied Toxicology*. 39(8):1215-1223.
- Goodsell, D.S., Morris, G.M., Olsen, A.J. 1996. Automated docking of flexible ligands. *Applications of Autodock*. *J. Mol. Recognition*, 9:1-5.
- Hagenaaars, A., Stinckens, E., Vergauwen, L., Bervoets, L., Knapen, D., 2014. PFOS affects posterior swim bladder chamber inflation and swimming performance of zebrafish larvae. *Aquat. Toxicol.* 157, 225–235, <http://dx.doi.org/10.1016/j.aquatox.2014.10.017>.

- Heijlen, M., Houbrechts, A., Bagci, E., Van Herck, S., Kersseboom, S., Esguerra, C., Blust, R., Visser, T., Knapen, D., Darras, V., 2014. Knockdown of type 3 iodothyronine deiodinase severely perturbs both embryonic and early larval development in zebrafish. *Endocrinology* 155, 1547-1559.
- Houbrechts, A.M., Delarue, J., Gabriels, I.J., Sourbron, J., Darras, V.M., 2016. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. *Endocrinology* 157, 3668-3681.
- Kurata, M., Ishibashi, Y., Takii, K., Kumai, H., Miyashita, S., Sawada, Y., 2014. Influence of initial swimbladder inflation failure on survival of Pacific bluefintuna, *Thunnus orientalis* (Temminck and Schlegel) larvae. *Aquacult. Res.* 45, 882-892.
- Lindsey, B.W., Smith, F.M., Croll, R.P., 2010. From inflation to flotation: contribution of the swimbladder to whole-body density and swimming depth during development of the zebrafish (*Danio rerio*). *Zebrafish* 7, 85-96, <http://dx.doi.org/10.1089/zeb.2009.0616>.
- Maack, G., Segner, H., 2003. Morphological development of the gonads in zebrafish. *Journal of Fish Biology* 62, 895-906.
- Massei R et al. (in preparation) Sublethal adverse effects of non-polar narcotics in the zebrafish embryo.
- Michiels, E.D.G., Vergauwen, L., Hagenaars, A., Fransen, E., Van Dongen, S., Van Cruchten, S.J., Bervoets, L., Knapen, D., 2017. Evaluating Complex Mixtures in the Zebrafish Embryo by Reconstituting Field Water Samples: A Metal Pollution Case Study. *International Journal of Molecular Sciences* 18, 539.
- Nagabhushana A, Mishra RK. 2016. Finding clues to the riddle of sex determination in zebrafish. *Journal of Biosciences*. 41(1):145-155.
- Olmstead AW, Villeneuve DL, Ankley GT, Cavallin JE, Lindberg-Livingston A, Wehmas LC, Degitz SJ. 2011. A method for the determination of genetic sex in the fathead minnow, *pimephales promelas*, to support testing of endocrine-active chemicals. *Environmental Science & Technology*. 45(7):3090-3095.
- Poppe, T.T., Hellberg, H., Griffiths, D., Mendal, H. 1977. Swim bladder abnormality in farmed Atlantic salmon, *Salmo salar*. *Diseases of aquatic organisms* 30:73-76.
- Roberston, G.N., McGee, C.A.S., Dumbarton, T.C., Croll, R.P., Smith, F.M., 2007. Development of the swim bladder and its innervation in the zebrafish, *Danio rerio*. *J. Morphol.* 268, 967-985, <http://dx.doi.org/10.1002/jmor>.
- Stewart, D.B., Gee, J.H., 1981. Mechanisms of buoyancy adjustments and effects of water velocity and temperature on ability to maintain buoyancy in fathead minnows, *Pimephales promelas*, Rafinesque. *Comparative Biochemistry and Physiology a-Physiology* 68, 337-347.
- Stinckens, E., Vergauwen, L., Blackwell, B.R., Anlley, G.T., Villeneuve, D.L., Knapen, D., 2020. Effect of Thyroperoxidase and Deiodinase Inhibition on Anterior Swim Bladder Inflation in the Zebrafish. *Environmental Science & Technology* 54, 6213-6223.
- Stinckens, E., Vergauwen, L., Schroeder, A.L., Maho, W., Blackwell, B., Witter, H., Blust, R., Ankley, G.T., Covaci, A., Villeneuve, D.L., Knapen, D., 2016. Disruption of thyroid hormone balance after 2-mercaptobenzothiazole exposure causes swim bladder inflation impairment—part II: zebrafish. *Aquat. Toxicol.* 173:204-17.
- Stoyek, M.R., Smith, F.M., Croll, R.P., 2011. Effects of altered ambient pressure on the volume and distribution of gas within the swimbladder of the adult zebrafish, *Danio rerio*. *Journal of Experimental Biology* 214, 2962-2972.
- van Aerle R, Runnalls TJ, Tyler CR. 2004. Ontogeny of gonadal sex development relative to growth in fathead minnow. *Journal of Fish Biology*. 64(2):355-369.
- Vergauwen, L., Schmidt, S.N., Stinckens, E., Maho, W., Blust, R., Mayer, P., Covaci, A., Knapen, D., 2015. A high throughput passive dosing format for the Fish Embryo Acute Toxicity test. *Chemosphere* 139, 9-17.

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

#### **AOPs Referencing Relationship**

| AOP Name                                                                                                            | Adjacency | Weight of Evidence | Quantitative Understanding |
|---------------------------------------------------------------------------------------------------------------------|-----------|--------------------|----------------------------|
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | adjacent  | Moderate           | Low                        |
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>  | adjacent  | Moderate           | Low                        |
| <a href="#">Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | adjacent  | Moderate           | Low                        |
| <a href="#">Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>  | adjacent  | Moderate           | Low                        |

[Thyropoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation](#)

AOP Name

Agency

Weight of Evidence

Quantitative Understanding

## Evidence Supporting Applicability of this Relationship

### Taxonomic Applicability

| Term           | Scientific Term     | Evidence | Links                |
|----------------|---------------------|----------|----------------------|
| zebrafish      | Danio rerio         | Moderate | <a href="#">NCBI</a> |
| fathead minnow | Pimephales promelas | Moderate | <a href="#">NCBI</a> |

### Life Stage Applicability

#### Life Stage Evidence

|          |          |
|----------|----------|
| Adult    | Moderate |
| Juvenile | Moderate |
| Larvae   | Moderate |

### Sex Applicability

#### Sex Evidence

|            |          |
|------------|----------|
| Unspecific | Moderate |
|------------|----------|

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

## Key Event Relationship Description

Reduced swimming performance is likely to affect essential endpoints such as predator avoidance, feeding behaviour and reproduction in taxa that rely on swimming to support these vital behaviours. These parameters are 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 5 dpf when the yolk is almost completely depleted, there was a lag period of around 4 days after which reduced feeding resulted in mortality. Obviously, in a laboratory setup there is no increased risk of being caught by a predator.

### References

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

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

#### AOPs Referencing Relationship

| AOP Name                                                                                                              | Adjacency | Weight of Evidence | Quantitative Understanding |
|-----------------------------------------------------------------------------------------------------------------------|-----------|--------------------|----------------------------|
| <a href="#">Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination &amp; Movement</a>   | adjacent  |                    |                            |
| <a href="#">Acetylcholinesterase inhibition leading to acute mortality</a>                                            | adjacent  | Moderate           | Moderate                   |
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>   | adjacent  | Moderate           | Moderate                   |
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>    | adjacent  | Moderate           | Moderate                   |
| <a href="#">Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a>   | adjacent  | Moderate           | Moderate                   |
| <a href="#">Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation</a>    | adjacent  | Moderate           | Moderate                   |
| <a href="#">Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation</a> | adjacent  | Moderate           | Moderate                   |
| <a href="#">Thyroperoxidase inhibition leading to increased mortality via altered retinal layer structure</a>         | adjacent  | High               | Moderate                   |
| <a href="#">Thyroperoxidase inhibition leading to increased mortality via decreased eye size</a>                      | adjacent  |                    |                            |
| <a href="#">Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning</a>        | adjacent  |                    |                            |
| <a href="#">Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)</a>             | adjacent  | High               | High                       |

#### Evidence Supporting Applicability of this Relationship

##### Taxonomic Applicability

| Term           | Scientific Term     | Evidence | Links                |
|----------------|---------------------|----------|----------------------|
| zebrafish      | Danio rerio         | High     | <a href="#">NCBI</a> |
| fathead minnow | Pimephales promelas | High     | <a href="#">NCBI</a> |

##### Life Stage Applicability

##### Life Stage Evidence

All life stages High

##### Sex Applicability

| Sex        | Evidence |
|------------|----------|
| Unspecific | Moderate |

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

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

**Sex:** This linkage is independent of sex.

### Key Event Relationship Description

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

### Evidence Supporting this KER

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

### Biological Plausibility

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

### Empirical Evidence

- According to empirical data, combined with population dynamic models, feeding larvae are the crucial life stage in zebrafish (and other r-strategists) for the regulation of the population. (Schäfers et al., 1993)
- In fathead minnow, natural survival of early life stages has been found to be highly variable and influential on population growth (Miller and Ankley, 2004)
- Rearick et al. (2018) used linked data from behavioural assays to survival trials and applied a modelling approach to quantify changes in antipredator escape performance of larval fathead minnows in order to predict changes in population abundance. This work was done in the context of exposure to an environmental oestrogen. Exposed fish had delayed response times and slower escape speeds, and were more susceptible to predation. Population modelling showed that this can result in population decline.
- In the context of fishing and fisheries, ample evidence of a link between increased mortality and a decrease of population size has been given. Important insights can result from the investigation of optimum modes of fishing that allow for maintaining a population (Alekseeva and Rudenko, 2018). Jacobsen and Essington (2018) showed the impact of varying predation mortality on forage fish populations.
- Boreman (1997) reviewed methods for comparing the population-level effects of mortality in fish populations induced by pollution or fishing.

### Uncertainties and Inconsistencies

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

### References

- Alekseeva SM, Rudenko AI. 2018. Modeling of optimum fishing population. *Marine Intellectual Technologies*. 3(4):142-146.
- Beaudouin, R., Goussen, B., Piccini, B., Augustine, S., Devillers, J., Brion, F., Pery, A.R., 2015. An individual-based model of zebrafish population dynamics accounting for energy dynamics. *PloS one* 10, e0125841.
- Boreman J. 1997. Methods for comparing the impacts of pollution and fishing on fish populations. *Transactions of the American Fisheries Society*. 126(3):506-513.
- Caswell, H., 2000. *Matrix population models*. Sinauer Sunderland, MA, USA.
- Eng, M.L., Stutchbury, B.J.M. & Morrissey, C.A. Imidacloprid and chlorpyrifos insecticides impair migratory ability in a seed-eating songbird. *Sci Rep* 7, 15176 (2017)
- Hazlerigg, C.R., Lorenzen, K., Thorbek, P., Wheeler, J.R., Tyler, C.R., 2012. Density-dependent processes in the life history of fishes: evidence from laboratory populations of zebrafish *Danio rerio*. *PLoS One* 7, e37550.
- Jacobsen NS, Essington TE. 2018. Natural mortality augments population fluctuations of forage fish. *Fish and Fisheries*. 19(5):791-797.
- MacArthur, R., Wilson, E., 1967. *The Theory of Island Biogeography*. Princeton: Princeton Univ. Press. 203 p.
- Miller, D.H., Ankley, G.T., 2004. Modeling impacts on populations: fathead minnow (*Pimephales promelas*) exposure to the endocrine disruptor 17 $\beta$ -trenbolone as a case study. *Ecotoxicology and Environmental Safety* 59, 1-9.
- Miller, D.H., Clark, B.W., Nacci, D.E. 2020. A multidimensional density dependent matrix population model for assessing risk of stressors to fish populations. *Ecotoxicology and environmental safety* 201, 110786
- Pinceel, T., Vanschoenwinkel, B., Brendonck, L., Buschke, F., 2016. Modelling the sensitivity of life history traits to climate change in a temporary pool crustacean. *Scientific reports* 6, 29451.
- Rearick, D.C., Ward, J., Venturelli, P., Schoenfuss, H., 2018. Environmental oestrogens cause predation-induced population decline in a freshwater fish. *Royal Society open science* 5, 181065.
- Schäfers, C., Oertel, D., Nagel, R., 1993. Effects of 3, 4-dichloroaniline on fish populations with differing strategies of reproduction. *Ecotoxicology and Ecophysiology*, 133-146.
- Stige, L.C., Rogers, L.A., Neuheimer, A.B., Hunsicker, M.E., Yaragina, N.A., Ottersen, G., Ciannelli, L., Langangen, Ø., Durant, J.M., 2019. Density- and size-dependent mortality in fish early life stages. *Fish and Fisheries* 20, 962-976.
- Hazlerigg, C.R.E., Tyler, C.R., Lorenzen, K., Wheeler, J.R., Thorbek, P., 2014. Population relevance of toxicant mediated changes in sex ratio in fish: An assessment using an individual-based zebrafish (*Danio rerio*) model. *Ecological Modelling* 280, 76-88.
- Stige, L.C., Rogers, L.A., Neuheimer, A.B., Hunsicker, M.E., Yaragina, N.A., Ottersen, G., Ciannelli, L., Langangen, O., Durant, J.M., 2019. Density- and size-dependent mortality in fish early life stages. *Fish and Fisheries* 20, 962-976.

## List of Non Adjacent Key Event Relationships

### [Relationship: 1042: Inhibition, Deiodinase 2 leads to Reduced, Posterior swim bladder inflation](#)

#### AOPs Referencing Relationship

| AOP Name                                                                                                            | Adjacency    | Weight of Evidence | Quantitative Understanding |
|---------------------------------------------------------------------------------------------------------------------|--------------|--------------------|----------------------------|
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | non-adjacent | Moderate           | Low                        |

#### Evidence Supporting Applicability of this Relationship

##### Taxonomic Applicability

| Term           | Scientific Term            | Evidence | Links                |
|----------------|----------------------------|----------|----------------------|
| zebrafish      | <i>Danio rerio</i>         | High     | <a href="#">NCBI</a> |
| fathead minnow | <i>Pimephales promelas</i> | High     | <a href="#">NCBI</a> |

##### Life Stage Applicability

Life Stage Evidence

|            |               |
|------------|---------------|
| Embryo     | High          |
| Life Stage | Evidence      |
| Sex        | Applicability |
| Sex        | Evidence      |
| Unspecific | Moderate      |

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

**Sex:** This KE/KER is plausibly applicable to both sexes. Sex differences are not often investigated in tests using early life stages of fish. In Medaka, sex can be morphologically distinguished as soon as 10 days post fertilization. Females appear more susceptible to thyroid-induced swim bladder dysfunction compared with males (Godfrey et al., 2019). In zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE/KER. Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the posterior chamber inflates around 5 days post fertilization in zebrafish, when sex differentiation has not started yet, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the posterior chamber inflates around 6 days post fertilization (1 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

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

### Key Event Relationship Description

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

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

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

### Evidence Supporting this KER

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

### Biological Plausibility

Inhibition of DIO 2 activity is widely accepted to reduce the conversion of T4 to the more biologically active T3. Thyroid hormones are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition and

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

### Empirical Evidence

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

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

- Chang et al., (2012) established a base-line for TH levels during zebrafish development and observed peaks in whole-body T3 content at 5 dpf when the posterior chamber of the swim bladder inflates.
- Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of DIO1+2 in zebrafish resulted in impairment of the inflation of the posterior chamber of the swim bladder.
- Permanent DIO 2 deficiency in zebrafish was shown to result in reduced posterior chamber inflation (Houbrechts et al., 2016).
- DIO1 and DIO2 mRNA has also been shown to be present in zebrafish swim bladder tissue at 96 hpf using whole mount in situ hybridization (Heijlen et al., 2013; Dong et al., 2013), suggesting a tissue-specific role of T3 in the inflation process of the posterior chamber.
- Propylthiouracil (PTU) decreased serum T3 levels in the rat (Frumess and Larsen, 1975) and resulted in effects on posterior chamber inflation in zebrafish (Jomaa et al., 2014; Stinckens et al., 2018). It should be noted that there are some uncertainties related to the species-specific susceptibility of DIO1 to inhibition by PTU, as teleostean DIO1 seems to be less sensitive to inhibition by PTU (Orozco and Valverde, 2005; Kuiper et al., 2006; Orozco et al., 2012).
- Stinckens et al. (2018) showed that effects on posterior chamber inflation in zebrafish could be predicted based on in chemico DIO2 inhibition potential with only few false positives and false negatives.
- After exposure of fathead minnows (*Pimephales promelas*) to the non-specific deiodinase inhibitor IOP from 1-6 dpf, incidence and length of inflated posterior swim bladders were significantly reduced (Cavallin et al., 2017).
- While DIO1 has a high Km and rT3 is its preferred substrate, DIO2 has a low Km and T4 is its preferred substrate, indicating that DIO2 is more important than DIO1 in converting T4 to T3 in a physiological situation (Darras and Van Herck, 2012). It follows that DIO2 inhibition is likely more important than DIO1 inhibition in reducing posterior chamber inflation.

### Uncertainties and Inconsistencies

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

It is currently unclear which aspect of swim bladder development and inflation is affected by TH disruption. Based on the developmental stages of the posterior chamber, several hypotheses could explain effects on posterior chamber inflation due to disrupted TH levels. A first hypothesis includes effects on the budding of the posterior chamber inflation. Secondly, the effect on posterior chamber inflation could also be caused by disturbing the formation and growth of the three tissue layers of this organ. It has been reported that the Hedgehog signalling pathway plays an essential role in swim bladder development and is required for growth and differentiation of cells of the swim bladder. The Wnt/ $\beta$ -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; Stelow 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.

### References

- Bagci, E., Heijlen, M., Vergauwen, L., Hagenaars, A., Houbrechts, A.M., Esguerra, C.V., Blust, R., Darras, V.M., Knapen, D., 2015. Deiodinase knockdown during early zebrafish development affects growth, development, energy metabolism, motility and phototransduction. *PLoS One* 10, e0123285, <http://dx.doi.org/10.1371/journal.pone.0123285>.
- Cavallin, J.E., Ankley, G.T., Blackwell, B.R., Blanksma, C.A., Fay, K.A., Jensen, K.M., Kahl, M.D., Knapen, D., Kosian, P.A., Poole, S.T., Randolph, E.C., Schroeder, A.L., Vergauwen, L., Villeneuve, D.L., 2017. Impaired swim bladder inflation in early life stage fathead minnows exposed to a deiodinase inhibitor, iopanoic acid. *Environmental Toxicology and Chemistry* 36, 2942-2952.
- Chang, J., Wang, M., Gui, W., Zhao, Y., Yu, L., Zhu, G., 2012. Changes in thyroid hormone levels during zebrafish development. *Zool. Sci.* 29, 181–184, <http://dx.doi.org/10.2108/zsj.29.181>.
- Darras, V.M., Houbrechts, A.M., Van Herck, S.L.J., 2015. Intracellular thyroid hormone metabolism as a local regulator of nuclear thyroid hormone receptor-mediated impact on vertebrate development. *Biochimica Et Biophysica Acta- Gene Regulatory Mechanisms* 1849, 130-141.
- Degitz, S.J., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., Tietge, J.E., 2005. Progress towards development of an amphibian-based screening assay using *Xenopus laevis*. Organismal and thyroidal responses to the model compounds 6-propylthiouracil, methimazole, and thyroxine. *Toxicol. Sci.* 87, 353–364.
- Dong, W., Macaulay, L., Kwok, K.W.H., Hinton, D.E., Stapleton, H.M., 2013. Using whole mount in situ hybridization to examine thyroid hormone deiodinase expression in embryonic and larval zebrafish: a tool for examining OH-BDE toxicity to early life stages. *Aquat. Toxicol.* 132–133, 190–199, <http://dx.doi.org/10.1016/j.biotechadv.2011.08.021>. Secreted.
- Frumess, R.D., Larsen, P.R. 1975. Correlation of serum triiodothyronine (T3) and thyroxine (T4) with biological effects of thyroid hormone replacement in propylthiouracil-treated rats. *Metabolism* 24:4.
- Galton, V.A., Schneider, M.J., Clark, A.S., St Germain, D.L., 2009. Life without thyroxine to 3,5,3'-triiodothyronine conversion: studies in mice devoid of the 5'-deiodinases. *Endocrinology* 150, 2957-2963.
- Godfrey A, Hooser B, Abdelmoneim A, Sepulveda MS. 2019. Sex-specific endocrine-disrupting effects of three halogenated chemicals in Japanese medaka. *Journal of Applied Toxicology*. 39(8):1215-1223.
- Heijlen, M., Houbrechts, A.M., Bagci, E., Van Herck, S.L.J., Kersseboom, S., Esguerra, C.V., Blust, R., Visser, T.J., Knapen, D., Darras, V.M., 2014. Knockdown of type 3 iodothyronine deiodinase severely perturbs both embryonic and early larval development in zebrafish. *Endocrinology* 155, 1547–1559, <http://dx.doi.org/10.1210/en.2013-1660>.
- Heijlen, M., Houbrechts, A.M., Darras, V.M., 2013. Zebrafish as a model to study peripheral thyroid hormone metabolism in vertebrate development. *Gen. Comp. Endocrinol.* 188, 289–296, <http://dx.doi.org/10.1016/j.ygcen.2013.04.004>.
- Houbrechts, A.M., Delarue, J., Gabriels, I.J., Sourbron, J., Darras, V.M., 2016. Permanent Deiodinase Type 2 Deficiency Strongly Perturbs Zebrafish Development, Growth, and Fertility. *Endocrinology* 157, 3668-3681.
- Jomaa, B., Hermesen, S.A.B., Kessels, M.Y., Van Den Berg, J.H.J., Peijnenburg, A.A.C.M., Aarts, J.M.M.J.G., Piersma, A.H., Rietjens, I.M.C.M., 2014. Developmental toxicity of thyroid-active compounds in a zebrafish embryotoxicity test. *ALTEX* 31, 303–317, <http://dx.doi.org/10.14573/altex.1402011>.
- Kress, E., Rezza, A., Nadjar, J., Samarut, J., Plateroti, M., 2009. The frizzled-related FRP2 gene is a target of thyroid hormone receptor  $\alpha 1$  and activates  $\beta$ -catenin signaling in mouse intestine. *J. Biol. Chem.* 284, 1234–1241, <http://dx.doi.org/10.1074/jbc.M806548200>.
- Kuiper, G., Klootwijk, W., Dubois, G.M., Destree, O., Darras, V.M., Van der Geyten, S., Demeneix, B., Visser, T.J., 2006. Characterization of recombinant *Xenopus laevis* type I iodothyronine deiodinase: substitution of a proline residue in the catalytic center by serine (Pro132Ser) restores sensitivity to 6-propyl-2-thiouracil. *Endocrinology* 147, 3519-3529.
- Mol, K.A., Van der Geyten, S., Burel, C., Kuhn, E.R., Boujard, T., Darras, V.M., 1998. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. *Fish Physiology and Biochemistry* 18, 253-266.
- Nagabhushana A, Mishra RK. 2016. Finding clues to the riddle of sex determination in zebrafish. *Journal of Biosciences*. 41(1):145-155.
- Olmstead AW, Villeneuve DL, Ankley GT, Cavallin JE, Lindberg-Livingston A, Wehmas LC, Degitz SJ. 2011. A method for the determination of genetic sex in the fathead minnow, *Pimephales promelas*, to support testing of endocrine-active chemicals. *Environmental Science & Technology*. 45(7):3090-3095.
- Orozco, A., Valverde, C., Olvera, A., Garcia, C., 2012. Iodothyronine deiodinases: a functional and evolutionary perspective. *Journal of Endocrinology* 215, 207-219.
- Orozco, A., Valverde, R.C., 2005. Thyroid hormone deiodination in fish. *Thyroid* 15, 799-813.
- Plateroti, M., Kress, E., Mori, J.I., Samarut, J., 2006. Thyroid hormone receptor  $\alpha 1$  directly controls transcription of the  $\beta$ -catenin gene in intestinal epithelial cells. *Mol. Cell. Biol.* 26, 3204–3214, <http://dx.doi.org/10.1128/MCB.26.8.3204>.
- Schneider, M.J., Fiering, S.N., Thai, B., Wu, S.Y., St Germain, E., Parlow, A.F., St Germain, D.L., Galton, V.A., 2006. Targeted

disruption of the type 1 selenodeiodinase gene (Dio1) results in marked changes in thyroid hormone economy in mice. *Endocrinology* 147, 580-589.

Stinckens, E., Vergauwen, L., Ankley, G.T., Blust, R., Darras, V.M., Villeneuve, D.L., Witters, H., Volz, D.C., Knapen, D., 2018. An AOP-based alternative testing strategy to predict the impact of thyroid hormone disruption on swim bladder inflation in zebrafish. *Aquatic Toxicology* 200, 1-12. 10.1016/j.aquatox.2018.04.009.

Stolow, M.A., Shi, Y.B., 1995. *Xenopus* sonic hedgehog as a potential morphogen during embryogenesis and thyroid hormone-dependent metamorphosis. *Nucleic Acids Res.* 23, 2555–2562, <http://dx.doi.org/10.1093/nar/23.13.2555>.

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

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

Walpita, C.N., Crawford, A.D., Darras, V.M., 2010. Combined antisense knockdown of type 1 and type 2 iodothyronine deiodinases disrupts embryonic development in zebrafish (*Danio rerio*). *General and Comparative Endocrinology* 166, 134-141.

Walpita, C.N., Crawford, A.D., Janssens, E.D., Van der Geyten, S., Darras, V.M., 2009. Type 2 iodothyronine deiodinase is essential for thyroid hormone-dependent embryonic development and pigmentation in zebrafish. *Endocrinology* 150, 530-539.

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

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

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

Woolley LD, Qin JG. 2010. Swimbladder inflation and its implication to the culture of marine finfish larvae. *Reviews in Aquaculture*. 2(4):181-190.

Yin, A., Korzh, S., Winata, C.L., Korzh, V., Gong, Z., 2011. Wnt signaling is required for early development of zebrafish swimbladder. *PLoS One* 6, <http://dx.doi.org/10.1371/journal.pone.0018431>.

Yin, A., Korzh, V., Gong, Z., 2012. Perturbation of zebrafish swim bladder development by enhancing Wnt signaling in Wif1 morphants. *Biochim.Biophys. Acta—Mol. Cell Res.* 1823, 236–244, <http://dx.doi.org/10.1016/j.bbamcr.2011.09.018>

### Relationship: 2213: Reduced, Posterior swim bladder inflation leads to Increased Mortality

#### AOPs Referencing Relationship

| AOP Name                                                                                                            | Adjacency    | Weight of Evidence | Quantitative Understanding |
|---------------------------------------------------------------------------------------------------------------------|--------------|--------------------|----------------------------|
| <a href="#">Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | non-adjacent | High               | Low                        |
| <a href="#">Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation</a> | non-adjacent | High               | Low                        |

#### Evidence Supporting Applicability of this Relationship

##### Taxonomic Applicability

| Term           | Scientific Term            | Evidence | Links                |
|----------------|----------------------------|----------|----------------------|
| zebrafish      | <i>Danio rerio</i>         | High     | <a href="#">NCBI</a> |
| fathead minnow | <i>Pimephales promelas</i> | Moderate | <a href="#">NCBI</a> |

##### Life Stage Applicability

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

##### Sex Applicability

| Sex | Evidence |
|-----|----------|
|-----|----------|

|            |          |
|------------|----------|
| Unspecific | Moderate |
|------------|----------|

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

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

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

### Key Event Relationship Description

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

### Evidence Supporting this KER

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

### Biological Plausibility

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

### Empirical Evidence

- Czesny et al. (2005) demonstrated that swim bladder non-inflation was associated with multiple phenotypic and behavioral outcomes that would be expected to adversely impact survival.
  - Yellow perch with non-inflated swim bladders grew more slowly than those with inflated swim bladders, both in the laboratory and in the field.
  - Yellow perch with non-inflated swim bladders always captured prey less efficiently than those with inflated swim bladders of the same size class.
  - Yellow perch with non-inflated swim bladders suffered from increased predation risk.
  - Yellow perch with non-inflated swim bladders experienced significantly increased mortality and lower time to mortality in a foodless environment compared to those with inflated swim bladders, indicating greater energy expenditure.
  - Yellow perch with non-inflated swim bladders had significantly greater oxygen consumption than fish of the same size class with inflated swim bladders, again indicating greater energy expenditure.
  - The authors hypothesized that failed swim bladder inflation occurs frequently in natural systems, but these individuals rarely survive in a natural environment where food resources are limited.
  - Note: yellow perch are a physoclistous species in which initial inflation can only occur during a narrow window of development in which the pneumatic duct is still connected to the gut, allowing the fish to gulp air and inflate its swim bladder. Once the pneumatic duct closes, normal inflation is no longer possible.
- In aquaculture systems, failure to inflate the swim bladder has been shown to reduce growth rates and cause high mortalities in a wide range of species (reviewed by Woolley and Qin, 2010).
- Pond-cultured walleye with non-inflated swim bladders were found to be smaller (weight and length) than fish with inflated swim bladders. There was also association with deformities (e.g., lordosis) that were expected to impair survival (Kindschi and Barrows, 1993).

- Review of failed swim bladder inflation in wild perch and 26 other physoclistous species showed that fish whose swim bladders failed to inflate had higher mortality, reduced growth, and increased incidence of spinal malformations stereotypical of persistent upward swimming (Egloff, 1996).
- Chatain (1994) reported that sea bream (*Sparus auratus*) and sea bass (*Dicentrarchus labrax*) with non-inflated swim bladders were 20-30% less in weight than those with inflated swim bladders and more susceptible to stress-induced mortality (e.g., associated with handling, hypoxia, etc.). It was suggested this was due to both increased energetic demands and decreased feeding efficiency.
- Marty et al. 1995 measured increased oxygen consumption in Japanese medaka (*Oryzias latipes*) with non-inflated swim bladders compared to those whose swim bladders had inflated.
- In zebrafish (*Danio rerio*) whose swim bladder inflation was prevented by holding in a closed chamber (preventing air gulping to inflate the swim bladder), larval survival was significantly less than that of fish held in open chambers whose swim bladders could inflate. There was also increased incidence of spinal curvature in the closed chamber fish whose swim bladders were prevented from inflating (Goolish and Oukutake, 1999).
- Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass (*Morone saxatilis*) lead to significant increases in both swim bladder inflation and survival (Brown et al., 1988).
- In striped bass, (*Morone saxatilis*) failure to inflate the swimbladder was reported to results in dysfunctional buoyancy control, deformities, and poor larval survival and growth (Martin-Robichaud and Peterson, 2008).
- All zebrafish larvae that failed to inflate the posterior chamber after exposure to 2 mg/L iopanoic acid (IOP), died by the age of 9 dpf (Stinckens et al., 2020). Since larvae from the same group that were able to inflate the posterior chamber survived, it is plausible to assume that uninflated posterior chambers limited the ability to swim and find food.
- MeHg and HgCl<sub>2</sub> exposure in medaka caused failure to inflate the swim bladder among other malformations, and also caused increased mortality. (Dong et al., 2016)
- Medaka embryos treated either with hypoxia or with a mixture of polyaromatic hydrocarbons showed higher occurrences of swim bladder non-inflation and decreased survival. (Mu et al., 2017)
- Triphenyltin (TPT) exposure in zebrafish embryos induced a high percentage of uninflated swim bladders and all affected larvae died within 9 dph. (Horie et al., 2021)

### Uncertainties and Inconsistencies

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

### References

- Brown, C. L., Doroshov, S. I., Nunez, J. M., Hadley, C., Vaneenennaam, J., Nishioka, R. S. and Bern, H. A. (1988), Maternal triiodothyronine injections cause increases in swimbladder inflation and survival rates in larval striped bass, *Morone saxatilis*. J. Exp. Zool., 248: 168–176. doi: 10.1002/jez.1402480207
- Chatain B (1994) Abnormal swimbladder development and lordosis in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*). Aquaculture 119 (4): 371-9
- Chatain, Beatrice. "Problems related to the lack of functional swimbladder in intensive rearing of the seabass *Dicentrarchus labrax* and the sea bream *Sparus auratus*." Advances in Tropical Aquaculture, Workshop at Tahiti, French Polynesia, 20 Feb-4 Mar 1989. 1989.
- Dong W, Liu J, Wei LX, Yang JF, Chernick M, Hinton DE. 2016. Developmental toxicity from exposure to various forms of mercury compounds in medaka fish (*oryzias latipes*) embryos. PeerJ. 4.
- Egloff, M. 1996. Failure of swim bladder inflation of perch, *Perca fluviatilis*, L. found in natural populations. Aquat. Sci. 58(1):15-23.
- Gary D. Marty, David E. Hinton & Joseph J. Cech Jr. (1995) Notes: Oxygen Consumption by Larval Japanese Medaka with Inflated or Uninflated Swim Bladders, Transactions of the American Fisheries Society, 124:4, 623-627, DOI: 10.1577/1548-8659(1995).
- Goolish, E. M. and Okutake, K. (1999), Lack of gas bladder inflation by the larvae of zebrafish in the absence of an air-water interface. Journal of Fish Biology, 55: 1054–1063. doi:10.1111/j.1095-8649.1999.tb00740.x
- Greg A. Kindschi & Frederic T. Barrows (1993) Survey of Swim Bladder Inflation in Walleyes Reared in Hatchery Production Ponds, The Progressive Fish-Culturist, 55:4,219-223, DOI: 10.1577/1548-8640(1993)055<0219:SOSBII>2.3.CO;2
- Horie Y, Chiba T, Takahashi C, Tatarazako N, Iguchi T. 2021. Influence of triphenyltin on morphologic abnormalities and the thyroid hormone system in early-stage zebrafish (*danio rerio*). Comparative Biochemistry and Physiology C-Toxicology & Pharmacology. 242.
- Kindschi GA, Barrows FT (1993) Survey of swim bladder inflation in walleyes reared in hatchery production ponds. Progressive Fish-Culturist 55 (4): 219-23
- Martin-Robichaud, D. J. and Peterson, R. H. (1998), Effects of light intensity, tank colour and photoperiod on swimbladder inflation success in larval striped bass, *Morone saxatilis* (Walbaum). Aquaculture Research, 29: 539–547. doi: 10.1046/j.1365-2109.1998.00234.

Mu JL, Chernick M, Dong W, Di Giulio RT, Hinton DE. 2017. Early life co-exposures to a real-world pah mixture and hypoxia result in later life and next generation consequences in medaka (*oryzias latipes*). *Aquatic Toxicology*. 190:162-173.

Sergiusz J. Czesny, Brian D. S. Graeb & John M. Dettmers (2005): Ecological Consequences of Swim Bladder Noninflation for Larval Yellow Perch, *Transactions of the American Fisheries Society*, 134:4, 1011-1020. <http://dx.doi.org/10.1577/T04-016.1>

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

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

Woolley, L. D. and Qin, J. G. (2010), Swimbladder inflation and its implication to the culture of marine finfish larvae. *Reviews in Aquaculture*, 2: 181–190. doi: 10.1111/j.1753-5131.2010.01035.x