Organisation for Economic Co-operation and Development

English - Or. English

Adverse Outcome Pathway Scientific Review Report

AOPs 155-159, Project 1.35: AOPs on Thyroperoxidase and/or deiodinase inhibition leading to impaired swim bladder inflation in fish during early life stages

- **AOP 155: Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation (DIO2i posterior swim bladder)**
- **AOP 156: Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation (DIO2i anterior swim bladder)**
- **AOP 157: Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation (DIO1i posterior swim bladder)**
- **AOP 158: Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation (DIO1i anterior swim bladder)**
- **AOP 159: Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (TPOi anterior swim bladder)**

This document is the final review report for AOPs 155-159. It was prepared by the UK National Centre for the 3Rs who organised and managed the review of these 5 AOPs.

The report compiles the views and comments of the reviewers for the AOP network and explains how the authors of the AOP network have addressed these comments.

It provides the basis to EAGMST for determining if the AOP network has been adequately revised by their authors following the review and if it can be released to the Working group of the National Coordinators of the Test Guidelines Programme and to the Working Party on Hazard Assessment for endorsement.

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Chapter 1. Introduction and background to specific AOP

1.1. Background

This is an AOP network consisting of five separate AOPs. AOPs 155 (Deiodinase 2 inhibition leading to reduced young of year survival via reduced posterior swim bladder inflation) and 159 (Thyroperoxidase inhibition leading to reduced young of year survival via reduced anterior swim bladder inflation) form the basis of the network and those two AOPs are described in full detail. AOPs 156, 157 and 158 are connected to the two main AOPs, and the descriptions of these AOPs are focused on highlighting those elements that are different from AOPs 155/159. Since there are many shared key events (KEs) it was suggested that the five AOPs be reviewed simultaneously as a network (see Figure 1).

This AOP network describes how inhibition of thyroperoxidase and/or deiodinase leads to reduced swim bladder inflation, resulting in reduced swimming performance, increased mortality and ultimately, decreased population trajectory (Knapen et al., 2018; Villeneuve et al., 2018; Knapen et al., 2020). Disruption of the thyroid hormone system is increasingly being recognized as an important toxicity pathway that can cause many adverse outcomes, including disruption of developmental processes. The network includes three molecular initiating events (MIEs) representing the inhibition of enzymes that are important for thyroid hormone (TH) synthesis and activation. Thyroperoxidase (TPO) is essential for TH synthesis and therefore inhibition of TPO leads to reduced levels of thyroxine (T4) and triiodothyronine (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. Type II deiodinase (DIO2) has T4 as a preferred substrate and is mostly important for converting T4 to the more biologically active T3, and type I deiodinase (DIO1) is capable of both converting T4 into T3 and converting rT3 to the inactive thyroid hormone 3,3' T2. Inhibition of DIO1 and/or DIO2 thus reduces T3 levels. Available evidence suggests that DIO2 is more important compared to DIO1 for proper swim bladder inflation in fish. The swim bladder is a gas-filled organ found in many bony fish species and typically consists of two gas-filled chambers. The posterior chamber inflates during early development (embryo), while the anterior chamber inflates during late development (larva). Both chambers are important for fish to control buoyancy and the anterior chamber has an additional role in hearing (Robertson et al., 2007). Evidence from chemical exposures (Jomaa et al., 2014; Nelson et al., 2016; Stinckens et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2018; Stinckens et al., 2020), as well as data from knockdowns, knockouts and TH supplementation (Walpita et al., 2009; Walpita et al., 2010; Heijlen et al., 2014; Bagci et al., 2015; Houbrechts et al., 2016; Chopra et al., 2019) have been instrumental in supporting the AOP network. An AOP initiated by TPO inhibition and leading to posterior chamber inflation was not included, since available evidence shows that TPO inhibition is less likely to cause effects on inflation of the posterior chamber during early embryonic development. The life stage specificity of the AOP network is explained in more detail below (see Domain of applicability).

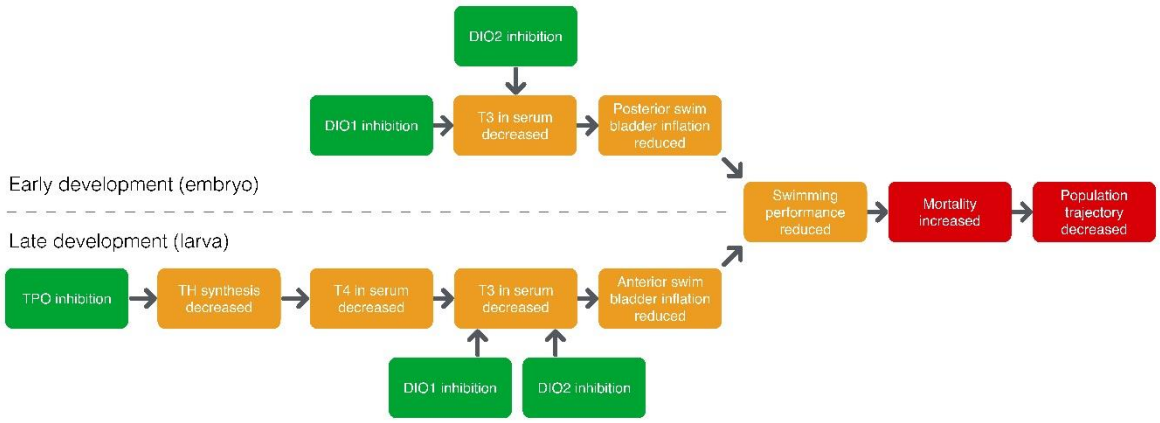

Figure 1. Graphical representation

Domain of applicability:

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

Life stage: Specific parts of the AOP network are relevant to different life stages. 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). This seems to limit the sensitivity to TPO inhibition during the earliest embryonic stages. When maternally derived THs are depleted during late development (larval stage), endogenous TH synthesis becomes more important and inhibition of TPO interferes with proper inflation of the anterior swim bladder chamber (Nelson et al., 2016; Stinckens et al., 2016; Godfrey et al., 2017; Stinckens et al., 2020). In all life stages however, the conversion of T4 into T3 is essential. Inhibition of deiodinase (DIO) therefore impacts swim bladder inflation in both early and late developmental life stages (Jomaa et al., 2014; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2018; Stinckens et al., 2020).

Maternal thyroid hormone levels in embryos have been demonstrated in zebrafish, fathead minnow, brown trout, striped bass, tilapia, rabbitfish, conger eel, sea bream and different species of salmon (Brown et al., 1988; Power et al., 2001; Walpita et al., 2007; Chang et al., 2012; Hsu et al., 2014; Ruuskanen and Hsu, 2018). Campinho et al. (2014) confirmed that maternal thyroid hormones are essential for normal brain development in zebrafish by knocking down MCT8,

responsible for transporting thyroid hormones into the cells. Alt et al. (2006) found a first differentiated thyroid follicle in zebrafish at 55 hours post fertilization. Elsalini et al. (2003) used immunohistochemistry to show the development of the first thyroid follicles producing thyroid hormone at 72 hours post fertilization (hpf) in zebrafish. Walter et al. (2019) showed decreased levels of T4 in 72 hpf embryos exposed to propylthiouracil (PTU), a TPO inhibitor, confirming embryonic TH synthesis at 72 hpf. At 24 hpf PTU had no effect on TH levels, confirming that at least at the whole body level, there is no detectable TH synthesis at 24 hpf. Time points between 24 and 72 hpf have not been investigated. Therefore, it is still uncertain when exactly embryonic TH synthesis is activated. As a result, early developmental processes (before thyroid activation) that are dependent on T4, such as posterior swim bladder inflation, may be less sensitive to chemicals reducing T4 synthesis. Nelson et al. (Nelson et al., 2016) and Stinckens et al. (2016) indeed found that 2-mercaptobenzothiazole (a thyroperoxidase inhibitor) decreased T4 levels in both zebrafish (5 days post fertilization) and fathead minnow (6 days post fertilization), which is after activation of the thyroid gland for both species, while it did not affect posterior chamber inflation. Godfrey et al. (2017) also reported normal posterior chamber inflation after exposure of zebrafish embryos to methimazole, a TPO inhibitor. In the latter study, only perfluorooctanoic acid (a deiodinase inhibitor in pig, Stinckens et al., 2018) affected posterior chamber inflation. Stinckens et al. (2018) also showed that sodium perchlorate, an inhibitor of the sodium iodide symporter (NIS), did not impact posterior chamber inflation. Since this symporter is essential for providing iodide for TH synthesis, this is indirect evidence for the hypothesis of life stage specificity. It is important to note that posterior chamber inflation should be observed until at least 6 days post fertilization (dpf) in zebrafish to exclude delayed swim bladder inflation (which may be due to a more general developmental delay, rather than a specific effect on the swim bladder). Because of the limited likelihood of TPO inhibition resulting in reduced posterior chamber inflation, an AOP representing this pathway has not been developed at this stage.

Sex: Sex differences are typically not investigated in tests using early life stages of fish and it is currently unclear whether sex-related differences are important in this AOP network. 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 both chambers of the swim bladder inflate before sex differentiation is finalized, sex differences are expected to play a minor role in this AOP network.

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1.2. AOP authors

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1.3. Scientific review

The project for the development of AOPs 155-159 was included on the AOPs Development Programme Workplan in 2015 (project 1.35) and is led by Belgium and the USA.

Based on completion of the internal review checklists in January 2021 (which ensured the AOPs are compliant with the Users' Handbook supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways), the draft AOP was ready for external expert review.

The internal review checklists can be found at:

https://aopwiki.org/aops/155/comments

https://aopwiki.org/aops/156/comments

https://aopwiki.org/aops/157/comments

https://aopwiki.org/aops/158/comments

https://aopwiki.org/aops/159/comments

This AOP network was reviewed between March and July 2021 by a panel of eight reviewers (see Annex 1). Two calls were put out for experts to apply to become reviewers (one from the OECD, and one from the review organisers). Candidates provided their CVs, outlining their skills and expertise relevant to the review role.

Selection to the joint scientific review panel was conducted by the review managers in accordance with the Draft Guidance Document for the scientific review of Adverse Outcome Pathways (published 27 July 2020). Selection was driven by the candidates' expertise in fish toxicology (including endocrine disruption and molecular biology, ensuring coverage across the different aspects of the AOPs, as well as expertise in AOP development). The first selection criterion was the skills of the reviewers to assess the AOP. Secondary criteria were balancing gender, sector (academy/industry/government) or origin from different countries. Each reviewed some, or all, of the AOPs in the network, depending on their area of expertise. There were a higher number of reviewers associated with this review than there normally would be for an external AOP review, due to there being several AOPs within the network for which not all reviewers reviewed every AOP (dependent on relevant expertise). Details of the review panel members can be found in Annex 1. No conflicts of interest were identified.

In line with Section 5.5 of the Draft Guidance Document for the scientific review of Adverse Outcome Pathways, the review panel was charged with reviewing the scientific evidence that has been presented to substantiate the AOP and to respond specifically to the following charge questions:

1. Scientific quality:
   - Does the AOP incorporate all appropriate scientific literature and evidence?
   - Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

2. Weight of evidence:
   - In your opinion, is the weight-of-evidence judgement/scoring well described and justified based on the evidence presented? If not please explain.
   - Please consider weight-of-evidence for each KER and for the AOP as a whole.

Chapter 2. Synthesis of main issues of the review

The reviewers were asked to reply to the charge questions outlined above regarding different aspects of the AOPs. Individual review comments are available in Annex 2 of this report. A summary of the answers for each charge question (as well as 'other considerations') is accessible in Annex 3, organised point by point under each question. The replies made by the authors to the reviewers' points are also accessible in Annex 3.

The versions used for the review were the snapshots provided by the OECD Secretariat and accessible at:

https://aopwiki.org/aopwiki/snapshot/pdf_file/155-2021-01-26T13:53:28+00:00.pdf

https://aopwiki.org/aopwiki/snapshot/pdf_file/156-2021-01-26T13:56:06+00:00.pdf

https://aopwiki.org/aopwiki/snapshot/pdf_file/157-2021-01-26T13:57:04+00:00.pdf

https://aopwiki.org/aopwiki/snapshot/pdf_file/158-2021-01-26T13:58:51+00:00.pdf

https://aopwiki.org/aopwiki/snapshot/pdf_file/159-2021-01-26T14:07:13+00:00.pdf

There were some general comments around the AOP process/concept, rather than scientific concerns. This included whether the five AOPs should be submitted as one AOP network containing all TH-related MIEs/KEs/AOs; however in keeping with the spirit and requirements of AOP development, they continue to be kept as separate AOPs in the AOP-wiki.

This section provides an overview of the main issues raised by the reviewers which required further discussion to reach consensus, and/or explanation or clarification further to the authors' responses detailed in Annex 3.

2.1. Scientific quality

Specific questions were raised around the MIEs for DIO1 and DIO2, and the applicability of the AOPs.

- It was suggested that the MIEs for DIO1 and DIO2 required further definition, as a specific enzymatic reaction catalyzed by DIO instead of DIO1/DIO2, e.g., conversion of T4 to T3. Chemicals often target more than one DIO isoform. Also, it was deemed that there is no convincing evidence that DIO1 results in a reduction of T2 levels, thus there was a query around whether the AOP can be published, or should be integrated into AOP 155.

- The question was raised regarding whether the taxonomic applicability could be refined. Should the list of species be based on experimental evidence or plausibility assessment? Is there any larger taxonomic category that could be used instead of (or in addition to) single species? (e.g. KE1003 T3).

- In terms of applicability of the AOPs, it was noted that AOP 158 (DIO1-AC) might be less relevant. T3 measurements are highly variable and can be unreliable, and the evidence may not be strong enough to suggest addition of T3 measurements to the fish embryo toxicity (FET) or fish early life stage (FELS) tests.

2.2. Weight of evidence

There were several queries related to the weight of evidence statements within the AOPs (across strength of evidence, and essentiality):

- For KER1034 (AC-swimming), the evidence was deemed weak (rather than moderate), there is no distinction between effect on AC or entire swim bladder system. It may be necessary to add uncertainties regarding other effects that may have influenced swimming performance.
- For KER1035 (T3-AC), the evidence was deemed weak (rather than moderate): only one study started exposure after PC inflation, with no evidence of primary effect. The taxonomic applicability should be changed from high to moderate for zebrafish.
- The essentiality for DIO2-anterior in AOP 156 is moderate; this was not fully supported.
- It was suggested the essentiality for DIO1-anterior in AOP158 be changed from moderate to low.
- The essentiality for TPO-anterior in AOP 159 was queried, as dual oxidase knockdown is indirect evidence: DUOX also plays a role in oxidative stress.

- In terms of KER2013 mortality-population, it was suggested that it would be better to harmonize with the AOP on AChE inhibition, i.e. change from high to moderate. There was disagreement that there is limited evidence to optimise population models.

2.3. Conclusions and next steps

This AOP was well written and documented. Nevertheless, additional work was required before finalising the process and submission at the OECD. The discussion points raised above were prioritised for discussion at a scientific review teleconference (see Section 3), to allow consensus on these points to be reached.

Chapter 3. Summary record of the teleconference

The scientific review teleconference (TC) was held on 15 July 2021 at 15.00-16.00 BST. It was attended by all reviewers, the authors of the AOP network and the review managers (Annex 1).

Before the TC, authors provided initial written responses to most of the comments (see Annex 3). Areas requiring further discussion to reach consensus between the authors and reviewers were prioritised for discussion at the TC (see also Section 2). One week prior to the call, the reviewers were provided with the authors' responses to their comments related to the charge questions. The authors and reviewers were given the opportunity to raise any particular issues of concern and input into the agenda. The agenda was shared for comment before the meeting. The agenda points provided the basis for the discussion.

The review manager and the authors thanked the reviewers who devoted significant amount of their time to provide constructive comments, editorial changes and additional literature.

3.1. TC agenda

1. Introductions
2. Scientific review process
3. Meeting aims
4. General comments ▪ MIEs DIO1 and DIO2

▪ Refine taxonomic applicability ▪ Refine applicability of the AOP
5. Comments regarding WoE evaluation ▪ KER1034 AC-swimming ▪ KER1035 T3-AC evidence is weak: only one study started exposure after PC inflation, no evidence of primary effect. Change to moderate for zebrafish ▪ Essentiality AOP156 DIO2-anterior: moderate is not fully supported ▪ Essentiality AOP158 DIO1-anterior change moderate to low ▪ Essentiality AOP159 TPO-anterior ▪ KER2013 mortality-population: harmonize with AOP on AChE inhibition, i.e. moderate
6. Actions and next steps
7. Meeting close

3.2. Main issues and responses/actions identified during the call

Refine taxonomic applicability - should the list of species be based on experimental evidence or plausibility assessment? Is there any larger taxonomic category that could be used instead of (or in addition to) a single species?

- It was noted that the concept or notion of plausible domain of applicability is not yet a formal concept that is supported by the AOP framework, but that this is under development. The authors agreed to update the AOP-wiki with the relevant information as soon as this became possible.
- In the meantime, it was agreed to add a broader "plausible domain of applicability" in the text field.
    - For upstream events taxonomic applicability would be broader than downstream events, e.g. reduction in T3 add plausibly applicable to vertebrates in general.
    - For more downstream events this would be more specific, e.g. for anterior Chamber inflation reduction this could be plausibly applicable to physostomous fish as a general group.

Refine considerations for applicability of the AOP - AOP158 is less relevant with regard to applicability because DIO1 is less important than DIO2; T3 measurements are highly variable/unreliable, the evidence is not strong enough to suggest addition of T3 measurements to the FET or FELS.

- Agreed to highlight that applications should be more focused on the DIO2 rather than DIO1, as DIO2 is more important.
- In the author's experience it was noted that T3 measurements are not very variable and are highly predictive of downstream effects, but since they can be variable in other studies it was agreed that a note would be added to acknowledge that more variability may be present in other studies.
- Additionally, it is envisioned that this endpoint would be part of a battery of endpoints, since T3 measurement alone is unlikely to be predictive. However, measurement of T3 levels in concert with other endpoints such as the MIE and the adverse outcome, as the causal link in between, is a more informative approach. Especially since thyroid hormone disruption leads to different effects, often not specific to thyroid hormone disruption.
- Discussion on whether the variability was due to technical issues, variability between individual fish, and/or due variable levels during development. It was suggested that specific timepoints could be added/specified for zebrafish for when these hormone levels should be measured, (where the variability is known), and that it is important to stay within specific life stages.

KER1034 AC-swimming - evidence is weak (rather than moderate), no distinction between effect on AC or entire swim bladder system, exposure concentration high. Add uncertainties regarding other effects that may have influenced swimming performance.

- Authors explained the reasons for the moderate WoE description (for plausibility, empirical and overall). Though the link between anterior swim bladder chamber inflation and swimming performance may not seem plausible initially the mechanism has been described in a detailed paper in the literature, proving that the anterior chamber is important for swimming performance. This is supported by the authors own considerable empirical evidence.
- Agreed to keep the description as moderate.

KER1035 T3-AC - Evidence is weak (rather than moderate): only one study started exposure after PC inflation, no evidence of primary effect. Change taxonomic applicability call from high to moderate for zebrafish

- Authors explained the reasons for moderate WoE evidence description. The role of thyroid hormones in swim bladder development and in developmental transitions (embryo-larval, larval-juvenile) is well established, and is supported by considerable empirical evidence from dedicated studies, including quantitative relations between the T3 levels and anterior chamber size.
- Agreed to keep the description as moderate.
- The taxonomic applicability call was set at high form zebrafish due to having a quantitative relationship but since this information is from only one study it was agreed to change to moderate until more information becomes available.

Essentiality AOP156 DIO2-anterior: moderate is not fully supported. Reviewer suggests changing to low evidence.

- The definitions of low and moderate evidence were explained. Low being '*no or contradictory experimental evidence of the essentiality of any of the KEs'* and moderate being '*indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE'*
- The authors explained that given the descriptions above they felt there was sufficient evidence to support moderate and that changing to low would not do justice to the evidence that is available i.e. evidence for several KEs within the AOP.
- It was agreed to keep the description of essentiality as moderate for all AOPs.
- Discussion on how to determine that DIO1/DIO2 are affecting AC and PC, and that the effect on the AC bladder inflation is not just a secondary effect due to limited PC swim bladder inflation. An explanation was provided (longer experiments where concentrations were specifically selected at which the PC inflated, problems with swimming seen when AC not inflated). Agreed to add further explanation about this evidence to support moderate essentiality decision.

Essentiality AOP158 DIO1-anterior change moderate to low

- Since it is not clear that DIO1 is essential it was agreed to change the essentiality to low for AOP158.

Essentiality AOP159 TPO-anterior

- Agreed to add the fact that DUOX also plays a role in oxidative stress to uncertainties and inconsistencies on the relevant pages.

KER2013 mortality-population - harmonize with AOP on AChE inhibition (high to moderate)

- Agreed to change description from high to moderate, because it has not yet been established what level of mortality will effectively affect the population, and survival long-term. However, it was noted that this could be changed to high when more evidence becomes available.
- Discussion around level of empirical data on the relationships between survival and population level effects in fish to optimize population models. It was agreed that there is more literature on fishing which could be exploited.

3.3. Conclusion

Overall, the authors agreed to implement most of the suggested changes by updating and making changes to specific sections of this AOP, as detailed above.

The reviewers supported the submission of the AOPs for approval and publication subject to modification of the AOPs according to the reviewers' recommendations.

As agreed, detailed changes were made to the AOPs which were also implemented in the respective Wiki pages.

The authors have provided a revision document (Annex 4), where the structure of the reviewer comments summary is used to detail the changes that have been made to the AOPs in response to the reviewer comments. Each of the general responses is shown first, followed by the changes that have been made in the relevant AOP-wiki pages in response. The responses have been further grouped into a first section including all responses that have been discussed during the scientific review meeting on 15 July 2021 where the author team as well as the reviewers and the review managers participated, and a second section including all remaining comments. To allow easy distinction between the original text and the changes that have been made, changes are marked in red.

Chapter 4. Outcome of the scientific review

The panel had agreed that once the AOP network had been modified according to the reviewers' recommendations, the AOPs would be submitted for approval and publication. The review managers have reviewed the authors' revisions following the scientific review and consider these modifications thoroughly address the comments from the panel.

Annex 1: List of Reviewers, Authors and Review manager

Function	Name	Affiliation	Country
Review manager/organiser	Fiona Sewell	NC3Rs	UK
Review manager/organiser	Natalie Burden	NC3Rs	UK
Review manager/organiser	Briony Labram	NC3Rs	UK
Author	Dries Knapen	University of Antwerp	Belgium
Author	Lucia Vergauwen	University of Antwerp	Belgium
Author	Evelyn Stinckens	University of Antwerp	Belgium
Author	Dan Villeneuve	US EPA	USA

Reviewer	David Du Pasquier	Laboratory Watchfrog	France
Reviewer	Ioanna Katsiadaki	Cefas	UK
Reviewer	Luigi Margiotta-Casaluci	Brunel University London	UK
Reviewer	Elke Eilebrecht	Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg	Germany
Reviewer	ZhiChao Dang	RIVM	Netherlands
Reviewer	Sebastian Eilebrecht	Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg	Germany
Reviewer	Andrea Zikova-Kloas	German Federal Institute for Risk Assessment	Germany
Reviewer	Audrey Bone	Bayer	Germany

Annex 2: Individual reviewers' comments, organised by AOP

Reviewer	AOP	Section	Comment
All AOPs			
Reviewer 1	All	General remarks	In principle, the presented AOPs 155-159 incorporate the appropriate scientific literature and at least to some extent they reflect the scientific knowledge concerning the specific topics. However, because the overall goal is to deal with disruption of the thyroid system being an endocrine system triggered by several MIEs at various organizational levels and also having multiple (negative) feedback mechanisms concerning its regulation it seems inappropriate to split it up in a large number of AOPs. Consequently, a growing number of further AOPs with different MIE but the same context (e.g. NIS, dual oxidase, deiodinase 3, TH binding proteins, TH excretion, TH membrane transporters, TH receptors, interactions with RX receptors, nuclear coactivators and repressors) could be expected. For each AOP a reviewing process will deal always with the same abstract, as it is the case for AOPs 155-159. The endocrine regulation of the thyroid system is highly complex. However, the recently provided AOPs (155-158) give an impression that there is one MIE only per AOP, followed by a linear pathway leading to an thyroid system specific KE (impaired inflation of swim bladder), which is hard to imagine considering the multiple feedback mechanisms that exist to countermeasure imbalances of the TH availabilities. Therefore, it might make more sense to submit only one AOP network concerning thyroid system disruption in zebrafish including all organizational levels where endocrine regulation can take place instead of submitting several separated AOPs (cf. the paper of Paul Friedman et al. (2016; doi: 10.1093/toxsci/kfw034) as an example for such an AOP network that can be modified for zebrafish). Such an AOP network should include more detailed description of the thyroid system referring also explicitly to species specific differences between mammals and teleost fish (for instance zebrafish do not have thyroxine binding globulin, the hypothalamic factors triggering TSH release by the pituitary differ from mammalian ones, also TH receptors vary).
Reviewer 1	All	More detailed remarks	According to these AOPs the KE inflation of the posterior (5 dpf) and anterior swim bladder (21 dpf) of the physostomous zebrafish is supposed to be specific due to thyroid system disruption. However, there is also evidence that further mechanisms could be involved in the impairment of swim bladder inflation in zebrafish. For instance, Yue et al. (2015, Aquat. Toxicol.) provided evidence that dioxin induces heart failure via arylhydrocarbon receptors and thus leading to an impaired inflation of swim bladder. Even the authors of these AOPs published a paper (Hagenaars et al. 2014; Aquat. Toxicol.) dealing with PFOS impairing swim

			bladder inflation via a yet unknown mechanism. In addition, if the zebrafish cannot swim to the surface to gulp some air for the first inflation of the posterior chamber of swim bladder (Goolish and Okutake, 2005) the inflation rate is significantly disturbed. Such effects seem to become also possible if zebrafish might be exposed to chemicals that change the tension of the water surface. Dumbarton et al. (2010; J. Exp. Biol.) suggest that swim bladder inflation can be also impaired via adrenergic substances. Further reports investigating the physoclistous Japanese medaka (Oryzias latipes) showed that swim bladder inflation could be also impaired by 17α-ethinylestradiol, levonorgestrel, and diclofenac (Pandelides, PhD thesis, 2017). Thus, it seems that the impaired inflation of swim bladder as a KE is not necessarily thyroid system specific, which should be seriously considered.
Reviewer 1	All	General: more specific	Each AOP (155-158) demonstrates a linear pathway associated with a single MIE (DIO 1 or 2 inhibition) leading to decreased T3 concentrations. However, each deiodinase type (1-3) is able to cause not only one but also at least two enzymatic reactions concerning inner and outer ring deiodinations and thus changes in T3 as well as in TH metabolites. DIO1 is able to perform all four possible metabolic conversions for TH such as T4 into T3, T3 into T2(diiodothyronine), T4 into rT3 and rT3 into T2. DIO2 can convert T4 into T3 and rT3 into T2, whereas DIO3 can change T4 into rT3 and T3 into T2. Thus, it is also evident that at least two (for DIO2 and 3) or four (DIO1) MIEs per DIO can occur and the complex interplay among DIOs is causing a varying output concerning TH and their metabolites (T4, T3, rT3, and T2). That might be the reason for endocrine regulatory measures of the thyroid system at various hierarchical levels (hypothalamus, pituitary, thyroid, transport of TH) or at the level of DIOs triggering the preferred conversion of each DIO. Therefore, DIOs should not be considered separately. Since one MIE per AOP is favored, the authors should then submit even four AOPs instead of one concerning DIO1 covering each potential enzymatic reaction of DIO1 as a MIE separately, which does not make really sense. Even the authors of the AOPs themselves claim, “The overall importance of DIO1 versus DIO2 in fish is not exactly clear” and therefore the suggestion to incorporate all DIOs into a AOP network might be advantageous. Another argument not to continue with single AOPs dealing with only one type of DIO is the fact that several chemicals affect not only one DIO type but two or even all three DIOs. Therefore, the resulting levels of TH and their metabolites are the outcome of the complex interplay of all DIOs as a whole.
Reviewer 2	All	Relationship: 1028: Reduced, Posterior swim bladder inflation leads to Reduced, Swimming performance + Relationship: 1034:	The WoE for this relationship has been classified as moderate due to some experimental inconsistencies observed in the literature, which prevent the establishment of a clear causal relationship. It is possible that some of those inconsistencies may be due to the limited ecological relevance of the methods used to quantify behaviour (e.g., 24 well plates). In my personal experience, behavioural assessment in multi-well plates and observation from the top may not be fully suitable to assess buoyancy-driven swimming performance disruption. Often, the swimming activity takes place mostly in proximity of the well bottom. The latter provides also a supporting surface for the steady larvae, hence even larvae without inflated chamber may not experience significant energy expenditure due to buoyancy issues. Tailored

		Reduced, Anterior swim bladder inflation leads to Reduced, Swimming performance	behavioural tests that, for example, employ a deeper water column plus stimuli that "push" the fish to swim towards the surface would highlight more easily possible disruptions of swimming performances due to buoyancy issues. Possible stimuli include virtual predators, food, light changes, startling noises, forced swimming. This approach could also be useful to elucidate relationship 1034, which takes place at later life stages. I am aware that previous studies tried to mathematically model the link between buoyancy and adult fish swimming behaviour. Those data may not be relevant to larval behaviour, but here I will share the details of one of those papers just in case the authors are interested in it (e.g., Strand et al., 2005 - https://www.sciencedirect.com/science/article/pii/S0304380005000049).
Reviewer 1	All	Weight of evidence	In principle, the authors described the WoE well but information that impairment of inflation of swim bladder is NOT a strictly TH dependent process is missing. The judgement of the WoE for each KER and for the AOPs as a whole is in agreement with the authors. However, as written in the first part covering scientific quality and content for such a complex endocrine system as the thyroidal one it would be more beneficial to merge all AOPs in an AOP network, which might allow also to consider all complex interplays among the various parts of the thyroid system using zebrafish as model.
Reviewer 8	All	General	AOPs 155-159 are similar and describe disruption of the HPT axis leading to alterations in development of the swim bladder in fish and ultimately to mortality. The AOPs follow the general outline of inhibition of either a DIO or TPO enzyme->reduction in serum T3-> effects on either the posterior or anterior swim bladder->reduction of swimming performance-> increase in mortality->decrease in population trajectory. The AOPs are well organized and described. The supporting literature is well-chosen and the authors do a good job in general in listing the uncertainties around specific KERs. The initial KERs have fairly strong evidence, i.e. the inhibition of the DIO or TPO enzymes leading to a decrease of serum T3; as do the final KERs of a reduction in swimming performance leading to mortality and ultimately a decrease in population. The key events that have the most uncertainty surrounding them are how the reduction in serum T3 relates to effects on the swim bladder and how the effects on the swim bladder relate to a reduction in swimming performance (particularly for the anterior swim bladder) and thus mortality. The overall relationship between reduced T3 leading to a swim bladder effect prolonged enough to impact predator avoidance, feeding etc. is lacking. The authors do point out these uncertainties. More information on how these effects on swimming performance and mortality were also accompanied by any other indicators of toxicity in order to understand the strength of the causal relationship would be useful. Effects on swim bladder inflation and size are commonly seen with many chemicals and can have many plausible mechanisms. In order to fully understand the utility of the AOP, it would be helpful to have more detailed information included on any other effects seen in the study from the Stinckens 2020 work and how they may have impacted swim performance, and whether any general developmental delay was observed.

			Given these uncertainties, I have some hesitation on the considerations for potential applications of the AOP. The quantitative understanding of how, in what tissue, and on what timescale alterations in T3 levels contribute to the AO in this AOP is very low, and T3 measurements are highly variable and can be unreliable. I don't think the evidence is strong enough to suggest addition of T3 measurements to the FET or FELS. An assessment of posterior chamber inflation and swimming performance could be interesting additions to the FELS, however, I think it should be clarified that these would not be diagnostic of a thyroid-specific mechanism. I think this section needs some editing to prevent misinterpretation of how robust the evidence is for the KERs that are applicable for these endpoints.
AOP 155			
Reviewer 3	155	General	AOP 155 is well described and included the critical literature, which reflects the current understanding of the relationship between swim bladder and the regulation of the HPT axis. This AOP is useful for both scientific and regulatory fields. In order to better understand this AOP and to increase the use of this AOP in the regulatory field, the following point are suggested. 1. As stated in this AOP, impaired posterior chamber inflation may not be solely regulated by the HPT axis. Except uncertainties, this point should also be emphasized in the other sections. This issue is especially important for the use of this AOP in the regulatory identification of thyroid disrupting chemicals, where triggering further testing and drawing a conclusion should be based on chemical specific effects. 2. In the stressor list, it was first mentioned iopanoic acid (IOP, p2) and then added PERFLUOROOCTANOIC ACID (p5) to the list. Iopanoic acid has been described in the text; whereas no description was found for PERFLUOROOCTANOIC ACID. There are more inhibitors for DIOs. Is it possible to list more chemicals that have been tested for the effects on swim bladder in fish? In the description of AOP, chemicals like PTU, BPS, F-53B etc. were also mentioned. Their mechanisms of action may not be via an inhibition of DIOs. An explanation of their mechanisms would be helpful for understanding the whole picture. Another issue is that the listed chemicals may influence DIO isoforms and not specific to DIO2. The proposed AOP is based on chemical exposure experiments. An explanation for the chemical exposure and the relationship with DIO2 is needed. 3. THs are typically measured at the whole body level in fish early life stages. The hypothesis is on the decreased plasma T3, which is different from the measurement of the whole body TH level. An explanation is needed for this discrepancy, which should be addressed, in addition to the section of uncertainties (p19), in the section of key event relationship.
Reviewer 4	155 157	General	The authors made an impressive high-quality job in setting up the AOPs and to provide rationale and supportive evidence. These AOPs incorporate the most important scientific literature and current

			scientific knowledge in this field. In general, supporting literature is appropriate for the proposed MIE, KEs and KERs, I could propose the following improvements: **General** The statement at the beginning of the abstract of the AOP 157 mentioning that the two APO are identical could be also placed in abstract of the AOP 155. The comment I made for AOP 159 regarding "sex applicability" and "specificity" are also valid for these two AOPs. The comments I made for AOP159 on the KE, KER and OA shared between these three AOPs are valid for these AOPs. Please see comments 106, 110, 113, 115, 124, 127 In Domains of applicability: it is mentioned "**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**." Medaka is not listed in the table of taxonomic applicability, I suggest to harmonize by listing medaka in the table with a low level of evidence and cite the available references showing a defect in swim bladder inflation in the appropriate KE or KER (see below).
Reviewer 4	155 157	Event 1004 Reduced posterior swim bladder inflation	Swim bladder inflation is not only influenced by the thyroid pathway, there is numerous publications reporting a reduction of swim bladder inflation that could be added to support this KE. • Normal zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), silent heart morphants (which lack cardiac contractility) and transiently transgenic cmlc2:caAHR-2AtRFP embryos (which mimic TCDD-induced heart failure via heart-specific, constitutive activation of AHR signaling), all developed hypoplastic swim bladders of comparable size and morphology. In all treatment groups swim bladder development was arrested during the growth/elongation phase. (Yue et al., 2016) • tris(2,3- dibromopropyl) isocyanurate (TBC) exposure caused defects in the inflation of the gas bladder of zebrafish larvae. (Li et al., 2011) • Adult female and male zebrafish (F0 generation) were exposed to polystyrene nanoparticules via diet for one week and bred to produce the F1 generation. F1 groups with a co-parental exposure (exposed males and females) show almost 100% uninflated swim bladder (Pitt, J., 2018) • Zebrafish larvae exposed to isoniazide (INH) showed morphological abnormalities, included swim bladder absence. *INH significantly increased the levels of reactive oxygen species and malondialdehyde and decreased the superoxide dismutase activity in zebrafish larvae, which suggested that oxidative stress was induced and that the antioxidant capacity was inhibited.* (Zou et al., 2017)

			• Embryonic ethanol exposure induces impaired swim bladder inflation. (Cadena et al., 2020) • Nitrite exposure in zebrafish embryos also cause swim bladder non-inflation, as other malformations. (Simmons et al., 2012) • Many of the embryos that had been incubated with RU486 (mifepristone, which is glucocorticoid receptor antagonist) had no detectable swim bladder at 120 hpf. (Wilson et al., 2013) • At the same concentration of PFOS, more uninflated swim bladder was observed in dechorionated-embryos than in embryos with chorion during their development. (Mylroie et al., 2021) *Oryzias latipes* could be added in taxonomic applicability as the number of publications showing defects of swim bladder inflation in this species is increasing. The following could be added in the KE description to support the occurrence of this KE: • Medaka embryos treated either with hypoxia or with a mixture of PAH sampled showed higher occurrences of swim bladder non-inflation. (Mu et al., 2018) • Medaka embryos treated with mercury compounds show abnormalities including swim bladder. (Dong et al., 2016). As mercury compounds is known to be a thyroid disruptor, this phenotype could appear via thyroid pathway, directly or indirectly. • Medaka embryos exposed to 17β-estradiol, 17α-ethinylestradiol, levonorgestrel, or diclofenac show significant increase in failure of swim bladder inflation with increasing exposure concentration. (Pandelides et al., 2021) • Exposure of medaka embryos to permethrin induce a lack of swim bladder inflation and inability to respond to stimuli. (Gonzalez-doncel M et al., 2003) • The percentage of embryos with failed swim bladders following hatch significantly increased following treatment with 5 μM SeMet at stages 9, 17, and 25 in medaka embryos. (Kupsco A et al., 2017)
Reviewer 4	155 157	Relationship: 1028: Reduced, Posterior swim bladder inflation leads to Reduced, Swimming performance	*In dose response relationship:* "Several studies have shown that larvae with inflated swim bladders have higher swimming activity compared to larvae that failed to inflate the swim bladder." References are lacking here. *In empirical evidence:* The following studies have shown that a defect in swim bladder inflation leads to reduced swimming performance and could be added to support this KER: • tris(2,3- dibromopropyl) isocyanurate (TBC) exposure caused defects in the inflation of the gas bladder of zebrafish larvae, resulting in the impairment of free motility. (Li J et al., 2011) • Zebrafish larvae treated by hypoxia showed less posterior swim bladder inflation at 120 hpf and reduction in velocity and in distance travelled. (Wilson KS et al., 2016) The general effects of reduced oxygen availability on swimming performance are well

			understood and could be linked with the absence of swim bladder that allows gas exchange. (Fry 1971, Beamish 1978) • Zebrafish larvae treated by glucocorticoids morpholino or with RU 486 (mifepristone) showed reduced frequency of swim bladder inflation at 120 hpf, and reductions in velocity and in distance travelled. (Wilson KS et al., 2016; 2013) • Nitrite exposure in zebrafish embryos induce impaired swim bladder inflation and immobility. (Simmons et al., 2012) • Embryonic ethanol exposure induces impaired swim bladder inflation and reduces the swimming activity. (Cadena PG et ., 2020) • Exposure of medaka embryos to permethrin induce a lack of swim bladder inflation and inability to respond to stimuli. (Gonzalez-doncel M et al., 2003)
Reviewer 4	155 157	Relationship: 1042: Inhibition, Deiodinase 2 leads to Reduced, Posterior swim bladder inflation	In empirical evidence, I suggest to remove the sentence "Exposure to PTU, a very potent DIO1 inhibitor, caused thyroid hypertrophy in X. leavis because of the inhibition of the peripheral conversion of T4 to T3 (Degitz et al., 2005)." Xenopus DIO1 has been shown to be insensitive to PTU (Kuiper GG et al. 2006 Endocrinology), the effect seen by Degitz et al. is likely to be due to the inhibition of TPO by PTU.
Reviewer 4	155 157	Relationship: 2213: Reduced, Posterior swim bladder inflation leads to Increased Mortality	In taxonomic applicability: The text states "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." This needs to be harmonized with the table indicating only two species zebrafish and fathead with respectively a high and moderate evidence level. Reading the text would rather lead to indicate in the table physoclistous fish (or yellow perch and Japanese Medaka) with a high evidence level and zebrafish and fathead minnows with a moderate/low evidence level. Other species are cited in reference: sea bream (Sparus auratus), sea bass (Dicentrarchus labrax) and striped bass (Morone saxatilis) shouldn't they be part of taxonomic applicability? In empirical evidence: The following studies have shown that a defect in swim bladder inflation leads to mortality and could be added to support this KER: • Swim bladder is crucial for survival in most fish species because it minimizes energy required to maintain vertical position in the water column (Alexander et al., 1972). • MeHg and HgCl2 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 PAH sampled showed higher occurrences of swim bladder non-inflation and decreased survival. (Mu et al., 2018)

			• Triphenyltin (TPT) exposure on zebrafish embryos induced high percentage of uninflated swim bladder, all affected larvae died within 9 dph. (Horie et al., 2021) Uncertainties and inconsistencies: this section is not present in this KER, some studies showed an absence of increased mortality but only by looking at short term effects. These are no relevant for long term effect on mortality but this could eventually be discussed here if the authors find it useful. • Adult female and male zebrafish (F0 generation) were exposed to polystyrene nanoparticules via diet for one week and bred to produce the F1 generation. F1 groups with a co-parental exposure (exposed males and females) show almost 100% uninflated swim bladder but no significative difference in survival measure from 24 to 96 hpf. (Pitt et al., 2018) • Nitrite exposure in zebrafish embryos induce swim bladder non-inflation but doesn't affect survival at 120 hpf. (Simmons et al. 2012) • PFECA-exposed zebrafish embryos, with high occurrence of posterior swim bladder uninflation, did not result in any notable increase in mortality compared with the control group after 5 days of exposure post-fertilization. (Wang et al., 2020) • In reference: the references are not in alphabetical order.
Reviewer 4	155 157	Relationship: 1044: Inhibition, Deiodinase 1 leads to Reduced, Posterior swim bladder inflation	In empirical evidence, I suggest to remove the sentence "Exposure to PTU, a very potent DIO1 inhibitor, caused thyroid hypertrophy in X. leavis because of the inhibition of the peripheral conversion of T4 to T3 (Degitz et al., 2005)." Xenopus DIO1 as been shown to be insensitive to PTU (Kuiper GG et al. 2006 Endocrinology), the effect seen by Degitz et al. is likely to be due to the inhibition of TPO by PTU.
Reviewer 4	155 157	Weight of evidence	The WOE judgment is well described and justified based on the evidence presented for each MOI, KER, OA and the AOP as a whole. I have the following comments to make in addition of the ones made for AOP159 (see comments 129, 130, 131, 132, 133) **Relationship: 1027: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Posterior swim bladder inflation:** The quantitative understanding has been rated "low" for this KER and "moderate" for the Relationship: 1035: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Anterior swim bladder inflation. I wonder if this difference of rating is really funded and suggest to harmonize.
Reviewer 3	155	MIE P6 Key Event Description	The description of the synthesis of the THs and TPO should be deleted because it diluted the attention of DIOs and did not include the whole picture of the synthesis of THs.
Reviewer 5	155	Abstract page 1	Instead of - disruption of developmental processes – perhaps developmental abnormalities TH - spell
Reviewer 5	155	Molecular Initiating Events (MIE), Key	Event ID 1005- KE isn't this an adverse outcome already

		Events (KE), Adverse Outcomes (AO) page 2	
Reviewer 5	155	Domain of Applicability page 3	Taxonomic applicability - This is extremely narrow; two regulatory species only. How many other AOPs are species specific? Can the authors comment of plausibility for relevance to all physostomus fish? Sex differences are typically not investigated in tests using early life stages of fish – they are if they are a sex marker. Since the posterior chamber inflates around 5 days post fertilization, when sex differentiation has not started yet, sex differences are expected to play a minor role in the current AOP. Although I agree this is almost certainly the case for zebrafish, the statement may not be true for other fish with a strong genetic element of sex determination
Reviewer 5	155	Considerations for Potential Applications of the AOP (optional) page 4	Fish Early Life Stage Toxicity (FELS) Test (OECD TG210) - Why not the TG243 (Fish Sexual Development Test)?
Reviewer 5	155	Domain of Applicability page 5	DIO2 seemed to be more important than DIO1. – in zebrafish Thyroid hormones (THs) - need to explain this earlier and use TH here
Reviewer 3	155	How it is Measured or Detected page 7	It is important to state whether it is possible to measure DIO2 inhibition in fish or fish cells/tissues?
Reviewer 5	155	Taxonomic Applicability page 9	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. - when is the best time to measure serum THs in fish? Extrapolation between species should be done with cautious. – with caution? One of the most unique characteristics of TH is their ability to regulate their own concentration, not only in the plasma level, but also in the individual cell level, to maintain their homeostasis. - This is the case for most hormones, including sex steroids; why unique?

Reviewer 3	155	KEs Short Name: Decreased, Triiodothyronine (T3) in serum, Taxonomic page 9	The description did not include fish. Relevant information in fish should be added.
Reviewer 3	155	Key Event Description page 9	The description is for humans/mammals but not for fish. It is important to include the description on fish specific issues, e.g. transport proteins in blood which are different from mammals; TRH may not be the key regulator for TSH in some fish species.
Reviewer 3	155	How it is Measured or Detected page 10	The current description is rather general. It is suggested that the measurement of THs in fish should be specified.
Reviewer 5	155	Domain of Applicability and Key Event Description page 12	96 h post fertilization (hpf) which is 2 days post hatch - Isn't this one day post hatch? Check with TG234-FET 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. - If this is correct (5 dpf), change the 96h pf above to 120hpf Days post fertilization - explain earlier and use dpf or dph thereafter 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 concentrations close to lethal concentrations - this statement doesn't make sense in my opinion. Please check the wording
Reviewer 5	155	Key Event Description page 14	Adequate swimming performance in fish is essential for behaviour such as foraging, predator avoidance and reproduction - ..and fishing!! After all is a major cause of 'mortality'
Reviewer 5	155	Regulatory Significance of the AO page18	Maintenance of sustainable fish and wildlife populations - There is huge relevant literature on this for fisheries research. Not sure why this is completely ignored when trying to link individual mortality to population trajectory. The Miller and Ankley paper is not relevant in my opinion as it deals with fecundity, not mortality.
Reviewer 3	155	Known Feedforward/Feedback loops influencing	Current description is based on mammalian results. Is there information specific for fish?

		this KER page 20	
Reviewer 5	155	Evidence Supporting Applicability of this Relationship: Sex page 20	Sex: Zebrafish - why all references to sex are on zebrafish? What about the FHM?
Reviewer 5	155	Biological Plausibility and Uncertainties and Inconsistencies page 22	Resorption of the yolk sac - This is interesting as yolk resorption would be very easy to measure. Do we have data that look into the relationship of swim bladder inflation versus yolk resorption? 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). - We have also observed loss of pigmentation after exposure to thyroid disrupting chemical. Another interesting avenue to follow for a readily measured key event.
Reviewer 5	155	Uncertainties and Inconsistencies page 23	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). - This is a key statement that worths highlighting-suggest bold?
Reviewer 5	155	Uncertainties and Inconsistencies page 26	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. - Is this an issue for the AOP? How can we discriminate between TDCs and Baseline toxicity on this basis?
Reviewer 5	155	Empirical Evidence and Timescale page 29	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). - This means that mortality was not 100%. What was it? This number is key in establishing the population trajectory and should be mentioned here clearly. Since zebrafish initiate exogenous feeding around day 5 - specify day 5
Reviewer 5	155	Uncertainties and Inconsistencies page 30	In general, there is not enough empirical data on the relationships between survival and population level effects in fish (Rearick et al., 2018) to optimize population models. - I fundamentally disagree. There is a massive literature on fishing which has not been exploited
Reviewer 5	155	Empirical Evidence page 32	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. - reduced pigmentation again!!! Why we are not orientating there as an endpoint?
Reviewer 5	155	Uncertainties and Inconsistencies page33	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). - Strong statement, changing the application of this AOP in the

			management of chemicals.
Reviewer 5	155	Empirical Evidence page 36	wide range of species - any inferences in mortality between the two major subtypes of fish in respect to bladder inflation?
Reviewer 2	155 157	Relationship: 1027: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Posterior swim bladder inflation	This section is very well developed. Despite the lack of understanding of the exact mechanisms through which altered TH levels result in impaired posterior chamber inflation, the phenotypic evidence generated using a diverse set of experimental approaches appears to be strong. Overall, I agree with the overall WoE classification, set as moderate.
Reviewer 2	155 156	Relationship: 1026: Inhibition, Deiodinase 2 leads to Decreased, Triiodothyronine (T3) in serum	Unsurprisingly, the characterization of effect direction and magnitude of hormonal perturbations are not simple due to the complex biological feedback involved in the regulation of such processes. This specific KER is defined as an inhibitory/negative relationship. The biological plausibility of this definition is strong, but the empirical evidence in different species appears to be mixed, in some cases. The authors described the existing uncertainties very well. Considered the evidence provided in the report, I do agree with the authors that the overall expected effect direction in fish (at the specific life-stage considered in this AOP) is “decreased serum T3”. Nonetheless, this may not always be the case as there may be age-specific, exposure window-specific, and exposure duration-specific effects that may deviate from that dynamic. Future experimental studies should be designed with such uncertainties in mind in order to clarify those important aspects. An alternative strategy would be to use the words “perturbation of T3 serum concentration” rather than “decrease”. However, I believe that this option may be evaluated at later stage if further experimental efforts fail to identify a clearly reproducible effect direction.
Reviewer 8	155	Weight of evidence	In general, the weight-of-evidence judgement and scoring is well described and justified. As mentioned in the general comments on the set of AOPs, the weakest evidence is present for how the effects on the swim bladder driven by the reduction in T3 translate to reduced swimming performance. This is reflected in the scoring given to the individual KERs.
AOP 156			
Reviewer 3	156	General	AOP 156 is well described and included the critical literature, which reflects the current understanding of the relationship between swim bladder and the regulation of the HPT axis. This AOP is useful for

			both scientific and regulatory fields. In order to better understand this AOP and to increase the use of this AOP in the regulatory field, the following point are suggested. 1.As stated in this AOP, the mechanism of the reduced anterior chamber inflation and the HPT axis remains unclear. The HPT axis is not the only pathway for regulation of the swim bladder. This point should be emphasized in the abstract and other relevant sections. It is especially important for the use of this AOP in the regulatory identification of thyroid disrupting chemicals, where triggering further testing and drawing a conclusion should be based on chemical specific effects. 2.In the stressor list, it was mentioned iopanoic acid and PERFLUOROOCTANOIC ACID. Iopanoic acid has been described in the text; whereas no description was found for PERFLUOROOCTANOIC ACID. As the support for this AOP is mainly based on chemical exposures, it is suggested that chemicals that have been tested for their effects on anterior chamber inflation in fish should be listed. Another issue is that the listed chemicals may influence DIO isoforms and not specific to DIO2. The proposed AOP is based on chemical exposure experiments. An explanation for the chemical exposure and the relationship with DIO2 is needed. 3.THs are typically measured on a whole body level in the fish early life stages. The hypothesis is on the decreased plasma T3, which is different from the measurement of the whole body TH level. An explanation is needed for this discrepancy, which should be addressed, in addition to the section of uncertainties (p19), in the section of key event relationship.
Reviewer 4	156 158	Scientific quality	The authors made an impressive high-quality job in setting up the AOPs and to provide rationale and supportive evidence. These AOPs incorporate the most important scientific literature and current scientific knowledge in this field. In general, supporting literature is appropriate for the proposed MIE, KEs and KERs, I could propose the following improvements:
Reviewer 4	156 158	General	The statement at the beginning of the abstract of the AOP 158 mentioning that the two AOP are identical could be also placed in abstract of the AOP 156.

			The comment I made for AOP 159 regarding “sex applicability” and “specificity” are also valid for these two AOPs. (see below comments 43 and 44 which are also under the AOP 159 heading as comments 101 and 102) The comments I made for AOP159 on the KE, KER and OA shared between these three AOPs are valid for these AOPs. (see below for comments 55, 56, 57, 58, 59, 61, 62, 63, 64 which are also under the AOP 159 heading as comments 106, 109, 110, 113, 115, 121, 123, 124, 127).
Reviewer 4	156 158 (159)	Sex applicability	For several Key events and in the APO abstract, the table for Sex Applicability indicates “unspecific” with a level of evidence indicated as “High”. This is supported by a paragraph emphasing that Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary after the swim bladder inflation, the paragraph concludes by “sex differences are expected to play a minor role in the current AOP.” First, as the AOP is applicable to Fathead minnow, I recommend that this should also be discussed for this species that is using a XY sex determination strategy (Olmstead AW et al. 2011 EST). Second, as no experiment have been published to decipher specifically if this event could be influenced by the sex of the animal, I wonder if the level of evidence could be really set to high rather that moderate. Even I feel unlikely that this process could be sex dependent, there is a publication in Medaka showing the extend of perturbation of medaka swim bladder inflation by halogenated chemicals including thyroid disruptors to be sex dependent (Godfrey A. 2019 J. Appl Toxicol.) which raise the question for other species.
Reviewer 4	156 158 (159)	Specificity	I feel important to mention somewhere in these APOs that swim bladder inflation is not under the sole control of TH and that not only thyroid disruption but also other mechanisms of toxicity could disrupt swim bladder inflation. Several publications show the absence of swim balder inflation to be not specific for thyroid disruption : inhibition of the glucocorticoid axis using RU486 lead to the absence of swim bladder (Wilson KS, 2013), increasing the levels of reactive oxygen species using isoniazide lead to the absence of swim bladder (Zou et al., 2017), zebrafish embryos exposed to TCDD, silent heart morphants (which lack cardiac contractility) and transiently transgenic cmlc2:caAHR-2AtRFP embryos (which mimic TCDD-induced heart failure via heart-specific, constitutive activation of AHR signaling), all developed hypoplastic swim bladders of comparable size and morphology showing the involvement of cardiac function in swim bladder inflation (Yue M., 2016). Autophagy (Morishita et al. 2021), folate signaling (Lee 2018) and WNT/hedgehog signaling are also important for swim bladder development or inflation (Xu et al., 2017). I suggest that these elements could be mentioned in the “Consideration for potential applications of the AOP” to emphases that other endpoints should be considered in parallel of the swim bladder inflation to be able to conclude on the thyroid activity of a test chemical.

Reviewer 4	156 158 (159)	Additional observations	There is an important heterogenicity in the writing of each KE or relationship. One could clearly see for the KE shared by multiple AOPs that authors from different AOP worked on a given KE, creating a diversity in the style, level of details and some redundancies that could complicate the understanding. I wonder if I some point the AOPs in general will need some editors that will harmonize the content and style between each MOI, KE and AO.
Reviewer 3	156	Essentiality of the Key Events page 3	Deiodinase knockdowns did not show the relationship between DIOs and anterior chamber inflation. The knockdown of dual oxidase reduced anterior swim bladder inflation. This indirect evidence, however, did not indicate the importance of DIOs. Based on the current description, the AOP labelled as moderate seems not evidence-based. It is suggested that the support evidence should be addressed by using data of chemical exposures in zebrafish and fathead minnows.
Reviewer 3	156	Considerations for Potential Applications of the AOP (optional) page 3	The Fish Embryo Acute Toxicity (FET) test (OECD TG 236) should be deleted because it is not in the life stage of the anterior chamber.
Reviewer 3	156	Key Event Description page 6	The description of the synthesis of the THs and TPO should be deleted because it diluted the attention of DIOs and did not include the whole picture of the synthesis of THs.
Reviewer 3	156	How it is Measured or Detected page 6	It is state that “Deiodination is the major pathway regulating T3 bioavailability in mammalian tissues”. What about fish?
Reviewer 3	156	How it is Measured or Detected page 6	It is important to state whether it is possible to measure DIO2 inhibition in fish or fish cells/tissues?
Reviewer 3	156	Short Name: Decreased, Triiodothyronine (T3) in serum, Taxonomic page 8	The description on transport proteins is related to humans and rats. No information was provided for fish. Relevant information in fish should be added. Similarly, the description on THs for fish should also be included.

Reviewer 3	156	Key Event Description page 8-9	The description is for humans/mammals but not for fish. It is important to include the description on fish specific issues, e.g. transport proteins in blood which are different from mammals; TRH may not be the key regulator for TSH in some fish species.
Reviewer 3	156	How it is Measured or Detected page 9	The current description is rather general. It is suggested that the measurement of THs in fish should be specified.
Reviewer 3	156	Known Feedforward/Feedback loops influencing this KER page 19	Current description is based on mammalian results. Is there information specific for fish?
Reviewer 4	156 158 (also 159)	Event 1003 Decreased, Triiodothyronine (T3) in serum.	There is a need to harmonize Event 1003 and 281, starting with the titles, I would expect 1003's title to be named "Triiodothyronine (T3) in serum, decreased" following 281 title or the titles to be harmonized in the other way. The key event description for T3 decrease is very detailed compared to the one for T4 decrease (even non genomic actions of the hormones are considered). Many information given in the T3 event are true for both T3 and T4 and I wonder to which extent both event description should reach the same level of details or being harmonized. In "how it is measured or detected": To my knowledge ELISA is difficult to use for aquatic organisms and RIA is the more reliable method to date. I would suggest to change the last sentence to "Amongst all these methods RIA is particularly repeatable and reproducible."
Reviewer 4	156 158 (also 159)	Event: 1007: Reduced, Anterior swim bladder inflation Short Name: Reduced, Anterior swim bladder inflation Key	In taxonomic applicability: Striped trumpeter Latris lineata is a typical transient physostome (physostomous as larvae and physoclistous as adults transient physostomes). Adult striped trumpeter is further defined as euphysoclistous, having a swim bladder with dual-chambers separated by a diaphragm. As Trotter et al. studied swim bladder malformation in this species, it may be interesting to investigate further to see if it could be part of the taxonomic applicability.

Reviewer 4	156 158 (159)	Event 1005 Reduced Swimming performance	In life stage “Importance of swimming performance for natural behavior is generally applicable across all life stage”. I suggest to modify to “all post-embryonic life stages” In references: references for fathead minnow are needed here. in how it is Measured or detected: The following reference could be cited: Little, E.E., Finger, S.E., 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environ. Toxicol. Chem.
Reviewer 4	156 158 (also 159)	Event 351 Increased mortality	In taxonomic applicability: Zebrafish, Chicken and Fathead are listed in the taxonomy table but “All living things are susceptible to mortality” is mentioned in the text. There is a need for harmonization between the text and table, I would suggest to indicate “All species” in the table. There is no reference for this KE, the need for references should be considered.
Reviewer 4	156 158 (also 159)	Event 360 Decreased Population trajectory	The taxonomic applicability indicates “All species” but the KE description and the only cited reference refer to Fish, the need for references for other species should be considered.
Reviewer 4	156 158	Relationship: 1026: Inhibition, Deiodinase 2 leads to Decreased, Triiodothyronine (T3) in serum	In empirical evidence, for the first, second and last bullet point the model specie needs to be indicated.
Reviewer 4	156 158 (also 159)	Relationship: 1035: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Anterior swim bladder inflation	In empirical evidence: a publication on striped bass is cited. Does this species should be then added to the taxonomic applicability (with a low evidence considering the sole publication cited)? In Uncertainties and Inconsistencies: the authors highlight that “inflation upon disruption of the thyroid hormone system is in most cases, but not always, accompanied by reduced whole body T3 levels” and that “The mechanism underlying the link between reduced T3 and reduced anterior chamber inflation remains unclear” several hypotheses are listed including effect on development, WNT or hedgehog

			signaling, etc. I would recommend to state that a possibility for the observed inconsistencies is that the tested compound act non only on thyroid axis but also in parallel directly on another target known to be linked to swim bladder inflation such as autophagy, ROS, cardiac function. For example, 2-mercaptobenzothiazole beside its action on TPO is known to induce an elevation in elevation in reactive oxygen species (ROS) levels in fish cells (Zeng 2016). This hypothesis could explain how swim bladder could be affected while T3 concentration remains constant. Alternatively, temporality between T3/T4 dosage (assessed at 32dpf and 120hpf), the moment when there is a need for T3 to inflate the swim bladder (unknown but probably in between 32dpf and 120hpf) and the observation of the phenotype (32dpf), could lead to the hypothesis that T3 concentration was reduced in between the two dosages. In Uncertainties and Inconsistencies: I would advise to add a bullet point explaining that it is unclear which aspect of swim bladder development and inflation is affected by TH disruption. This is present in the KER 1027 (Decreased T3 leads to reduced posterior swim bladder) and could be adapted for this KER.
Reviewer 4	156 158 (also 159)	Relationship: 1034: reduced anterior swim bladder inflation leads to reduced swimming performance	In empirical evidence: the following reference showing a reduced swimming performance could be added: Lihua Yang, Emma Ivantsova , Christopher L Souders, Christopher J Martyniuk. The agrochemical S-metolachlor disrupts molecular mediators and morphology of the swim bladder: Implications for locomotor activity in zebrafish (Danio rerio)/ Ecotoxicol Environ Saf 2021 Jan 15;208:111641. doi: 10.1016/j.ecoenv.2020.111641
Reviewer 4	156 158 (also 159)	Relationship: 2212: reduced swimming performance leads to increased mortality	There is only one reference and focusing on reduced swimming performance linked to swim bladder defect. Swimming performance could be affected by a lot of stressors or mutation, I wonder if more references could be added to support this KER but no necessary linked to swim bladder. Regarding taxonomic applicability, only zebrafish is in the table but the text mentioned “generally applicable to all hatched fish”. There is probably a need to harmonize here.
Reviewer 4	156 158 (also 159)	Relationship: 2013: Increased mortality leads to decrease population trajectory	Regarding taxonomic applicability, only zebrafish and fathead are in the table but the text mentioned “all organism must survive…” “consideration made above are applicable to other fish species”. There is

			probably a need to harmonize here. Most of the reference cited in the KER focused on fish rather that especially on zebrafish or fathead, supporting the possibility to enlarge the taxonomic applicability.
Reviewer 4	156 158	Weight of evidence	The WOE judgment is generally well described and justified based on the evidence presented for each MOI, KER, OA and the AOP as a whole. I have no further comments to make in addition of the ones made for AOP159. WOE comment numbers: 129, 130, 131, 132
Reviewer 8	156	Weight of evidence	In general, yes the weight-of-evidence judgement and scoring is well described and justified. As mentioned in the general comments on the set of AOPs, the weakest evidence is present for how the effects on the swim bladder driven by the reduction in T3 translate to reduced swimming performance. This is reflected in the scoring given in to the individual KERs.
AOP 157			
Reviewer 6	157	General	The authors of the AOP 157 (Knapen et al.) incorporated the scientific literature and evidence to a high extend. The AOP 157 itself is incorporated in a wider AOP network, including the AOPs 155, 156, 157, 158, and 159, which all relate to effects of thyroid disruption on swim bladder inflation in zebrafish embryos and larvae, finally resulting in increased mortality. Generally, the authors thoroughly discuss the current literature, starting from the scientific background of the AOP in the abstract (i.e. thyroid signaling and its effects on development or metabolism, and the influence of swim bladder inflation for buoyancy and swimming performance). In the abstract, the AOP is discussed across taxonomic boundaries and the integration into the thyroidal AOP network is highlighted. The cited literature in the abstract is sufficient for giving a general overview of the topic. One critique is that the authors mainly focus on their own literature, which might be owned to the fact that the authors are the main drivers of this scientific topic. There are two main aspects which have to be considered

			1.The uncertainties of the MIE and following, the KER to the first KE. As there is no convincing evidence that DIO1 results in a reduction of T2 levels, I would assume that the AOP cannot be published, or should be integrated into the AOP 155. 2.The authors mainly focus on own research. I would suggest performing another literature review including other recent literature, e.g. Reinwald et al., 2021, Spaan et al., 2019, Noyes et al., 2019.
Reviewer 6	157	Domain of applicability	The domains of applicability are adequately reflected and focus on zebrafish and fathead minnow. As the posterior swim bladder inflation is restricted to the embryonic development, also the life stage evidence is correctly set. The taxonomic applicability is restricted to physostomous fish, and excludes physoclystous fish. This exclusion is based on limited knowledge for physoclystous fish. The taxonomic evidence could be extended to other physostomous fish, and set to “low evidence”, as laboratory data would be lacking. The evidence for the applicability with respect to the sex of the fish is also correctly set, however, the explanation is that at this developmental stage no sex differentiation has occurred. Irrespective of the correctness of this statement I would assume that the applicability to both sexes would also been given if sex differentiation has already occurred, as proper swim bladder inflation and maintenance of swimming performance is independent of the sex of the fish.
Reviewer 6	157	Essentiality of the Key Events	The classification of the essentiality of KEs as moderate is adequate. The highest concern is at the level of the MIE, as DIO1 is considered less relevant than DIO2, and most of the studies cited were performed with inhibitors addressing both, DIO1 and DIO2. Regarding the following building blocks of the AOP, the evidence for essentiality would be high.

Reviewer 6	157	KE is Decrease, T3 in serum	The first KE is Decrease, T3 in serum. This KE is involved in a number of AOPs, i.e., in all AOPs of the here evaluated network and in additional two AOPs. The applicability to the taxa is high for zebrafish, fathead minnow, and African clawed frog. Furthermore the applicability is high for all life stages and both sexes. There is no doubt that thyroid hormones are essential across taxa, life stages, and sexes. The authors thoroughly describe the function of THs, however not focusing on fish but including all taxa (including mammals) even though the AOP is specific for fish. I would appreciate if the essential role of TH in fish would be described in more detail, including all relevant literature of this aspect. The methods for TH measurements are mentioned, including RIA, ELISA and mass spectrometry.
Reviewer 6	157	KE is Reduced, Posterior swim bladder inflation	This KE is the central one in the here described AOP. It is involved in two AOPs (155, 157). The taxonomic applicability is restricted to zebrafish and fathead minnow, the life stage applicability is restricted to embryos, and the applicability with respect to sex is unspecific. In all cases, the evidence for this is high. The literature cited for this AOP is limited to literature of the authors, and other current literature is not considered. I assume that based on the importance of this topic in ecotoxicology and the number of labs dealing with this more current literature is available. I would suggest performing another literature research as this might also help interpreting the KER between T3 levels decreased and impaired posterior swim bladder inflation, a link which is still missing. The authors describe how the inflation can be measured (stereomicroscope) and point out that is must be ensured that the concentrations tested are well below the concentrations causing systemic toxicity. This is a crucial aspect, which is true for all endocrine disruptors, as otherwise, it cannot be ensured that the endocrine MoA is the primary MoA.
Reviewer 6	157	KE: Reduced, Swimming performance	This KE is involved in a number of AOPs, not restricted to TH signaling. The taxonomic applicability to fish is high, and the evidence is high for all life stages and unspecific for sex. This is true except for very early phases of development (before hatch and shortly thereafter). Thus, it might be relevant to mention that this KE is also temporarily located after the adjacent KE. For measurement, the authors suggest automated observation and tracking systems.

Reviewer 6	157	KE: Increased Mortality	This KE is included in a high number of AOPs. The taxonomic applicability should not be restricted, but be true for all taxa. Correctly the applicability is high for all life stages and unspecific for sex
Reviewer 6	157	KE: Decrease, Population trajectory	This KE is also involved in a high number of AOPs. Even though this could not be measured but mainly modelled, it is assumed that increased mortality would influence the populations.
Reviewer 6	157	KER is between DIO1 inhibition and T3 levels decreased	The taxonomic evidence for zebrafish and fathead minnow is low. As this is the first and most relevant KER for this AOP, a low evidence weakens the AOP substantially. This relates to the uncertain role of DIO1 in fish. If neither the literature nor own observations strengthen this KER, I want to stress once again that this MIE cannot be defined as it is. I still want to suggest that the two AOPs for DIO inhibition should be integrated into one AOP. This assumption is supported as the empirical evidence provided by the authors is not specific for DIO1 but is rather true for both, DIO1 and DIO2 inhibition. The authors address these uncertainties. However, the conclusion for me is that this AOP is in summary based on this uncertainty.
Reviewer 6	157	KER: Decreased T3 levels lead to reduced posterior swim bladder inflation	The taxonomic evidence for zebrafish is high, and for fathead minnow moderate. The authors indicate that there is only an indirect relationship, and that further events might be in between. The evidence is defined as moderate, however, information on TH receptor activation and signaling downstream are missing. There is no information on how the T3 levels directly influence swim bladder inflation. The authors postulate that effects on budding could be involved or that an effect on posterior chamber inflation could be caused by disturbing the formation and growth of the three tissue layers. There is current literature (Reinwald et al., 2021) aiming at solving these uncertainties, and I would suggest to carefully consider this publication and publications cited here in describing the plausibility of this KER. The authors are however aware that there is more information needed in order to resolve all uncertainties.

Reviewer 8	157	Weight of evidence	In general, yes the weight-of-evidence judgement and scoring is well described and justified. As mentioned in the general comments on the set of AOPs, the weakest evidence is present for how the effects on the swim bladder driven by the reduction in T3 translate to reduced swimming performance. This is reflected in the scoring given in to the individual KERs.
AOP 158			
Reviewer 6	158	General	The authors of the AOP 158 (Knapen et al.) incorporated the scientific literature and evidence to a high extend. The AOP 158 itself is incorporated in a wider AOP network, including the AOPs 155, 156, 157, 158, and 159, which all relate to effects of thyroid disruption on swim bladder inflation in zebrafish embryos and larvae, finally resulting in increased mortality. Generally, the authors thoroughly discuss the current literature, starting from the scientific background of the AOP in the abstract (i.e. thyroid signaling and its effects on development or metabolism, and the influence of swim bladder inflation for buoyancy and swimming performance). In the abstract, the AOP is discussed across taxonomic boundaries and the integration into the thyroidal AOP network is highlighted. The cited literature in the abstract is sufficient for giving a general overview of the topic. One critique is that the authors mainly focus on their own literature, which might be owned to the fact that the authors are the main drivers of this scientific topic.
Reviewer 6	158	Domain of applicability	The domains of applicability are adequately reflected and focus on zebrafish and fathead minnow. As the anterior swim bladder inflation is restricted to the larval development, also the life stage evidence is correctly set. The taxonomic applicability is restricted to physostomous fish, and excludes physoclystous fish. In contrast to the posterior swim bladder inflation, this restriction is based on the fact that physoclystous fish do not possess an anterior swim bladder, while the restriction to physostomous fish with respect to the posterior swim bladder inflation is due to limited knowledge for physoclystous fish. The taxonomic evidence could be extended to other physostomous fish, and set to “low evidence”, as laboratory data would be lacking. The evidence for the applicability with respect to the sex of the fish is also correctly set, however, the explanation is that at this developmental stage no sex differentiation has occurred. Irrespective of the correctness of this statement I would assume that the applicability to both sexes would also been given if sex differentiation has already occurred, as proper swim bladder inflation and maintenance of swimming performance is independent of the sex of the fish.

Reviewer 6	158	Essentiality of the Key Events	The classification of the essentiality of KEs as moderate is likely not adequate, as there are several KEs with only low provement of essentiality. The highest concern is at the level of the MIE, as DIO1 is considered less relevant than DIO2, and most of the studies cited were performed with inhibitors addressing both, DIO1 and DIO2. Another weak evidence is at the level of essentiality of the anterior swim bladder inflation for swimming behavior. There is limited knowledge if there is a clear link or if this is a secondary effect e.g. of posterior swim bladder inflation of due to systemic toxicity. The next weakness is for the KE increased mortality, as it is indicated that the swimming behavior in embryonic stages is directly related to increased mortality. However, as for this AOP the swimming behavior at larval stages, i.e., after anterior swim bladder inflation, should be judged, there is no evidence provided for the essentiality of this KE.
Reviewer 6	158	Considerations for potential applications of the AOP	The authors sufficiently describe the applicability of the AOP to be used in fish studies, i.e. with the aim to reduce the numbers of animal studies required. However, as described above, the applicability is not restricted to this AOP, but might be extended to all AOPs of this network. The AOP 158 might be the less relevant one with regard to applicability, especially, if most of the substances address both, DIO1 and DIO2, and evidence that posterior swim bladder inflation is more relevant than anterior swim bladder inflation.
Reviewer 6	158	List of MIEs in this AOP	The listed stressors are all not specific for DIO1. This is a limitation for this AOP, as all effects following could be also assigned to other AOPs of this network. With regard to the taxonomic applicability, the evidence is only moderate for zebrafish, and no evidence is listed for fathead minnow. This is a crucial fact, as the AOP is defined for zebrafish and fathead minnow, and the evidence for the MIE is only moderate. The authors describe that DIO1 is the main supplier of T3 in mammals and birds, however, its function in fish and amphibian T3 regulation is less clear. It is postulated that DIO2 is responsible for T3 levels in these vertebrates. As the authors themselves have some doubt on the essentiality of this MIE for the AOP, I am wondering if this AOP should be described individually, or if a more general AOP (i.e. DIO1/DIO2 inhibition leading to increased mortality via reduced posterior swim bladder inflation) would be more appropriate. In order to identify if DIO1 alone is essential for posterior swim bladder inflation, I would suggest to generate a DIO1 morpholinos in order to determine if DIO2 function is sufficient to result in properly inflated posterior swim bladders. The study can also be performed vice versa, i.e., DIO2 morpholinos, in

			order to see if DIO1 function alone also results in posterior swim bladder inflation. If the one or the other is not the case, the AOP has to be reorganized. The corresponding KE description includes a general description of thyroid signaling in vertebrates and its function in a diverse range of biological processes. The authors also list at which points of the signaling chemicals can interfere. At the end, the authors describe the functions of the DIO enzymes, they mention that DIO1 is rather involved in the conversion of rT3 to 3,3' T2, and the high Km of DIO1 compared to DIO2. DIO2 is more involved in T4 conversion to T3 and is thus the more active form. The authors further describe methods to measure the DIO1 inhibition by stressors, which is restricted to in vitro assays with pig liver tissue or rat liver tissue. Another method utilizes an adenovirus expression system producing DIO1 enzyme, which was applied in a non-radioactive measurement of iodide release in a 96 well format. This assay was used to screen the ToxCast Phase 1 chemical library. This method seems to be appropriate for determining the activity, however, it does not give any hint on the specificity, and if this finally results in any effect in fish. An appropriate assay for fish is missing.
Reviewer 6	158	KE 1003 Decrease, T3 in serum	This KE is involved in a number of AOPs, i.e., in all AOPs of the here evaluated network and in additional two AOPs. The applicability to the taxa is high for zebrafish, fathead minnow, and African clawed frog. Furthermore the applicability is high for all life stages and both sexes. There is no doubt that thyroid hormones are essential across taxa, life stages, and sexes. The authors thoroughly describe the function of THs, however not focusing on fish but including all taxa (including mammals) even though the AOP is specific for fish. I would appreciate if the essential role of TH in fish would be described in more detail, including all relevant literature of this aspect. The methods for TH measurements are mentioned, including RIA, ELISA and mass spectrometry.
Reviewer 6	158	KE 1007 Reduced, Anterior swim bladder inflation	This is the central KE of this AOP. It is involved in three AOPs (156, 158, 159). The taxonomic applicability is restricted to zebrafish and fathead minnow, the life stage applicability is restricted to embryos, and the applicability with respect to sex is unspecific. In all cases, the evidence for this is high. The taxonomic restriction to physostomous fish is more applicable, as physoclystous fish do not possess an anterior swim bladder.

			The inflation of the anterior swim bladder is part of the larval-to-juvenile transition in fish. The AC itself has closely packed and highly organized bundles of muscle fibers. This observation suggests to examine muscle impairment as additional KE in this AOP, which might provide a link between T3 levels and anterior swim bladder inflation. There is already a publication available providing evidence for this link (Reinwald et al., 2021). The measurement of anterior swim bladder inflation can be performed with a stereomicroscope. However, it is only possible if fish are still not fully pigmented.
Reviewer 6	158	KE 1005 Reduced, Swimming performance	This KE is involved in a number of AOPs, not restricted to TH signaling. The taxonomic applicability to fish is high, and the evidence is high for all life stages and unspecific for sex. This is true except for very early phases of development (before hatch and shortly thereafter). Thus, it might be relevant to mention that this KE is also temporarily located after the adjacent KE. For measurement, the authors suggest automated observation and tracking systems.
Reviewer 6	158	AO 351 Increased Mortality	This KE is included in a high number of AOPs. The taxonomic applicability should not be restricted, but be true for all taxa. Correctly the applicability is high for all life stages and unspecific for sex. Next KE: Decrease, Population trajectory This KE is also involved in a high number of AOPs. Even though this could not be measured but mainly modelled, it is assumed that increased mortality would influence the populations.
Reviewer 6	158	Key Event Relationships in the AOP DIO1 inhibition and T3 levels decreased	The taxonomic evidence for zebrafish and fathead minnow is low. As this is the first and most relevant KER for this AOP, a low evidence weakens the AOP substantially. This relates to the uncertain role of DIO1 in fish. If neither the literature nor own observations strengthen this KER, I want to stress once again that this MIE cannot be defined as it is. I still want to suggest that the two AOPs for DIO inhibition should be integrated into one AOP. This assumption is supported as the empirical evidence provided by the authors is not specific for DIO1 but is rather true for both, DIO1 and DIO2 inhibition.

			The authors address these uncertainties. However, the conclusion for me is that this AOP is in summary based on this uncertainty.
Reviewer 6	158	Decreased T3 levels lead to reduced anterior swim bladder inflation	The taxonomic evidence for zebrafish and fathead minnow is high. The empirical evidence the authors provided is however not convincing. Only one cited publication performed a study which allows a direct relationship between T3 levels and anterior swim bladder inflation, i.e. the study of Nelson et al., 2016). The other studies were performed with fish exposed from fertilization, or even as F1 generation of exposed parental fish. Thus, from my perspective, there is no evidence that the effect is really a primary effect on anterior swim bladder inflation, or if the effect is secondary to an effect on posterior swim bladder inflation. Thus, I would assume that the evidence for this connection is not high for zebrafish, but maximum moderate. The described uncertainties rely on the link between T3 and AC inflation. As this could not be a direct connection, some intermediate KE is unknown. Transcriptomics or proteomics approaches would help identifying the MoA of substances. One recent paper identified an effect on muscle development in response to T3 and 6-PTU treatment (Reinwald et al., 2021). Furthermore, the authors should consider that there might be species-specific differences in this AOP.
Reviewer 6	158	Reduced anterior swim bladder inflation leads to reduced swimming performance	The taxonomic evidence for zebrafish and fathead minnow is high. However, the authors indicate that the weight of evidence for a direct linkage between these two KEs is weak. This indication is reflected by the description of the biological plausibility. The authors barely could differentiate between a direct effect resulting from anterior swim bladder inflation of an effect resulting from a more general effect on the swim bladder system. As the MIE for both, the anterior and posterior swim bladder inflation is identical, I would assume that these two effects are not independent from each other and cannot be judged separately. The empirical evidence is also not clearly focused on the anterior swim bladder inflation. Furthermore, the concentrations of the stressor mentioned here are way too high to result in specific effects and not in general toxic effects.
Reviewer 6	158	Reduced swimming performance leads to increased mortality	The description here is identical to the description for AOP 157. However, in AOP157, the swimming performance is evaluated with respect to early larval development (e.g. 6-9 dpf). At this age, an influence of swimming performance on mortality as a result of anterior swim bladder inflation cannot be determined. Thus, I suggest to perform an additional literature research focusing on mortality as a result in a failure of swimming performance in later larval stages.

Reviewer 6	158	Overall comment	Taken together, there are several main aspects which have to be considered: The uncertainties of the MIE and following, the KER to the first KE. As there is no convincing evidence that DIO1 results in a reduction of T2 levels, I would assume that the AOP cannot be published, or should be integrated into the AOP 155. The authors mainly focus on own research. I would suggest performing another literature review including other recent literature, e.g. Reinwald et al., 2021, Spaan et al., 2019, Noyes et al., 2019. The role of the anterior swim bladder is not fully convincing. Furthermore, it is unclear if the effects described are a result of anterior swim bladder inflation alone or of failure of the whole system. Swimming performance was discussed with respect to early larval stages, and not to later stages, which are important for this AOP.
Reviewer 8	158	Weight of evidence	In general, yes the weight-of-evidence judgement and scoring is well described and justified. As mentioned in the general comments on the set of AOPs, the weakest evidence is present for how the effects on the swim bladder driven by the reduction in T3 translate to reduced swimming performance. This is reflected in the scoring given in to the individual KERs.
AOP 159			
Reviewer 7	159	General	The authors have well described he key events and key event relationships within this AOP. However, some points need to be addressed particularly to further provide scientific evidence on certain key events and key event relationships in fish. As a general point, the vast majority of literature, which has been cited by the authors as scientific evidence for the early key events and key event relationships (until KER 1035: Decreased, Triiodothyronien (T3) in serum leads to Reduced, Anterior swim bladder inflation) describes studies in mammals, humans or amphibians. With regard to this AOP, this assumes a certain degree of read-across and cross-species conservation of key events and their relationships. For certain key events of key event relationships this may be due to the fact that these had been reviewed previously in the context of the development of AOPs in mammals or amphibians (as stated in the response to the internal review report). However, as AOP159 is supposed to describe key events and their relationships in fish, a particular focus should be put on scientific evidence in fish by citing the corresponding literature. This approach should also be applied to previously reviewed key events and key event relationships in mammals, by adding literature providing scientific evidence in fish. For this, the authors should also mine data of molecular studies, which have been performed in fish.

Reviewer 7	159	AOP summary	One study inducing a knockdown of dual oxidase is reported as indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation (Chopra et al., 2019). Dual oxidase is not only involved in thyroid hormone synthesis, but also in the production of reactive oxygen species (ROS) (Flores et al. 2010; Niethammer et al. 2009). In zebrafish, ROS can also be induced e.g. by copper (Zhou et al. 2016), which has also been shown to impair swim bladder development (Xu et al. 2017). Therefore, also an impaired production of ROS after dual oxidase knockdown may contribute to an impairment of swim bladder development.
Reviewer 7	159	Molecular Initiating Event	It may be beneficial to cite literature regarding the expression (onset) of TPO in fish early life stages. There may be a sufficient number of gene expression studies (either RT-qPCR or transcriptomics), which provide this information. In the description of the MIE thyroid peroxidase inhibition, the authors cite a study comparing genistein-induced TPO inhibition in different species (Doerge and Chang 2002). However, given that genistein has been shown to act in a variety of mechanisms, such as estrogen receptor agonism (Patisaul et al. 2002; Prossnitz and Arterburn 2015; Prossnitz and Barton 2014), inhibition of DNA methyltransferase (Fang et al. 2007), modulation of nicotinic acetylcholine receptor activity (Glushakov et al. 1999), and others, this evidence should be taken with care. More reliable evidence for cross-species comparability of the MIE would be provided using more specific TPO inhibitors.
Reviewer 7	159	Adverse Outcome	The authors may consider listing all signs of death assessed in OECD guideline tests with fish. The texts for key event description and regulatory significance of the AO are identical.
Reviewer 4	159	Scientific quality	The authors made an impressive high-quality job in setting up the AOPs and to provide rationale and supportive evidence. This AOP incorporate the most important scientific literature and current scientific knowledge in this field. In general, supporting literature is appropriate for the proposed MIE, KEs and KERs
Reviewer 7	159	Scientific quality Does the AOP incorporate all appropriate scientific literature and evidence?	In particular for the early key events and key event relationships, the authors should mine molecular studies in fish in order to cite them as scientific evidence for the corresponding events and relationship in that species. In the current version of the AOP, a significant proportion of the cited literature with regard to fish has been published by the authors themselves. The references of this AOP require to be updated, as a part of the recent literature, which deals with thyroid disruption in fish, has not been cited yet (Peng et al. 2020; Reinwald et al. 2021; Spaan et al. 2019; Vergauwen et al. 2018).
Reviewer 4	159	Species Applicability	There are some contradictions arising from the use of references using single swim bladder species in this AOP applicable to species with two chambers. The AOP states in taxonomy page 3: “This AOP is not applicable to fish that do not have a second swim bladder chamber that inflates during larval development, e.g., the Japanese rice fish (Oryzias latipes)”. However, some references using sea bass (Chatain et al., 1994) and striped bass (Brown et al., 1988) could be found in KER 1034 and KER 1035 for instance, despite the fact that they are physoclistous species with one chamber. (Peruzzi et al., 2007; Doroshev et al., 1981; Kitajima et al., 1994). The same comment could be made for the Atlantic salmon (Poppe et al., 1997). This article mentioned that Salmonidae are physostomatous but that the “swim

			bladder itself is of a simple type, i.e. not divided into chambers as in cyprinids" (The year of the reference is no correct, it should be 1997 but not 1977). This is also the case for Bluefin tuna (Brill et al., 2014; Gleiss et al. 2019). I think that the reference using these species could be mentioned in KER 2212 (Reduced, Swimming performance leads to Increased Mortality) but not in KER 1034 (Reduced, Anterior swim bladder inflation leads to Reduced, Swimming performance) or KER 1035 (Decreased, Triiodothyronine (T3) in serum leads to Reduced, Anterior swim bladder).
Reviewer 5	159	Taxonomic page 3	This AOP is not applicable to fish that do not have a second swim bladder chamber that inflates during larval development, e.g., the Japanese rice fish (Oryzias latipes) - Is it only the medaka in this category? Any other taxonomic division that is wider than a species example?
Reviewer 5	159	Taxonomic Applicability page 6	Evidence strength missing for mouse
Reviewer 5	159	How it is Measured or Detected page 9	Line 1 – double full stop
Reviewer 5	159	Key Event Description page 11	Zoeller and Tan, 2007 – Zoeller et al?
Reviewer 4	159	Sex applicability	For several Key events and in the APO abstract, the table for Sex Applicability indicates "unspecific" with a level of evidence indicated as "High". This is supported by a paragraph emphasing that Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary after the swim bladder inflation, the paragraph concludes by "sex differences are expected to play a minor role in the current AOP." First, as the AOP is applicable to Fathead minnow, I recommend that this should also be discussed for this species that is using a XY sex determination strategy (Olmstead AW et al. 2011 EST). Second, as no experiment have been published to decifer specifically if this event could be influenced by the sex of the animal, I wonder if the level of evidence could be really set to high rather that moderate. Even I feel unlikely that this process could be sex dependent, there is a publication in Medaka showing the extend of perturbation of medaka swim bladder inflation by halogenated chemicals including thyroid disruptors to be sex dependent (Godfrey A. 2019 J. Appl Toxicol.) which raise the question for other species.
Reviewer 4	159	Specificity	I feel important to mention somewhere in these APOs that swim bladder inflation is not under the sole control of TH and that not only thyroid disruption but also other mechanisms of toxicity could disrupt swim bladder inflation. Several publications show the absence of swim balder inflation to be not specific for thyroid disruption : inhibition of the glucocorticoid axis using RU486 lead to the absence of swim bladder (Wilson KS, 2013), increasing the levels of reactive oxygen species using isoniazide lead to the absence of swim bladder (Zou et al., 2017), zebrafish embryos exposed to TCDD, silent heart morphants (which lack cardiac contractility) and transiently transgenic cmlc2:caAHR-2AtRFP embryos (which mimic TCDD-induced heart failure via heart-specific, constitutive activation of AHR signaling),

			all developed hypoplastic swim bladders of comparable size and morphology showing the involvement of cardiac function in swim bladder inflation (Yue M., 2016). Autophagy (Morishita et al. 2021), folate signaling (Lee 2018) and WNT/hedgehog signaling are also important for swim bladder development or inflation (Xu et al., 2017). I suggest that these elements could be mentioned in the "Consideration for potential applications of the AOP" to emphases that others endpoints should be considered in parallel of the swim bladder inflation to be able to conclude on the thyroid activity of a test chemical.
Reviewer 4	159	Event 277 Thyroid hormone synthesis decreased	In taxonomy availability: the scientific name for "Pig" should be Sus Scrofa. TPO, NIS and iodine deficiency are listed as potential molecular initiating events, I suggest to add DUOX inhibition (Chopra 2019) and iodotyrosine deiodinase (dehalogenase, IYD) inhibition (Olker 2018).
Reviewer 7	159	Key event 277: Thyroid hormone synthesis, decreased	As indicated above, the major knowledge about thyroid hormone synthesis comes from studies in mammals. Thus, it may be beneficial to include recent publication on cross-species AOP networks with regard to thyroid disruption (Noyes et al. 2019) in order to transfer this knowledge to fish.
Reviewer 4	159	Event 281 Thyroxine (T4) in serum, decreased	*In evidence for perturbation by stressor:* information on methimazole could be harmonized to the ones on PTU and Perchlorate *In taxonomy availability:* -the scientific name for "Pig" should be Sus Scrofa in the table. -First paragraph page 15. I suggest to add "As such extrapolation regarding TH action across species **and developmental stages** should be done with caution." -Second paragraph page 15. I feel that this long paragraph focusing on TH transport is useless here.
Reviewer 4	159	Event 1003 Decreased, Triiodothyronine (T3) in serum.	There is a need to harmonize Event 1003 and 281, starting with the titles, I would expect 1003's title to be named "Triiodothyronine (T3) in serum, decreased" following 281 title or the titles to be harmonized in the other way. The key event description for T3 decrease is very detailed compared to the one for T4 decrease (even non genomic actions of the hormones are considered). Many information given in the T3 event are true for both T3 and T4 and I wonder to which extent both event description should reach the same level of details or being harmonized. In "how it is measured or detected": To my knowledge ELISA is difficult to use for aquatic organisms and RIA is the more reliable method to date. I would suggest to change the last sentence to "Amongst all these methods RIA is particularly repeatable and reproducible."
Reviewer 7	159	Key event 1003: Decreased, triiodothyronine (T3) in serum	As mentioned above, the key event description is predominantly based on studies in humans or mammals. Moreover, no particular focus is put on T3 serum levels, but rather on T4 as well as T3. Are any studies available showing that decreased T4 serum levels induce decreased T3 concentrations in fish? I miss a description of processes involved in decreased T3 serum levels as a consequence of decrease serum T4 levels.
Reviewer	All	Event 1003:	This is a central KE within the network.

2		Decreased, Triiodothyronine (T3) in serum	The taxonomic applicability section of this KE indicates only three species (two fish, one amphibian). However, it appears that this KE can occur, in principle, in any vertebrate species that uses T3. Should the list of species be based on experimental evidence or plausibility assessment? Is there any larger taxonomic category that could be used instead of (or in addition to) single species? The first paragraph of the description of the taxonomic applicability provides information on THs binding proteins and the influence of this process on THs' half-life. Information is provided for rat and human, but not for the species listed in the same KE. Are THs binding proteins also conserved in fish and amphibians?
Reviewer 4	159	Event: 1007: Reduced, Anterior swim bladder inflation Short Name: Reduced, Anterior swim bladder inflation Key	In taxonomic applicability : Striped trumpeter Latris lineata is a typical transient physostome (physostomous as larvae and physoclistous as adults transient physostomes). Adult striped trumpeter is further defined as euphysoclistous, having a swim bladder with dual-chambers separated by a diaphragm. As Trotter et al. studied swim bladder malformation in this species, it may be interesting to investigate further to see if it could be part of the taxonomic applicability.
Reviewer 4	159	Event 1005 Reduced Swimming performance	In life stage "Importance of swimming performance for natural behavior is generally applicable across all life stage". I suggest to modify to "all post-embryonic life stages" In references: references for fathead minnow are needed here in how it is Measured or detected: The following reference could be cited: Little, E.E., Finger, S.E., 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environ. Toxicol. Chem.
Reviewer 7	159	Key event 1005: Reduced, swimming performance	The authors should cite some additional literature besides their own publications with regard to tracking systems for measuring swimming performance.
Reviewer 2	All	Event 1005: Reduced, Swimming performance	This KE description is very brief. That is ok, but the authors may consider to further develop the description in future development efforts. In the current version, it is not fully clear what swimming performance means. The meaning is partially explained in the "How it is measured" section, but I think it would be helpful to provide an explicit definition of swimming performance also in the KE description.
Reviewer 4	159	Event 351 Increased mortality	In taxonomic applicability: Zebrafish, Chicken and Fathead are listed in the taxonomy table but "All living things are susceptible to mortality" is mentioned in the text. There is a need for harmonization between the text and table, I would suggest to indicate "All species" in the table. There is no reference for this KE, the need for references should be considered.
Reviewer 2	All	Event 351: Increased mortality	The relevance of the list of species indicated in the taxonomic applicability domain is unclear. The list includes two fish species as well as Gallus gallus. I think this is a general problem linked to the re-use of

			KEs, but I wonder whether and how it can be fixed. The list should only indicate "All species". The "How it is measured section" seems to be related to fish only (e.g., it mentions gill movements, mesocosms, ponds, capture-tag-recapture(?)). As this KE is applicable to any living being, an alternative strategy would be to use a general biological definition of death plus a general definition of mortality rate. This issue will not affect the present AOPs (which are developed for fish species), hence I am not sure who should be responsible for the amendment of the KE. For example, the same KE has recently been used for AOP 320, which is related to Covid-19 and mortality in humans.
Reviewer 4	159	Event 360 Decreased Population trajectory	The taxonomic applicability indicates "All species" but the KE description and the only cited reference refer to Fish, the need for references for other species should be considered.
Reviewer 7	159	Key Event Relationship 309: Thyroperoxidase, inhibition leads to TH synthesis, decreased	In the evidence supporting this KER, the authors should also cite the respective studies in fish. In "Uncertainties and Inconsistencies", the authors report the results of a study on genistein to be inconsistent with the expected outcome of TPO inhibition. Although apparently a TPO inhibition of 80% was observed in this study, it is worth noting that genistein is far from being a specific TPO inhibitor. Thus, the observed inconsistency may be a result from feedback mechanisms of its primary mode of action. Also, in "Response-response relationship", the authors mention genistein as a TPO inhibitor. This statement needs to be tempered. It should be critically stated that e.g. data about a quantitative understanding mainly result from a study in Xenopus laevis (Haselman et al., 2020), as this AOP is supposed to be developed in fish. Transferring results generated in amphibians to fish requires a certain degree of read-across.
Reviewer 4	159	Relationship 309: Thyroperoxidase inhibition leads to TH synthesis, decreased	In Life stage, line 4: "As a results TPO inhibition is not expected to decrease TH synthesis during these early stages". I suggest to modify "TH synthesis" to "TH total amount" because of the absence of synthesis in at these early developmental stages. In empirical evidence, line 7 "Tietge et al (2010) recently showed", "recently" could be omitted considering the year of publication. In temporal evidence: "The temporal nature of this KER is applicable to all life stages, including development". I suggest to add: "but excluding early developmental stages relying on maternal transfer of TH." Alternatively the whole sentence could be removed as this information is already in the "life stages" section.
Reviewer 4	159	Relationship 309: TH synthesis leads to T4 in serum, Decreased	In temporal evidence: "The temporal nature of this KER is applicable to all life stages, including development". I suggest to add: "but excluding early developmental stages relying on maternal transfer of TH." There is no reference for Fish in this KER, publications such as Nelson 2016 and Stinckens 2016 could be added showing the decrease in T4 concentration following TPO inhibition. Some empirical evidence

			taken from Relationship 2038 could be used in this KER: "A decrease in whole-body T4 and T3 was observed in zebrafish exposed to methimazole from fertilization until the age of 21 and 32 days and to propylthiouracil until the age of 14, 21 and 32 days (Stinckens et al., 2020). Additionally, a strong correlation was observed between T4 and T3 levels. Both compounds are thyroperoxidase inhibitors expected to inhibit thyroid hormone synthesis. While T4 measurements could not be acquired in fathead minnows exposed to 1 mg/L 2-mercaptobenzothiazole, a thyroperoxidase inhibitor, for 14 days, a significant decrease in T3 was observed (Nelson et al., 2016). The decreased T3 levels were likely the result of reduced T4 synthesis."
Reviewer 4	159	Relationship 2038: T4 in serum, decreased leads to decreased T3 in serum	In taxomic: This KER was apparently first written for the fish AOP 159, it leads to this surprising statement: "The evidence for a relationship between circulating T4 and T3 levels currently comes from work on zebrafish and fathead minnow". It is obviously true in the context of the AOP 159 but not historically. I feel that a more general wording would be more appropriate such as "The evidence for a relationship between circulating T4 and T3 levels is present in many species including human, rat, xenopus, zebrafish and fathead minnow". One or several review paper on thyroid hormones could be cited here to support this statement.
Reviewer 7	159	Key event relationship 2038: T4 in serum, Decreased leads to Decreased, Triiodothyronine (T3) in serum	In "Uncertainties and Inconsistencies", the authors mention that this KER depends on the MIE that is causing the decrease in T3. If this is the case, the authors should include this KER only, in case it has been observed as a consequence of TPO inhibition. Based on the cited studies by Stinckens et al., 2016 and Crane et al., 2006, TPO inhibition may not necessarily result in reduced T3 levels, even though T4 levels were decreased.
Reviewer 4	159	Relationship: 1035: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Anterior swim bladder inflation.	In empirical evidence: a publication on striped bass is cited. Does this species should be then added to the taxonomic applicability (with a low evidence considering the sole publication cited)? In Uncertainties and Inconsistencies: the authors highlight that "inflation upon disruption of the thyroid hormone system is in most cases, but not always, accompanied by reduced whole body T3 levels" and that "The mechanism underlying the link between reduced T3 and reduced anterior chamber inflation remains unclear" several hypotheses are listed including effect on development, WNT or hedgehog signaling, etc. I would recommend to state that a possibility for the observed inconsistencies is that the tested compound act non only on thyroid axis but also in parallel directly on another target known to be linked to swim bladder inflation such as autophagy, ROS, cardiac function. For example, 2-mercaptobenzothiazole beside its action on TPO is known to induce an elevation in elevation in reactive oxygen species (ROS) levels in fish cells (Zeng 2016). This hypothesis could explain how swim bladder could be affected while T3 concentration remains constant. Alternatively, temporality between T3/T4 dosage (assessed at 32dpf and 120hpf), the moment when there is a need for T3 to inflate the swim bladder (unknown but probably in between 32dpf and 120hpf) and the observation of the phenotype (32dpf), could lead to the hypothesis that T3 concentration was reduced in between the two dosages. In Uncertainties and Inconsistencies: I would advise to add a bullet point explaining that it is unclear which aspect of swim bladder development and inflation is affected by TH disruption. This is present in

			the KER 1027 (Decreased T3 leads to reduced posterior swim bladder) and could be adapted for this KER.
Reviewer 7	159	Key event relationship 1035: Decreased, triiodothyronine (T3) in serum leads to reduced, anterior swim bladder inflation	This is one of the most important key event relationships in the AOP, as it connects the molecular and the physiological level. The texts provided for key event description and biological plausibility are identical. In "Uncertainties and Inconsistencies", the authors should cite the corresponding literature with regard to the potential mechanism underlying the link between reduced T3 and reduced anterior chamber inflation and they should update the corresponding references (Reinwald et al. 2021).
Reviewer 4	159	Relationship: 1034: reduced anterior swim bladder inflation leads to reduced swimming performance	In empirical evidence: the following reference showing a reduced swimming performance could be added: Lihua Yang, Emma Ivantsova, Christopher L Souders, Christopher J Martyniuk. The agrochemical S-metolachlor disrupts molecular mediators and morphology of the swim bladder: Implications for locomotor activity in zebrafish (Danio rerio)/ Ecotoxicol Environ Saf 2021 Jan 15;208:111641. doi: 10.1016/j.ecoenv.2020.111641
Reviewer 4	159	Relationship: 2212: reduced swimming performance leads to increased mortality	There is only one reference and focusing on reduced swimming performance linked to swim bladder defect. Swimming performance could be affected by a lot of stressors or mutation, I wonder if more references could be added to support this KER but no necessary linked to swim bladder. Regarding taxonomic applicability, only zebrafish is in the table but the text mentioned "generally applicable to all hatched fish". There is probably a need to harmonize here.
Reviewer 7	159	Key event relationship 2212: Reduced, swimming performance leads to increased mortality	The texts for evidence supporting this KER and empirical evidence of this KER are identical.
Reviewer 2	All	Relationship: 2212: Reduced, Swimming performance leads to Increased Mortality	I do agree with the authors that this relationship is plausible but difficult to quantify, as it is influenced by a high number of confounding factors.
Reviewer 4	159	Relationship: 2013: Increased mortality leads to decrease population trajectory.	Regarding taxonomic applicability, only zebrafish and fathead are in the table but the text mentioned "all organism must survive…" "consideration made above are applicable to other fish species". There is probably a need to harmonize here. Most of the reference cited in the KER focused on fish rather that especially on zebrafish or fathead, supporting the possibility to enlarge the taxonomic applicability.
Reviewer 2	All	Relationship: 2013: Increased Mortality leads to Decrease,	I am not a big supporter of the use of population effects within AOPs, unless population effects are ecologically plausible. The upstream relationship (2212, leading to increased mortality) is not characterised from a quantitative perspective. Consequently, no modelling approach can be used to

		Population trajectory	predict population effects in KER 2013. I believe that such extrapolation is too weak. This is only my personal opinion of course.
Reviewer 7	159	Weight of evidence:	Yes, the weight-of-evidence judgement/scoring is well described and justified. However, in particular for the early (molecular) key events and their relationships the references in large parts describe studies in humans, mammals or amphibians. Therefore, weight-of-evidence for these key events and relationships in fish requires read-across. Another critical point is the limitation in highly specific TPO inhibitors. The vast majority of studies supporting this AOP applied mainly three TPO inhibitors, namely methimazole, 2-mercaptobenzothiazole and 6-PTU. In mammals (in which most of these studies have been performed), the latter chemical has been shown to also inhibit Deiodinases, besides it's activity on TPO. Therefore, most relationships and conclusions are drawn from studies with a very limited number of specific TPO inhibitors, which may be critical as the AOP should not be substance-specific, but MIE-specific. The authors should point out relevant studies applying specific TPO inhibitors, particularly in fish. Further evidence can also be provided by genomic studies, which have been published rather recently.
Reviewer 4	159	WoE Relationship 309: Thyroperoxidase inhibition leads to TH synthesis, decreased	In AOP referencing relationship: the weight of evidence for this KER is high for AOP 159 but only "moderate" for the AOP "Inhibition of TPO leading to impaired fertility in fish". The quantitative understanding is also different despite that the two AOP are applicable to fish. I would recommend to align the weight of evidence to "high" considering the existing publications on zebrafish such as Raldua 2009, Thienpont 2011, Reheberger 2018 linking TPO inhibition to T4 synthesis in the thyroid gland. I feel that the quantitative understanding could be align to low considering the few available quantitative data for fish.
Reviewer 4	159	WoE Relationship: 1035: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Anterior swim bladder inflation.	The quantitative understanding has been rated "moderate" for this KER and "low" for the Relationship: 1027: Decreased, Triiodothyronine (T3) in serum leads to Reduced, Posterior swim bladder inflation: I wonder if this difference of rating is really funded and suggest to harmonize.
Reviewer 4	159	WoE Relationship: 2013: Increased Mortality leads to Decrease, Population trajectory	The WOE is different for this KER in the AOP "Acetylcholinesterase inhibition leading to acute mortality" compared to the others AOP. I wonder if it should be harmonized, to my point of view, the KER WOE should be independent from each AOP.
Reviewer 4	159	CQ3. Additional observations:	There is an important heterogenicity in the writing of each KE or relationship. One could clearly see for the KE shared by multiple AOPs that authors from different AOP worked on a given KE, creating a diversity in the style, level of details and some redundancies that could complicate the understanding. I wonder if I some point the AOPs in general will need some editors that will harmonize the content and

			style between each MOI, KE and AO.
Reviewer 8	159	Weight of evidence	In general, yes the weight-of-evidence judgement and scoring is well described and justified. As mentioned in the general comments on the set of AOPs, the weakest evidence is present for how the effects on the swim bladder driven by the reduction in T3 translate to reduced swimming performance. This is reflected in the scoring given in to the individual KERs.

Annex 3: Individual reviewers' comments grouped by charge question, with initial responses from authors

Scientific quality

Comment	Response
• Stressor: Harmonize MIE and AOP page; Is it possible to list more chemicals that have been tested for the effects on swim bladder in fish?	• When a stressor is known to target the MIE of an AOP, this does not necessarily mean that there is evidence for the perturbation of every KE along the AOP. Adding stressors was not our focus during AOP development.

• Include all relevant literature on role of TH in fish
• Add references
  ○ Add specific references
  ○ Add references besides your own
	• The development of these AOPs was mainly based on a series of dedicated experiments in zebrafish and fathead minnows that form the core of the empirical evidence. Specific literature searches were used to add evidence from other studies, mainly in zebrafish and fathead minnows. No systematic review approach was applied and the aim was not to cite all the relevant literature. We aimed at including as much literature as needed to support the AOPs. We could include a methods description to clarify this.
	• Some literature on fish will be added where it is currently limited to other vertebrates and it will be highlighted as relevant to fish. Some of the suggested references will also be included.

▪ Harmonize/expand taxonomic applicability
  ○ Taxonomic applicability domain is extremely narrow; How many other AOPs are species specific?
  ○ List every species that is mentioned in empirical evidence under taxonomic applicability
  ○ Harmonize text in domain of applicability with table listing species
  ○ Add references on other species if taxonomic applicability indicates "all species"
  ○ Should the list of species be based on experimental evidence or plausibility assessment? Is there any larger taxonomic category that could be used instead of (or in addition to) single species? (e.g. KE1003 T3)
  ○ Use "All species" instead of single species
  ○ KE351: "How it is measured section" seems to be related to fish only. As this KE is applicable to any living being, an alternative strategy would be to use a general biological definition of death plus a general definition of mortality rate.
  ○ Requests to add specific species: e.g., Medaka
	▪ Harmonization of the table listing taxonomic applicability, the text under domain of applicability and empirical evidence is not necessarily the goal. Very often AOP development is based on one or a few key species. In this case, strongest evidence is available for zebrafish and fathead minnow. Information on additional taxa can be added later in the spirit of collaborative AOP development.
	▪ Nonetheless, we will double check the taxonomic applicability sections and evaluate which changes can be made.

▪ Impaired swim bladder inflation is not TH-specific ▪ T3 measurements are highly variable and can be unreliable, don't think the evidence is strong enough to suggest addition of T3 measurements to the FET or FELS	▪ Add to abstract: Other mechanisms can contribute to impaired swim bladder inflation. ▪ Add to 'potential applications of AOP': Thyroid hormone system disruption causes multiple unspecific effects. Addition of TH measurements, although they can be variable, 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.
▪ MIEs DIO1 and DIO2 ○ Aggregate DIO1 and DIO2 or leave out DIO1	▪ Assays are available to **measure** the DIO isoforms separately. This is our main reason to split the isoforms.

○ Define MIE as specific enzymatic reaction catalyzed by DIO instead of DIO1/DIO2, e.g., T4→T3 ○ Chemicals often target more than one DIO isoform	▪ At this point there is uncertainty regarding DIO1 vs DIO2 importance which is highlighted in several places in the AOPs. Keeping DIO1 and DIO2 as separate MIEs allows for better definition as more knowledge becomes available in the future. ▪ Need to consider re-use and cross-species AOP network development. Distinct AOPs for DIO isoforms are also under development in amphibians. ▪ It is true that chemicals often target more than one DIO isoform. Chemicals often target multiple MIEs. This is not contradictory with the AOP framework. ▪ Splitting up in MIEs per catalyzed reaction: current assays are not aiming at distinguishing the different reactions, but rather at distinguishing the different isoforms.
• Information on fish is limited in early KEs and KERs, especially those that have been initially developed for mammals	• We will add more information on fish to KE277, KE279, KE281, KE1002, KE1003, KE1009, KER309, KER305, KER1026 and make it more visible.
• Refine sex applicability ○ Add comment on sex differentiation in fathead minnow ○ Sex differences are investigated in species with sex markers ○ Change evidence for unspecific sex to moderate	• We will refine sex applicability in all AOPs: add a comment for fathead minnow and species with sex markers. • We will change the WoE call for unspecific sex from high to moderate.
• Refine life stage applicability: Swimming performance is applicable to post-embryonic life stages)	• We will refine life stage applicability in KE1005.
• Refine “uncertainties and inconsistencies” ○ Oxidative stress among other mechanisms may contribute to impaired swim bladder inflation ○ KER2038 T4→T3: TPO inhibition may not necessarily result in reduced T3 levels, even though T4 levels were decreased ○ KER2213: section is absent	• Refine “uncertainties and inconsistencies” sections across AOPs ○ Add other contributing mechanisms + need to measure several KEs to evaluate thyroid hormone system disruption ○ It is not a general criterion that the downstream KE should always occur when the upstream KE occurs, this can be dose and time dependent.
• Improve description	• We will improve the descriptions of KE277, KE351, KE1003, KER2038
• RIA is the more reliable method to date	• KE 1003: We will refine the section “how it is measured or detected”.
• Refine applicability of the AOP: The AOP 158 might be the less relevant one with regard to applicability	• We will refine the section ‘applicability of AOPs’ in AOP 158 to highlight potential differences in relevance compared to other AOPs (DIO1 vs DIO2, posterior vs anterior)
• KE1005 swimming performance: isn’t this an AO already?	• We agree that this is debatable. We chose to limit AOs to outcomes that are considered of direct regulatory relevance in ecotoxicology.
• Suggestions for small modifications	• We will implement small modifications.

Weight of evidence

Comment AC: anterior chamber, PC: posterior chamber	Response
	• General: We will re-evaluate these WoE calls in light of the reviewer comments

• KER1034 AC→swimming
  ○ Evidence is weak, no distinction between effect on AC or entire swim bladder system, exposure concentration high (87)
  ○ Add uncertainties regarding other effects that may have influenced swimming performance (Rev8)
	• KER1034: The swim bladder system is indeed affected. Since the larger PC does not alleviate the effect on swimming, an effect of AC on swimming is plausible. Therefore we opted for defining the available evidence as moderate, i.e., biologically plausible with some evidence.
	• We will address this uncertainty in the text: effects of AC not necessarily distinguished from PC effects
	• Sublethal exposure concentrations were selected where the PC inflates.
	• Add uncertainties regarding other effects that may have influenced swimming performance

• KER1035 T3→AC evidence is weak: only one study started exposure after PC inflation, no evidence of primary effect. Change to moderate for zebrafish. (86)	• KER1035: For evaluation of AC inflation, concentrations were selected where the PC inflated. We will refine the description of evidence to highlight the difference in experimental setup: two studies with larval exposure (Nelson et al., 2016; Cavallin et al., 2017); one study with continuous exposure (Stinckens et al., 2020). Additionally, Stinckens et al. (2020, supplementary information) established a very convincing linear quantitative relationship between reduced T3 levels and reduced anterior chamber volume (measured as surface in 2D images) in 32 day old zebrafish across three compound exposures. Therefore we opted for defining the available evidence as moderate, i.e., biologically plausible with some evidence. • The latter quantitative relationship in zebrafish was the basis to select high level of evidence for zebrafish.

• Essentiality AOP156 DIO2-anterior: moderate is not fully supported (46)
• Essentiality AOP158 DIO1-anterior change moderate to low
  ○ DIO1 low
  ○ anterior chamber low: secondary to posterior chamber? Systemic toxicity?
  ○ Swimming performance at larval stage low
  ○ Dual oxidase knockdown as indirect evidence: DUOX also plays a role in oxidative stress
	• Although there is less convincing and indirect evidence for essentiality of inhibition of DIO and reduced T3 levels for AC inflation compared to PC inflation, we do have direct evidence for essentiality of reduced AC inflation for reduced swimming performance. We believe that moderate 'Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE' is a better call than low 'No or contradictory experimental evidence of the essentiality of any of the KEs'.
	• We will refine the text to include the uncertainties that have been raised.

• Essentiality AOP159 TPO-anterior ○ Dual oxidase knockdown as indirect evidence: DUOX also plays a role in oxidative stress	• The DUOX knockdown (Chopra et al., 2019) is used as evidence for the essentiality of KE277 'TH synthesis decreased'. Chopra et al. specifically included evidence of decreased TH synthesis. In the overall evaluation of the essentiality of KEs in AOP 159 this is referred to as indirect evidence. • We will add the fact that DUOX also plays a role in oxidative stress to uncertainties and inconsistencies on the relevant pages.
• KER2013 mortality→population: harmonize with AOP on AChE inhibition • Disagree with limited evidence	• KER2013: Agreed to change evidence from high to moderate
• KER309 TPO→TH: change evidence from moderate to high	• KER309: Agreed to change evidence from moderate to high
• KERs1027 (T3→PC)-1035(T3→AC): Is there an actual difference between evidence?	• KERs1027-1037: there is indeed less data for T3-PC compared to T3-AC
• KER1028 PC→swimming: evidence is moderate, limited ecological relevance of the methods used to quantify behavior, could be increased by tailored behavioural tests	• Agreed that evidence could be improved in the future

• Refine KER1027 T3→PC: link uncertain, add impaired muscle development	• KER1027: We will add impaired muscle development

Other considerations, including related to general AOP concepts

Comment	Response

• Submit as one AOP network containing all TH-related MIEs/KEs/AOs
  ○ Inappropriate to split up in large number of AOPs; growing number of AOPs with different MIEs could be expected; AOPs give the impression that there is only 1 MIE per AOP; submit one AOP network highlighting specific differences
  ○ Why not look at yolk resorption, pigmentation?
  ○ Add impaired muscle development as KE
  ○ Not a supporter of adding population level effects to AOPs, no data available for modelling
	• An AOP does not intend to be complete in terms of literature nor to include all MIEs, KEs, species, stressors The approach to development and description of these AOPs follows the guidance in the Users Handbook (see page 47) where it is indicated that including AOPs with different MIEs on a single AOP page is not encouraged.
	• This AOP network is already quite complicated and includes the MIEs for which most evidence is available. AOPs with additional MIEs can be developed later.
	• Focusing on yolk resorption or pigmentation would lead to the development of different AOPs. These could be developed in the future.
	• Currently, there is not enough evidence for addition of muscle development as a KE, but available info will be added.
	• In ecotoxicology, the population level is considered of highest regulatory relevance. As reviewer 5 (34) indicates, the link between mortality and population size has been described in other contexts such as fisheries research. If quantitative data in the current, specific context becomes available later, modelling can be applied to better support this relationship.

• Heterogenicity of KEs/KERs
  ○ Heterogenicity in writing of shared versus unique KEs/KERs
  ○ Harmonize KE descriptions in terms of level of detail
  ○ Remove part on TH transport in KE281 because irrelevant
	• Need to consider collaborative AOP development and re-use of KEs/KERs
	• Cannot remove parts of endorsed AOPs (KE281)

• WoE calls should be independent of the AOP, harmonize calls across AOPs	• AOP pages allow for AOP-specific WoE evaluation which is sometimes needed to include differences, e.g., between taxa.

Annex 4: Revision summary document detailing changes that have been made to the AOPs in response to the reviewer comments

Table of Contents

Introduction

We would like to thank the reviewers for their massive efforts and enthusiasm in reviewing these AOPs and for their insightful comments that have greatly improved this set of AOPs.

The reviewer comments were first categorized and grouped and general responses were formulated to facilitate discussion. This resulted in a summary of comments and responses. In this revision document, the structure of the summary is used to detail the changes that have been made to the AOPs in response to the reviewer comments. Below, each of the general responses is shown first, followed by the changes that have been made in the relevant AOP-wiki pages in response. The responses have been further grouped into a first section including all responses that have been discussed during the scientific review meeting on 15 July 2021 where the author team as well as the reviewers and the review managers participated, and a second section including all remaining comments.

In order to allow easy distinction between the original text and the changes that have been made, changes are marked in red.

Section 1: Comments discussed during the scientific review meeting of 15 July 2021

1. Taxonomic domain of applicability

☐ Refine taxonomic applicability ○ Should the list of species be based on experimental evidence or plausibility assessment? Is there any larger taxonomic category that could be used instead of (or in addition to) single species? (e.g. KE1003 T3)	As a consequence of how the present AOP network was developed, the strongest evidence is available for zebrafish and fathead minnow. We will double check the taxonomic applicability sections and evaluate whether we have enough confidence to add other fish species to the tables. There are recognized limitations in the current guidance related to description of domain of applicability using the structured fields and free text of the AOP-Wiki. We agree that a broader description of the taxonomic applicability domain based on biological and phylogenetic plausibility would be a very welcome addition to AOP descriptions in the AOP-wiki. Updates to the AOP-wiki to accomplish this are currently under development. For the time being, we will mention a broader "plausible domain of applicability" in the free text field under domain of applicability for selected cases and we will update the AOP-wiki as soon as the relevant formal procedures become available. We would welcome any relevant specific information in this respect from the review team, should they have such information available.
Summary of response: Evidence for taxonomic applicability broader for upstream events Strongest evidence available for 2 fish species for downstream events Add plausible domain of applicability in text field T3: vertebrates Anterior chamber: physostomous fish	

☐ Changes made to KE1003

Domain of applicability

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 Isihara, 2009). Specifically, the majority of supporting data for TH decreases in serum come from rat studies, and the predominant iodothyronine binding protein in rat serum is transthyretin (TTR). TTR demonstrates a reduced binding affinity for T4 when compared with thyroxine binding globulin (TBG), the predominant serum binding protein for T4 in humans. This difference in serum binding protein affinity for THs is thought to modulate serum half-life for T4; the half-life of T4 in rats is 12-24 hr, wherease the half-life in humans is 5-9 days (Capen, 1997). While these species differences impact hormone half-life, possibly regulatory feedback mechanisms, and quantitative dose-response relationships, measurement of serum THs is still regarded as a measurable key event causatively linked to downstream adverse outcomes.

THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in amphibian and larbean metamorphoses ~~is well established~~ (Manzon and Youson, 1997; Yaoita and Brown, 1990) 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 differrent animal and plant kingdoms (Eales, 1997; Heyland and Moroz, 2005), while their role as environmental messenger via exogenous routes in echinoderms confirms the hypothesis that these molecules are widely distributed among the living organisms (Heyland and Hodin, 2004). However, the role of TH in the different species may differ depending on the expression or function of specific proteins (e.g receptors or enzymes) that are related to TH function, and therefore extrapolation between species should be done with caution.Changes made to KE1007: anterior chamber

References added

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

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.

- Changes made to KE1007

Domain of applicability

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). The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2016; Nelson et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020). While zebrafish and fathead minnows are physostomous fish with a two-chambered swim bladder, the Japanese rice fish (Oryzias latipes) is a physoclistous fish with a single chambered swim bladder that inflates during early development. The key event 'reduced anterior chamber inflation' is not applicable to such fish species. Therefore, the current key event is plausibly applicable to physostomous fish in general.

- Changes made to KE279

Domain of applicability

Taxonomic: This KE is plausibly applicable across vertebrates. TPO inhibition is a MIE conserved across taxa, with supporting data from experimental models and human clinical testing. …

- Changes made to KE 277

Domain of applicability

Taxonomic: This KE is plausibly applicable across vertebrates. Decreased TH synthesis resulting from TPO or NIS inhibition is conserved across vertebrate taxa, with in vivo evidence from humans, rats, amphibians, some fish specis, and birds, and in vitro evidence from rat and porcine microsomes. Indeed, TPO and NIS mutations result in congenital hypothyroidism in humans (Bakker et al., 2000; Spitzweg and

Morris, 2010), demonstrating the essentiality of TPO and NIS function toward maintaining euthyroid status. Though decreased serum T4 is used as a surrogate measure to indicate chemical-mediated decreases in TH synthesis, clinical and veterinary management of hyperthyroidism and Grave's disease using propylthiouracil and methimazole, known to decrease TH synthesis, indicates strong medical evidence for chemical inhibition of TPO (Zoeller and Crofton, 2005).

- Changes made to KER309

Domain of applicability

Taxonomic: This KER is plausibly applicable across vertebrates. Inhibition of TPO activity is widely accepted to directly impact TH synthesis. This is true for both rats and humans, as well as some fishes, frogs and birds. Most of the data supporting a causative relationship between TPO inhibition and altered TH synthesis is derived from animal studies, in vitro thyroid microsomes from rats or pigs, and a limited number of human ex vivo (Nagasaka and Hidaka, 1976; Vickers et al., 2012) and clinical studies. There are data to support that gene mutations in TPO result in congenital hypothyroidism, underscoring the essential role of TPO in human thyroid hormone synthesis.

- Changes made to KE 281

Domain of applicability

Taxonomic: This KE is plausibly applicable across vertebrates and ~~T~~the overall evidence supporting taxonomic applicability is strong. THs are evolutionarily conserved molecules present in all vertebrate species (Hulbert, 2000; Yen, 2001). Moreover, their crucial role in zebrafish development, embryo-tolarval transition and larval-to-juvenile transition (Thienpont et al., 2011; Liu and Chan, 2002), and amphibian and lamprey metamorphoses is well established (Manzon and Youson, 1997; Yaoita and Brown, 1990; Furlow and Neff, 2006). Their existence and importance has also been 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 depends on the expression and function of specific proteins (e.g receptors or enzymes) under TH control and may vary across species and tissues. As such extrapolation regarding TH action across species should be done with caution.

- Changes made to KER 305

Domain of applicability

Taxonomic: This KER is plausibly applicable across vertebrates. While a majority of the empirical evidence comes from work with laboratory rodents, there is a large amount of supporting data from humans (with anti-hyperthyroidism drugs including propylthiouracil and methimazole), some amphibian species (e.g., frog), fish species (e.g., zebrafish and fathead minnow), and some avian species (e.g, chicken). The following are samples from a large literature that supports this concept: Cooper et al. (1982; 1983); Hornung et al. (2010); Van Herck et al. (2013); Paul et al. (2013); Nelson et al. (2016); Alexander et al. (2017); Stinckens et al. (2020).

- Changes made to KER 366

Domain of applicability

Taxonomic: Use of TPO inhibitors as anti-hyperthyroidism drugs in humans and pets (Emiliano et al., 2010; Trepanier, 2006) and effects of these drugs on serum TH concentrations in rats (US EPA, 2005), amphibian, fish and avian species (Coady et al., 2010; Grommen et al., 2011; Nelson et al., 2016; Rosebrough et al., 2006; Stinckens et al.; 2020; Tietge et al., 2012), strongly supports a causative linkage between inhibition of TPO and decreased serum T4 across vertebrate species. Therefore, this KER is plausibly applicable across vertebrates.

- Changes made to KER 2038

Domain of applicability

Taxonomic: Thyroid follicles mainly produce T4 and to a lesser extent T3 across vertebrates. When serum T4 levels are decreased, less T4 is available for conversion to the more biologically active T3. This key event relationship is not always evident. This could be due to feedback/compensatory mechanisms that in some cases seem to be able to maintain T3 levels even though T4 levels are reduced, for example through increased conversion of T4 to T3 by deiodinases. These feedback mechanisms can also differ across species. Therefore, although this KER is plausibly applicable across vertebrates, variation can be expected. In zebrafish and fathead minnow, several studies reported~~The~~ evidence for a relationship between circulating T4 and T3 levels (Nelson et al., 2016; Stinckens et al., 2020, Wang et al., 2020) ~~currently comes from work on zebrafish and fathead minnow~~.

- Changes made to KER 1035

Domain of applicability

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

- Changes made to KER 1039

Domain of applicability

Taxonomic: Teleost fish can be divided in two groups according to swim bladder morphology: physoclistous (e.g., yellow perch, sea bass, striped bass) and physostomus (e.g., zebrafish and fathead minnow). Physostomus fish retain a duct between the digestive tract and the swim bladder during

adulthood allowing them to gulp air at the surface to fill the swim bladder. In contrast, in physoclistous fish, once initial inflation by gulping atmospheric air at the water surface has occurred, the swim bladder is closed off from the digestive tract and swim bladder volume is regulated by gas secretion into the swim bladder (Woolley and Qin, 2010). The evidence for impaired inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow (Stinckens et al., 2016; Nelson et al., 2016; Cavallin et al., 2017; Godfrey et al., 2017; Stinckens et al., 2020). While zebrafish and fathead minnows are physostomous fish with a two-chambered swim bladder, the Japanese rice fish (Oryzias latipes) is a physoclistous fish with a single chambered swim bladder that inflates during early development. This KER is not applicable to such fish species. Therefore, the current key event is plausibly applicable to physostomous fish in general.~~The evidence for a relationship between circulating T4 levels and inflation of the anterior chamber of the swim bladder currently comes from work on zebrafish and fathead minnow.~~

References added

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

- Changes made to KE 1002

Domain of applicability

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 ~~using zebrafish~~. Stinckens et al. (2018) showed that chemicals with DIO inhibitory potential in pig liver impaired swim bladder inflation in zebrafish, a thyroid hormone regulated process. Based on these results, DIO2 seemed to be more important than DIO1. 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).

References added

Walpita CN, Grommen SV, Darras VM, 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(1):18-25.

- Changes made to KE 1009

Domain of applicability

Taxonomic: Deiodination by DIO enzymes is known to exist in a wide range of vertebrates and invertebrates. Therefore, this KE is plausibly applicable across vertebrates. Studies reporting DIO1 inhibition have used human liver (Kuiper et al., 2006), human recombinant DIO1 enzyme (Olker et al., 2019), rat (Rattus norvegicus) liver (Klaren et al., 2005; Freyberger and Ahr, 2006; Kuiper et al., 2006; Pavelka, 2010) and thyroid gland (Ferreira et al., 2002), mouse (Mus musculus) brain (hernandez et al., 2006), hog (Sus scrofa domesticus) liver (Stinckens et al., 2018), sheep (Ovis orientalis aries) fetal hepatic, renal and perirenal adipose tissue (Forhead et al., 2006), tadpole (Xenopus laevis) liver (Kuiper et al., 2006), fathead minnow (Pimephales promelas) whole fish (Noyes et al., 2011), Nile tilapia (Oreochromis niloticus) liver (Walpita et al., 2007), Gilthead Seabream (Sparus aurata) kidney (Klaren et al., 2005), and killifish (Fundulus heteroclitus) liver (Orozco et al., 2003) among others. The latter teleostean DIO1 enzymes as well as amphibian enzymes differ from other vertebrate DIO1 enzymes in their lower sensitivity to propylthiouracil (PTU), a typical DIO1 inhibitor in mammals.

Deiodinase 1 in liver is the main supplier of T3 to circulation in mammals (Marsili et al., 2011), and the same appears to be true for birds. By contrast, DIO1 function in teleostean and amphibian T3 plasma regulation is less clear (Finnson et al. 1999, Kuiper et al. 2006). The presence of DIO1 in the liver of teleosts has been a controversial issue, and both the high level of DIO2 activity and its expression in the liver of teleosts are unique among vertebrates (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).

- Changes made to KER 1026

Domain of applicability

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.

- Changes made to KER 1037

Domain of applicability

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. It is generally assumed that deiodinase 1 in liver is the main supplier of T3 to circulation in mammals (Leonard et al., 1986), and the same appears to be true for birds (Freeman et al., 1991), while DIO2 is assumed to regulate intracellular concentrations of T3. In contrast to the general assumptions however, 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. By contrast, DIO1 function in teleostean and amphibian T3 plasma regulation is less clear (Finnson et al. 1999, Kuiper et al. 2006). The presence of DIO1 in the liver of teleosts has been a controversial issue, and both the high level of DIO2 activity and its expression in the liver of teleosts are unique among vertebrates (Orozco and Valverde, 2005). These differences make it difficult to exactly evaluate the importance of DIO1 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.

- Changes made to KE 1004

Domain of applicability

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 (e.g., Stinckens et al., 2018; Cavallin et al., 2017; Wang et al., 2020). Increasing evidence is becoming available on defects of swim bladder inflation in Medala (*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.

References added

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.

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.

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.

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.

- Changes made to KER 1027, KER 1042, KER 1044

Domain of applicability

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

References added

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

- Changes made to KE 1005

Domain of applicability

Taxonomic: Importance of swimming performance for natural behaviour is plausibly ~~generally~~ applicable to fish in general.

- Changes made to KER 2212

Domain of applicability

Taxonomic: 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.

- Changes made to KE 351

'all species' was added as taxonomic term and specific species were removed.

- Changes made to KE 360

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, ~~M~~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.

2. Considerations for potential applications of the AOP

Refine applicability of the AOP: The AOP 158 (DIO1-AC) might be the less relevant one with regard to applicability T3 measurements are highly variable and can be unreliable, don't think the evidence is strong enough to suggest addition of T3 measurements to the FET or FELS (Rev8)	☐ We will refine the section 'Considerations for Potential Applications of the AOP' in AOP 158 to highlight potential differences in relevance compared to other AOPs (DIO1 vs DIO2) ☐ To be added to 'Considerations for Potential Applications of the AOP': Thyroid hormone system disruption causes multiple unspecific effects. Addition of TH measurements, although they can be variable, 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".
Summary of response: 1. Suggest to focus applications on DIO2	
2. T3 levels Not that variable and highly predictive of effects in our datasets Add note/warning that more variability may be present in other studies Are suggested to be part of a battery of endpoints	

☐ Changes made to AOP 155

Considerations for Potential Applications of the AOP

A growing number of environmental pollutants are known to adversely affect the thyroid hormone system, and major gaps have been identified in the tools available for the identification, and the hazard and risk assessment of these thyroid hormone disrupting chemicals. Villeneuve et al. (2014) discussed the relevance of swim bladder inflation as a potential key event and endpoint of interest in fish tests. Knapen et al. (2020) provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. 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), ~~as well as~~ 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).

- Changes made to AOP 156

Considerations for Potential Applications of the AOP

A growing number of environmental pollutants are known to adversely affect the thyroid hormone system, and major gaps have been identified in the tools available for the identification, and the hazard and risk assessment of these thyroid hormone disrupting chemicals. Villeneuve et al. (2014) discussed the relevance of swim bladder inflation as a potential key event and endpoint of interest in fish tests. Knapen et al. (2020) provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. A suite of assays covering all the essential biological processes involved in the underlying toxicological pathways can be implemented in a tiered screening and testing approach for thyroid hormone disruption, using the levels of assessment of the OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals as a guide. Specifically, for this AOP, deiodinase inhibition can be assessed using an in chemico assay, measurements of T3 levels could be added to the Fish Embryo Acute Toxicity (FET) test (OECD TG 236), ~~and to~~ the Fish Early Life Stage Toxicity (FELS) Test (OECD TG210) and the Fish Sexual Development Test (FSDT) (OECD TG 234), and assessments of anterior chamber inflation and swimming performance could be added to the FELS Test and FSDT.

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- Changes made to AOP 157

Considerations for Potential Applications of the AOP

A growing number of environmental pollutants are known to adversely affect the thyroid hormone system, and major gaps have been identified in the tools available for the identification, and the hazard

and risk assessment of these thyroid hormone disrupting chemicals. Villeneuve et al. (2014) discussed the relevance of swim bladder inflation as a potential key event and endpoint of interest in fish tests. Knapen et al. (2020) provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. A suite of assays covering all the essential biological processes involved in the underlying toxicological pathways can be implemented in a tiered screening and testing approach for thyroid hormone disruption, using the levels of assessment of the OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals as a guide. Specifically, for this AOP, deiodinase inhibition can be assessed using an in chemico assay, measurements of T3 levels could be added to the Fish Embryo Acute Toxicity (FET) test (OECD TG 236), ~~and to~~ the Fish Early Life Stage Toxicity (FELS) Test (OECD TG210) and 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.

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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 AOP 155 may be more relevant for applications compared to the AOP that is described here.

- Changes made to AOP 158

Considerations for Potential Applications of the AOP

A growing number of environmental pollutants are known to adversely affect the thyroid hormone system, and major gaps have been identified in the tools available for the identification, and the hazard and risk assessment of these thyroid hormone disrupting chemicals. Villeneuve et al. (2014) discussed the relevance of swim bladder inflation as a potential key event and endpoint of interest in fish tests. Knapen et al. (2020) provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. A suite of assays covering all the essential biological processes involved in the underlying toxicological pathways can be implemented in a tiered screening and testing approach for thyroid hormone disruption, using the levels of assessment of the OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals as a guide. Specifically, for this AOP, deiodinase inhibition can be assessed using an in chemico assay, measurements of T3 levels could be added to the Fish Embryo Acute Toxicity (FET) test (OECD TG 236), ~~and to~~ the Fish Early Life Stage Toxicity (FELS) Test (OECD TG210) and the Fish Sexual Development Test (FSDT, OECD TG 234), and assessments of anterior chamber inflation and swimming performance could be added to the FELS Test and FSDT.

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

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 AOP 156 may be more relevant for applications compared to the AOP that is described here.

- Changes made to AOP 159

Considerations for Potential Applications of the AOP

A growing number of environmental pollutants are known to adversely affect the thyroid hormone system, and major gaps have been identified in the tools available for the identification, and the hazard and risk assessment of these thyroid hormone disrupting chemicals. Villeneuve et al. (2014) discussed the relevance of swim bladder inflation as a potential key event and endpoint of interest in fish tests. Knapen et al. (2020) provide an example of how the adverse outcome pathway (AOP) framework and associated data generation can address current testing challenges in the context of fish early-life stage tests, and fish tests in general. 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, thyroperoxidase inhibition can be assessed using an in chemico assay, measurements of T4 and T3 levels could be added to the Fish Embryo Acute Toxicity (FET) test (OECD TG 236), ~~and to~~ the Fish Early Life Stage Toxicity (FELS) Test (OECD TG210) and the Fish Sexual Development Test (FSDT), and assessments of anterior chamber inflation and swimming performance could be added to the FELS Test and FSDT.

Thyroid hormone system disruption causes multiple unspecific effects. Addition of TH measurements could aid in increasing the diagnostic capacity of a battery of endpoints since they are specific to the TH system. A battery of endpoints would ideally include the MIE, the AO and TH levels as the causal link. It is also in this philosophy that TH measurements are currently being considered as one of the endpoints in project 2.64 of the OECD TG work plan, "Inclusion of thyroid endpoints in OECD fish Test Guidelines". While thyroid hormone 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 thyroid hormone levels within specific developmental stages. For example, clear changes in thyroid hormone levels have been observed in zebrafish at 5, 14, 21 and 32 dpf (Stinckens et al., 2016; 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).

3. WoE call KER 1034

KER1034 AC→swimming Evidence is weak, no distinction between effect on AC or entire swim bladder system, exposure concentration high Add uncertainties regarding other effects that may have influenced swimming performance	KER1034: The entire swim bladder system is indeed affected. Since the larger PC does not alleviate the effect on swimming, an effect of AC on swimming is plausible. Most of the empirical evidence is specific for the AC and some more general evidence about the swim bladder system was added. Therefore we opted for defining the available evidence as moderate, i.e., biologically plausible with some evidence. We will add the observation that after IOP and PTU exposure, swimming performance was only affected in fish that had no inflated AC while swimming performance of fish with inflated AC from the same treatment was similar to controls. (Stinckens et al., 2020) We will address this uncertainty in the text: effects of AC not always distinguishable from PC effects Sublethal exposure concentrations were selected where the PC inflates. Regarding other effects that may have influenced swimming performance: In some treatments reduced length and/or condition factor was observed. No morphological effects were observed. However, reduced swimming performance after 32 days of IOP exposure to medium concentrations was not accompanied by reduced length or condition factor. (Stinckens et al., 2020)
Current WoE calls: Plausibility: moderate Empirical evidence: moderate Overall: moderate Summary of response: Understand that this may not seem plausible initially, but mechanism has been described in literature Considerable empirical evidence for link between AC and swimming from dedicated studies Overall moderate: mechanism described and empirically supported	

- Changes made to KER 1034

Specific lines of evidence supporting the link between reduced AC inflation and reduced swimming performance were highlighted.

Empirical evidence

Methimazole (MMI) and propylthiouracil (PTU), two thyroperoxidase inhibitors, and iopanoic acid (IOP), a deiodinase inhibitor, each reduced both anterior chamber inflation and swimming distance in zebrafish exposed from fertlization until the age of 32 days (Stinckens et al., 2020). Stinckens et al. (2020) showed a specific, direct link between reduced anterior chamber inflation and reduced swimming performance.

First, after 21 d of exposure to 111 mg/L propylthiouracil around 30% of anterior chambers were not inflated and swimming distance was reduced, while by 32 days post fertilization all larvae had inflated their anterior chamber (although chamber surface was still smaller) and the effect on swimming distance had disappeared. ○ The most direct way to assess the role of anterior chamber inflation in swimming performance, however, is to compare larvae with and without inflated anterior chamber at the same time point and within the same experimental treatment. Both in the propylthiouracil exposure at 21 days post fertilization and in the iopanoic acid exposure at 21 and 32 days post fertilization, swimming distance was clearly reduced in larvae lacking an inflated anterior

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

Exposure concentrations were selected where the posterior chamber inflates. Even though the posterior chamber was generally larger when anterior chamber inflation was reduced, this did not remove the effect on swimming performance, confirming a direct link between proper anterior chamber inflation and swimming performance.

No morphological effects were observed, but in some treatments reduced length and/or condition factor was observed. However, reduced swimming performance after 32 days of IOP exposure to medium concentrations was not accompanied by reduced length or condition factor. Therefore, at least in this study no evidence was found that the effect on swimming performance was an indirect consequence of effects other than reduced swim bladder inflation.

Uncertainties and Inconsistencies

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

4. WoE call KER 1035

KER1035 T3→AC evidence is weak: only one study started exposure after PC inflation, no evidence of primary effect. Change to moderate for zebrafish.	KER1035: For evaluation of AC inflation, concentrations were selected where the PC inflated. We will refine the description of evidence to highlight the difference in experimental setup: two studies with larval exposure started after PC inflation (Nelson et al., 2016; Cavallin et al., 2017); one study with continuous exposure (Stinckens et al., 2020). Additionally, Stinckens et al. (2020, supplementary information) established a convincing linear quantitative relationship between reduced T3 levels and reduced AC surface in 32 day old zebrafish across three compound exposures. Therefore we opted for defining the available evidence as moderate, i.e., biologically plausible with some evidence. The latter quantitative relationship in zebrafish was the basis to select a high level of evidence for zebrafish. We will change this to moderate pending additional evidence.
Current WoE calls: Plausibility: moderate Empirical evidence: moderate Overall: moderate Summary of response: Role of TH in SB development and in developmental transitions (embryo-larval, larval-juvenile) is well established Considerable empirical evidence for link between T3 and AC inflation from dedicated studies	

Overall moderate: mechanism described and empirically supported

- Changes made to KER 1035

Empirical evidence

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

1. Studies applying larval exposures initiated after posterior chamber inflation:

   In a study in which ~~embryo~~ larval fathead minnows (Pimephales promelas) were exposed to the thyroid peroxidase inhibitor 2-mercaptobenzothiazole (MBT), T3 concentrations measured at 14dpf were reduced at the same concentration (1 mg/L) that significantly reduced anterior swim bladder inflation at the same time-point (Nelson et al. 2016).

   ~~Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass (Morone saxatilis) lead to significant increases in both swim bladder inflation and survival (Brown et al., 1988).~~

   In the study of Cavallin et al. (2017) fathead minnow larvae were exposed to IOP, a model iodothyronine deiodinase inhibitor that is assumed to inhibit all three deiodinase enzymes (DIO1,2,3). The authors observed pronounced decreases of whole body T3 concentrations and increases of whole body T4 concentrations, together with impaired inflation of the anterior swim bladder chamber. More specifically, inflation was delayed and the size of the swim bladder chamber was reduced until the end of the exposure experiment.

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

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

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

Taxonomic applicability

Level of evidence was changed from high to moderate for zebrafish.

5. Essentiality calls

Essentiality calls:

Current WoE calls: moderate

Definition of low evidence: No or contradictory experimental evidence of the essentiality of any of the KEs.
Definition of moderate evidence: Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE

Essentiality AOP156 DIO2-anterior: moderate is not fully supported Essentiality AOP158 DIO1-change moderate to low DIO1 low anterior chamber low: secondary to posterior chamber? Systemic toxicity? Swimming performance at larval stage low Dual oxidase knockdown as indirect evidence: DUOX also plays a role in oxidative stress	AOP 156: Although there is less convincing and indirect evidence for essentiality of inhibition of DIO and reduced T3 levels for AC inflation compared to PC inflation, we do have direct evidence for essentiality of DIO2 inhibition for reduced T3 levels and of reduced AC inflation for reduced swimming performance. We believe that moderate 'Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE' is a better call than low 'No or contradictory experimental evidence of the essentiality of any of the KEs'. AOP158: Because of the additional uncertainty concerning DIO1 importance, we will change the essentiality call to low. DUOX knockdown is mentioned as indirect evidence. We will refine the text to include the uncertainties that have been raised.
Essentiality AOP159 TPO anterior Dual oxidase knockdown as indirect evidence: DUOX also plays a role in oxidative stress	The DUOX knockdown (Chopra et al., 2019) is used as evidence for the essentiality of KE277 'TH synthesis decreased'. Chopra et al. specifically included evidence of decreased TH synthesis. In the overall evaluation of the essentiality of KEs in AOP 159 this is referred to as indirect evidence. We will add the fact that DUOX also plays a role in oxidative stress to uncertainties and inconsistencies on the relevant pages.

Changes made to the evaluation of essentiality in AOP 156

2. Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE 1002 (MIE): Inhibition, deiodinase 2	Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of Dio1+2 in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Permanent Dio2 knockout also impaired swim bladder inflation and locomotor activity in zebrafish (Houbrechts et al., 2016). Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. This confirms that DIO2 is essential for causing downstream effects. These effects were rescued after T3 supplementation but not after T4			
	supplementation, confirming the importance of T4 to T3 conversion by Dio2 and the essentiality of DIO2 inhibition for causing downstream effects (Walpita et al., 2009, 2010).			

KE 1003: Decreased triiodothyronine (T3) in serum	There is ample evidence confirming the essentiality of decreased T3 levels for the occurrence of reduced posterior chamber inflation, confirming a direct link between T3 levels and the swim bladder system in general. (1) from zebrafish knockdown/knockout studies: Knockdown of deiodinase 1 and 2 (Bagci et al., 2015; Heijlen et al., 2013, 2014), knockdown of TH transporter MCT8 (de Vrieze et al., 2014), knockdown of thryoid hormone receptor alpha or beta (Marelli et al., 2016), and permanent knockout of deiodinase 2 (Houbrechts et al., 2016) in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Marelli et al. (2016) additionally showed that high T3 doses partially rescued the negative impact in mutants with partially resistant thyroid hormone receptors. Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. These effects were rescued after T3 supplementation, but not after T4 supplementation. While swim bladder inflation was not among the assessed endpoints in this study, this generally confirms the essentiality of decreased T3 in causing downstream effects upon disruption of DIO1 and 2 function (Walpita et al., 2009, 2010). (2) from chemical exposures: Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids and exogeneous T3 or T4 supplementation partly rescued this effect. Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass lead to significant increases in posterior swim bladder inflation (Brown et al., 1988). Similarly, Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. Less information is available about the essentiality of reduced T3 levels for reduced anterior chamber inflation in particular. • Chopra et al. (2019) provided indirect evidence showing that knockdown of dual oxidase - expected to lead to reduced T4 and T3 levels since dual oxidase is important for thyroid hormone synthesis - reduced anterior swim bladder inflation. Proving essentiality of reduced T3 levels for reduced anterior chamber inflation is further complicated by the complexity of the swim bladder system and the difficulty of distinguishing effects resulting from altered anterior chamber inflation from those resulting from altered posterior chamber inflation.
KE 1007: Reduced, anterior swim bladder inflation	Stinckens et al. (2020) showed that at the time point where control zebrafish inflate the anterior chamber, larvae exposed to PTU have a lower frequency of inflated anterior chambers together with reduced swimming distance. Later during the exposure the frequency of non-inflated anterior chambers decreased and the effect on swimming distance disappeared confirming the essentiality of reduced anterior chamber inflation for the downstream effect on swimming performance.
KE 1005: Reduced, swimming performance	Experimental blocking of this KE is difficult to achieve.
KE 351: Increased mortality	By definition, increased mortality ~~reduces~~ is essential for reduced population size.
AOP as a whole	Moderate Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is evidence from deiodinase knockdowns showing the link with reduced posterior chamber inflation and the essentiality for downstream effects, but anterior chamber inflation was not studied. There is additional indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation. It should be noted that dual oxidase also plays a role in oxidative stress. There is also evidence that alleviation of the effect on anterior chamber inflation reduces the effect on swimming performance.

The corresponding text field on the AOP page was updated.

- Changes made to the evaluation of essentiality in AOP 158

2. Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)

	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE 1009 (MIE): Inhibition, deiodinase 1	Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of Dio1+2 in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. This suggests that DIO1 is less important than DIO2 in causing downstream effects. These effects were rescued after T3 supplementation but not after T4 supplementation,			
	confirming the importance of T4 to T3 conversion by Dio2 and perhaps also Dio1 (Walpita et al., 2009, 2010).			
KE 1003: Decreased triiodothyronine (T3) in serum	There is ample evidence confirming the essentiality of decreased T3 levels for the occurrence of reduced posterior chamber inflation, confirming a direct link between T3 levels and the swim bladder system in general. (1) from zebrafish knockdown/knockout studies: Knockdown of deiodinase 1 and 2 (Bagci et al., 2015; Heijlen et al., 2013, 2014), knockdown of TH transporter MCT8 (de Vrieze et al., 2014), knockdown of thryoid hormone receptor alpha or beta (Marelli et al., 2016), and permanent knockout of deiodinase 2 (Houbrechts et al., 2016) in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Marelli et al. (2016) additionally showed that high T3 doses partially rescued the negative impact in mutants with partially resistant thyroid hormone receptors. Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. These effects were rescued after T3 supplementation, but not after T4 supplementation. While swim bladder inflation was not among the assessed endpoints in this study, this generally confirms the essentiality of decreased T3 in causing downstream effects upon disruption of DIO1 and 2 function (Walpita et al., 2009, 2010). (2) from chemical exposures: Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids and exogeneous T3 or T4 supplementation partly rescued this effect. Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass lead to significant increases in posterior swim bladder inflation (Brown et al., 1988). Similarly, Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. Less information is available about the essentiality of reduced T3 levels for reduced anterior chamber inflation. • Chopra et al. (2019) provided indirect evidence showing that knockdown of dual oxidase - expected to lead to reduced T4 and T3 levels since dual oxidase is important for thyroid hormone synthesis - reduced anterior swim bladder inflation. Proving essentiality of reduced T3 levels for reduced anterior chamber inflation is further complicated by the complexity of the swim bladder system and the difficulty of distinguishing effects resulting from altered anterior chamber inflation from those resulting from altered posterior chamber inflation.			
KE 1007: Reduced, anterior swim bladder inflation	Stinckens et al. (2020) showed that at the time point where control zebrafish inflate the anterior chamber, larvae exposed to PTU have a lower frequency of inflated anterior chambers together with reduced swimming distance. Later during the exposure the frequency of non-inflated anterior chambers decreased and the effect on swimming distance disappeared confirming the essentiality of reduced anterior chamber inflation for the downstream effect on swimming performance.			
KE 1005: Reduced, swimming performance	Experimental blocking of this KE is difficult to achieve.			
KE 351: Increased mortality	By definition, increased mortality ~~reduces~~ is essential for reduced population size.			

AOP as a whole	Low~~Moderate~~ Overall, the support for essentiality of the KEs is ~~moderate~~ low since there is limited direct evidence from specifically designed experimental studies illustrating essentiality ~~for several of the important KEs in the AOP~~. This includes evidence from combined DIO1 and DIO2 knockdown studies in zebrafish showing the link with reduced posterior chamber inflation, but anterior chamber inflation was not studied. There is additional indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation. It should be noted that dual oxidase also plays a role in oxidative stress. There is no specific evidence for the essentiality of DIO1 inhibition independent of DIO2 inhibition and ~~It should be noted that~~ DIO2 seems more important than DIO1 in providing sufficient T3 for proper swim bladder inflation. Therefore the overall evidence for essentiality is considered low.

The corresponding text field on the AOP page was updated.

- Changes made to the evaluation of essentiality in AOP 159

2. Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.
KE 279 (MIE): Thyroperoxidase, inhibition	There is evidence of recovery of serum T4 levels after cessation of exposure to a TPO inhibitor in rats (Cooper et al., 1982; 1983; AOP 42), but not in fish.			
KE 277: Thyroid hormone synthesis, decreased	There is evidence of recovery of serum T4 levels in athyroid mice following grafting of in-vitro derived follicles (Antonica et al., 2012; AOP 42). Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation.			
KE 281: Thyroxine (T4) in serum, decreased	There is ample evidence of recovery of phenotypes after cessation of exposure to TPO inhibitors and subsequent T4 recovery in mammals (Cooke et al., 1993; Goldey et al., 1995; Sawin et al., 1998; Axelstad et al., 2008; Shibutani et al., 2009; Lasley and Gilbert, 2011; AOP 42), but not in fish.			

KE 1003: Decreased triiodothyronine (T3) in serum	There is ample evidence confirming the essentiality of decreased T3 levels for the occurrence of reduced posterior chamber inflation, confirming a direct link between T3 levels and the swim bladder system in general. (1) from zebrafish knockdown/knockout studies: Knockdown of deiodinase 1 and 2 (Bagci et al., 2015; Heijlen et al., 2013, 2014), knockdown of TH transporter MCT8 (de Vrieze et al., 2014), knockdown of thryoid hormone receptor alpha or beta (Marelli et al., 2016), and permanent knockout of deiodinase 2 (Houbrechts et al., 2016) in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Marelli et al. (2016) additionally showed that high T3 doses partially rescued the negative impact in mutants with partially resistant thyroid hormone receptors. Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. These effects were rescued after T3 supplementation, but not after T4 supplementation. While swim bladder inflation was not among the assessed endpoints in this study, this generally confirms the essentiality of decreased T3 in causing downstream effects upon disruption of DIO1 and 2 function (Walpita et al., 2009, 2010). (2) from chemical exposures: Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids and exogeneous T3 or T4 supplementation partly rescued this effect. Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass lead to significant increases in posterior swim bladder inflation (Brown et al., 1988). Similarly, Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae. Less information is available about the essentiality of reduced T3 levels for reduced anterior chamber inflation. • Chopra et al. (2019) provided indirect evidence showing that knockdown of dual oxidase - expected to lead to reduced T4 and T3 levels since dual oxidase is important for thyroid hormone synthesis - reduced anterior swim bladder inflation. It should be noted that dual oxidase also plays a role in oxidative stress. Proving essentiality of reduced T3 levels for reduced anterior chamber inflation is further complicated by the complexity of the swim bladder system and the difficulty of distinguishing effects resulting from altered anterior chamber inflation from those resulting from altered posterior chamber inflation.
KE 1007: Reduced, anterior swim bladder inflation	Stinckens et al. (2020) showed that at the time point where control zebrafish inflate the anterior chamber, larvae exposed to PTU have a lower frequency of inflated anterior chambers together with reduced swimming distance. Later during the exposure the frequency of non-inflated anterior chambers decreased and the effect on swimming distance disappeared confirming the essentiality of reduced anterior chamber inflation for the downstream effect on swimming performance.
KE 1005: Reduced, swimming performance	Experimental blocking of this KE is difficult to achieve.
KE 351: Increased mortality	By definition, increased mortality ~~reduces~~ is essential for reduced population size.
AOP as a whole	Moderate Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation from a study where a similar MIE was targeted: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation. Additionally, there is indirect evidence from deiodinase knockdowns supporting the downstream part of the AOP linking decreased T3 levels to reduced swim bladder inflation (targeted at posterior chamber inflation, not specifically at anterior chamber inflation). There is also evidence that alleviation of the effect on anterior chamber inflation reduces the effect on swimming performance.

The corresponding text field on the AOP page was updated.

- Changes made to the evaluation of essentiality in AOP 157

2. Essentiality of KEs	Defining question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an	Direct evidence from specifically designed experimental studies	Indirect evidence that sufficient modification of an expected	No or contradictory experimental evidence

	upstream KE is blocked?	illustrating essentiality for at least one of the important KEs	modulating factor attenuates or augments a KE	of the essentiality of any of the KEs.
KE 1009 (MIE): Inhibition, deiodinase 1	Bagci et al. (2015) and Heijlen et al. (2013, 2014) reported that knockdown of Dio1+2 in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. This suggests that DIO1 is less important than DIO2 in causing downstream effects. These effects were rescued after T3 supplementation but not after T4 supplementation, confirming the importance of T4 to T3 conversion by Dio2 and perhaps also Dio1 (Walpita et al., 2009, 2010).			
KE 1003: Decreased triiodothyronine (T3) in serum	There is ample evidence confirming the essentiality of decreased T3 levels for the occurrence of reduced posterior chamber inflation (1) from zebrafish knockdown/knockout studies: Knockdown of deiodinase 1 and 2 (Bagci et al., 2015; Heijlen et al., 2013, 2014), knockdown of TH transporter MCT8 (de Vrieze et al., 2014), knockdown of thryoid hormone receptor alpha or beta (Marelli et al., 2016), and permanent knockout of deiodinase 2 (Houbrechts et al., 2016) in zebrafish resulted in impaired inflation of the posterior swim bladder chamber. Marelli et al. (2016) additionally showed that high T3 doses partially rescued the negative impact in mutants with partially resistant thyroid hormone receptors. Walpita et al. (2009, 2010) reported reduced pigmentation, otic vesicle length and head-trunk angle in the same Dio1+2 and also Dio2 knockdown fish. These effects were rescued after T3 supplementation, but not after T4 supplementation. While swim bladder inflation was not among the assessed endpoints in this study, this generally confirms the essentiality of decreased T3 in causing downstream effects upon disruption of DIO1 and 2 function (Walpita et al., 2009, 2010). (2) from chemical exposures: Wang et al. (2020) observed a decrease of whole-body T3 as well as impaired posterior chamber inflation in zebrafish exposed to perfluorooctanoic acid and perfluoropolyether carboxylic acids and exogeneous T3 or T4 supplementation partly rescued this effect. Maternal injection of T3, resulting in increased T3 concentrations in the eggs of striped bass lead to significant increases in posterior swim bladder inflation (Brown et al., 1988). Similarly, Molla et al. (2019) showed that T3 supplementation increased posterior chamber diameter in zebrafish larvae.			
KE 1004: Reduced, posterior swim bladder inflation	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), confirming the essentiality of posterior swim bladder inflation for the occurrence of the downstream key event 'reduced young of year survival'.			
KE 1005: Reduced, swimming performance	Experimental blocking of this KE is difficult to achieve.			
KE 351: Increased mortality	By definition, increased mortality ~~reduces~~ is essential for reduced population size.			
AOP as a whole	~~Moderate~~Low Overall, the support for essentiality of the KEs is ~~moderate~~ low. ~~since there is direct evidence from specifically designed experimental studies illustrating essentiality for several of the important KEs in the AOP. This includes~~There is ample evidence from combined DIO1 and DIO2 knockdown studies in zebrafish that shows 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. There is no specific evidence for the essentiality of DIO1 inhibition independent of DIO2 inhibition and ~~It should be noted that~~ DIO2 seems more important than DIO1 in providing sufficient T3 for proper swim bladder inflation. Therefore the overall evidence for essentiality is considered low.			

The corresponding text field on the AOP page was updated.

- Changes made to KER 1039

In some cases indirect effects may play a role in the impact of chemical exposure or genetic knockdown/knockout on swim bladder inflation. For example, dual oxidase also plays a role in oxidative

stress.

6. Response A18: link between mortality and population

A18	☐ KER2013 mortality→population: harmonize with AOP on AChE inhibition, i.e. moderate	☐ KER2013: Agreed to change evidence from high to moderate ☐ A discussion of evidence for population models could be added later in collaboration with additional AOP developers who have more expertise in this area (as the
	☐ Disagree with limited evidence to optimize population models	reviewer suggests, these developers are likely to be working on different AOPs converging to the same AO). We would be happy to add any relevant input in this respect from the review team.
Current WoE calls Plausibility: high Empirical evidence: moderate Overall: high Summary of response: ☐ Agree to change to overall moderate		

☐ Changes made to KER 2013

Evidence for this KER was changed from high to moderate in all AOPs.

Uncertainties and inconsistencies

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

Empirical evidence

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

(Schäfers et al., 1993) ○ In fathead minnow, natural survival of early life stages has been found to be highly variable and influential on population growth (Miller and Ankley, 2004)

Rearick et al. (2018) used linked data from behavioural assays to survival trials and applied a modelling approach to quantify changes in antipredator escape performance of larval fathead minnows in order to predict changes in population abundance. This work was done in the context of exposure to an environmental oestrogen. Expsoed fish had delayed response times and slower escape speeds, and were more susceptible to predation. Population modelling showed that his can result in population decline.

In the context of fishing and fisheries, ample evidence of a link between increased mortality and a decrerase of population size has been given. Important insights can result from the investigation of optimum modes of fishing that allow for maintaining a population (Alekseeva and Rudenko, 2018). Jacobsen and Essington (2018) showed the impact of varying predation mortality on forage fish populations.

Boreman (1997) reviewed methods for comparing the population-level effects of mortality in fish populations induced by pollution or fishing.

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Section 2: Remaining comments

1. MIEs DIO1 and DIO2

MIEs DIO1 and DIO2 Define MIE as specific enzymatic reaction catalyzed by DIO instead of DIO1/DIO2, e.g., T4→T3. Chemicals often target more than one DIO isoform. As there is no convincing evidence that DIO1 results in a reduction of T2 levels, I would assume that the AOP cannot be published, or should be integrated into the AOP 155.	The choice to define DIO1 and DIO2 inhibition as separate MIEs is based on the definition of a KE, i.e. the fact that a KE is a measureable change in biological state. Different assays are indeed available to measure inhibition of the DIO isoforms separately. The current assays are not aiming at distinguishing the different reactions, but rather at distinguishing the different isoforms. Even though our current description of the toxicological process does not distinguish between effects of DIO1 versus DIO2 inhibition, defining both as separate MIEs does allow further refinement of the AOP network in the future, should evidence in that sense become available. This is of particular importance for the re-use of KEs in a crossspecies AOP network development context: distinct AOPs for different DIO isoforms are also under development in amphibians. Chemicals often target multiple MIEs. The concept of AOP networks allows to capture that level of complexity. The current AOP network only describes the conversion T4 → T3 (as indicated by the link to KE1003: decreased T3). Effects related to rT3 and T2 are currently not considered by any of the AOPs in the network. A statement acknowledging the role of DIO1 in rT3 to T2 conversion is present in the descriptions on the relevant pages. Overall, our current understanding of the AOP network suggests that DIO2 is more important than DIO1 in the context of impaired swim bladder inflation, and this is emphasized in several places in the AOP descriptions. DIO1 is capable of catalyzing the conversion of T4 into T3, but there is still uncertainty about the physiological role of DIO1 in this conversion.

We believe that the information the reviewers are referring to is present in the relevant pages in the AOP-Wiki. See highlights below as an example. No further changes were made.

Abstract of AOP 157

Other than the difference in deiodinase (DIO) isoform, the current AOP is identical to the corresponding AOP leading from DIO2 inhibition to increased mortality via posterior swim bladder inflation (https://aopwiki.org/aops/155). The overall importance of DIO1 versus DIO2 in fish is not exactly clear. The current state f the art suggests that DIO2 is more important than DIO1 in regulatring swim bladder inflation. Therefore AOP 155 may be of higher biological relevance compared to the AOP that is described here.

This AOP describes the sequence of events leading from deiodinase inhibition to increased mortality via reduced posterior swim bladder inflation. Thyroid hormones (THs) are critical during embryonic development and disruption of the TH system can interfere with normal development. 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. While 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), type I deiodinase is capable of both converting T4 into T3 and converting rT3 to the inactive thyroid hormone 3,3' T2. Inhibition of DIO1 thus reduces T3 levels. However, partly because rT3, rather than T4, is the preferred substrate for DIO1, DIO1 inhibition is probably less important in causing reduced T3 levels when compared to DIO2 inhibition.

2. Information on fish

☐	Information on fish is limited in early KEs and KERs, especially those that have been initially developed for mammals	☐	We agree. We will add more information on fish to KE277, KE279, KER309, KER305, KE281, KE1003, KE1002, KE1009, KER1026, and make the fish-specific information more visible.

☐ Changes made to KE 277

Key event description

While the thyroid hormone system is highly conserved across vertebrates, there are some taxonspecific considerations.

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 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).

Decreases in TH synthesis can only occur after initiation of embryonic TH synthesis. The components of the TH system responsible for TH synthesis are highly conserved across vertebrates and therefore interference with the same molecular targets compared to mammals can lead to decreased TH synthesis (TPO, NIS, etc.). Endogenous transcription profiles of thyroid-related genes in zebrafish and fathead minnow showed that mRNA coding for these genes is also maternally transferred and increasing expression of most transcripts during hatching and embryo-larval transition indicates a fully functional HPT axis in larvae (Vergauwen et al., 2018). 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). Also, in most fish species thyroid follicles are more diffusely located in the pharyngeal region rather than encapsulated in a gland.

How It Is Measured or Detected

Techniques for in vivo analysis of thyroid hormone system disruption among other drug-related effects

in fish were reviewed by Raldua and Piña (2014). TIQDT (Thyroxine-immunofluorescence quantitative disruption test) is a method that provides an immunofluorescent based estimate of thyroxine in the gland of zebrafish (Raldua and Babin, 2009; Thienpont et al., 2011; Jomaa et al., 2014; Rehberger et al., 2018~~Thienpont et al 2011~~). Thienpont used ~~T~~this method ~~has been used for~~with ~25 xenobiotics (e.g., amitrole, perchlorate, methimazole, PTU, DDT, PCBs). The method detected changes for all chemicals known to directly impact TH synthesis in the thyroid gland (e.g., NIS and TPO inbibitors), but not those that upregulate hepatic catabolism of T4. Rehberger et al. (2018) updated the method to enable simultaneous semi-quantitative visualization of intrafollicular T3 and T4 levels. Most often, whole body thyroid hormone level measurements in fish early life stages are used as indirect evidence of decreased thyroid hormone synthesis (Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020). Analytical determination of thyroid hormone levels by LC-MS is becoming increasingly available (Hornung et al., 2015).

More recently, transgenic zebrafish with fluorescent thyroid follicles are being used to visualize the compensatory proliferation of the thyroid follicles following inhibition of thyroid hormone synthesis (Opitz et al., 2012).

Domain of applicability

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. The earliest life stages of teleost fish (e.g., fathead minnow, zebrafish) rely on maternally transferred THs to regulate certain developmental processes until embryonic TH synthesis is active (Power et al., 2001). In externally developing fish species, decreases in TH synthesis can only occur after initiation of embryonic TH synthesis. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. Therefore, it is still uncertain when exactly embryonic TH synthesis is activated and thus when exactly this process becomes sensitive to disruption. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It currently remains unclear when exactly embryonic thyroid hormone production is initiated in zebrafish.

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- Changes made to KE 279

Key event description

The components of the TH system responsible for TH synthesis are highly conserved across vertebrates. In fish and amphibians TPO and NIS inhibition result in an expected decrease of TH synthesis (Hornung et al., 2010; Tietge et al., 2013; Nelson et al., 2016; Stinckens et al., 2016; Stinckens et al., 2020) like in mammals. Although the thyroid hormone system is highly conserved across vertebrates, there are some taxon-specific considerations.

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 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).

Inhibition of thyroperoxidase can only occur after activation of embryonic TH synthesis mediated by thyroperoxidase. Endogenous transcription profiles of thyroid-related genes in zebrafish and fathead minnow showed that mRNA coding for thyroid peroxidase is maternally transferred in relatively high amounts with subsequent mRNA degradation followed by initiation of embryonic transcription around hatching (Vergauwen et al., 2018).

How it is measured or detected

In fish, increases of TPO mRNA levels are often used as indirect evidence of TPO inhibition in in vivo experiments (Baumann et al., 2016; Nelson et al., 2016; Wang et al., 2020).

Domain of applicability

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. 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, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf and not at 24 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first

observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TPO inhibition.

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- Changes made to KER 309

Key Event Relationship Description

Thyroperoxidase (TPO) is a heme-containing apical membrane protein within the follicular lumen of thyrocytes that acts as the enzymatic catalyst for thyroid hormone (TH) synthesis (Taurog, 2005) across vertebrates. Two commonly used reference chemicals, propylthiouracil (PTU) and methimazole (MMI), are drugs that inhibit the ability of TPO to: a) activate iodine and transfer it to thyroglobulin (Tg) (Davidson et al., 1978); and, b) couple thyroglobulin (Tg)-bound iodotyrosyls to produce Tg-bound thyroxine (T4) and triiodothyronine (T3) (Taurog, 2005).

Empirical evidence

Empirical support for this KER is strong. There are several papers that have measured alterations in TPO and subsequent effects on TH synthesis across vertebrates. …

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. Several other studies have also shown that chemically induced Inhibition of TPO results in reduced TH synthesis in zebrafish (Van der Ven et al., 2006; Raldua and Babin, 2009; Liu et al., 2011; Thienpont et al., 2011; Rehberger et al., 2018). A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Temporal Evidence: In **mammals**, ~~T~~the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). The impact of decreased TPO activity on thyroidal hormone synthesis is similar across all ages in mammals. …

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal thyroid hormones transferred to the eggs. Embryonic thyroid hormone synthesis is activated later during embryo-larval development. (See Domain of applicability)

Domain of Applicability

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. 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, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production

already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). ~~Therefore, i~~It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH disruptors.

Time-scale

In Xenopus laevis, Haselman et al. (2020) showed a decrease in thyroidal iodinated species after only 2 days of exposure to potent TPO inhibitor MMI during thyroid-mediated metamorphosis and within 4 days for PTU and MBT, both model TPO inhibitors. In zebrafish, Walter et al. (2019) reported a similar time frame, namely a decrease in T4 levels at 72 hpf after starting the exposure to PTU at 0-2 hpf. It should be noted that the time-scale is probably depending on the developmental stage and whether the embryo is capable of thyroid hormone synthesis, rather than on the exposure duration.

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- Changes made to KER 305

Key Event Relationship Description

Thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3) are synthesized by NIS and TPO in the

thyroid gland as iodinated thyroglobulin (Tg) and stored in the colloid of thyroid follicles across vertebrates. Secretion from the follicle into serum is a multi-step process. The first involves thyroid stimulating hormone (TSH) stimulation of the separation of the peptide linkage between Tg and TH. The next steps involve endocytosis of colloid, fusion of the endosome with the basolateral membrane of the thyrocyte, and finally release of TH into blood. More detailed descriptions of this process can be found in reviews by Braverman and Utiger (2012) and Zoeller et al. (2007).

Empirical evidence

It is widely accepted that TPO inhibition leads to declines in serum T4 levels in adult **mammals**. …

Additionally, evidence is available from studies investigating responses to TPO inhibitors in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. Several other studies have also shown that chemically induced Inhibition of TPO results in reduced TH synthesis in zebrafish (Van der Ven et al., 2006; Raldua and Babin, 2009; Liu et al., 2011; Thienpont et al., 2011; Rehberger et al., 2018). A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

Temporal Evidence: In **mammals**, ~~T~~the temporal nature of this KER is applicable to all life stages, including development (Seed et al., 2005). There are currently no studies that measured both TPO synthesis and TH production during development. However, the impact of decreased TH synthesis on serum hormones is similar across all ages in mammals. …

In oviparous **fish** such as zebrafish and fathead minnow, the nature of this KER depends on the life stage since the earliest stages of embryonic development rely on maternal thyroid hormones transferred to the eggs. Embryonic thyroid hormone synthesis is activated later during embryo-larval development. (See Domain of applicability)

Domain of Applicability

Life stage: Applicability to certain life stages may depend on the species and their dependence on maternally transferred thyroid hormones during the earliest phases of development. 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, TPO inhibition is not expected to decrease TH synthesis during these earliest stages of development. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid

follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH disruptors.

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- Changes made to KE 281

Key Event Description

Serum T4 is used as a biomarker of TH status because the circulatory system serves as the major transport and delivery system for TH delivery to tissues. The majority of THs in the blood are bound to transport proteins (Bartalena and Robbins, 1993). In serum, it is the unbound, or 'free' form of the hormone that is thought to be available for transport into tissues. Free hormones are approximately 0.03 and 0.3 percent for T4 and T3, respectively. There are major species differences in the predominant binding proteins and their affinities for THs (see below). However, there is broad agreement that changes in serum concentrations of THs is diagnostic of thyroid disease or chemicalinduced disruption of thyroid homeostasis across vertebrates (DeVito et al., 1999; Miller et al., 2009; Zoeller et al., 2007; Carr and Patiño, 2011).

Normal serum T4 reference ranges can be species and lifestage specific. In **rodents**, …

Additionally, ample evidence is available from studies investigating responses to inhibitors of thyroid hormone synthesis in **fish**. For example, Stinckens et al. (2020) showed reduced whole body T4 concentrations in zebrafish larvae exposed to 50 or 100 mg/L methimazole, a potent TPO inhibitor, from immediately after fertilization until 21 or 32 days of age. Exposure to 37 or 111 mg/L propylthiouracil also reduced T4 levels after exposure up to 14, 21 and 32 days in the same study. Walter et al. (2019) showed that propylthiouracil had no effect on T4 levels in 24h old zebrafish, but decreased T4 levels of 72h old zebrafish. This difference is probably due to the onset of embryonic TH production between the age of 24 and 72 hours (Opitz et al., 2011). Stinckens et al. (2016) showed that exposure to 2-mercaptobenzothiazole (MBT), an environmentally relevant TPO inhibitor, decreased whole body T4 levels in continuously exposed 5 and 32 day old zebrafish larvae. A high concentration of MBT also decreased whole body T4 levels in 6 day old fathead minnows, but recovery was observed at the age of 21 days although the fish were kept in the exposure medium (Nelson et al., 2016). Crane et al. (2006) showed decreased T4 levels in 28 day old fathead minnows continuously exposed to 32 or 100 µg/L methimazole.

How is it measured or detected

Serum T3 and T4 can be measured as free (unbound) or total (bound + unbound). Free hormone concentrations are clinically considered more direct indicators of T4 and T3 activities in the body, but in animal studies, total T3 and T4 are typically measured. Historically, the most widely used method in toxicology is the radioimmunoassay (RIA). The method is routinely used in rodent endocrine and toxicity studies. The ELISA method is commonly used as a human clinical test method. Analytical determination of iodothyronines (T3, T4, rT3, T2) and their conjugates, through methods employing HPLC, liquid chromatography, immuno luminescence, and mass spectrometry are less common, but are becoming increasingly available (Hornung et al., 2015; DeVito et al., 1999; Baret and Fert, 1989; Spencer, 2013; Samanidou V.F et al., 2000; Rathmann D. et al., 2015). In fish early life stages most evidence for the ontogeny of thyroid hormone synthesis comes from measurements of whole body thyroid hormone levels 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). It is important to note that thyroid hormones concentrations can be influenced by a number of intrinsic and extrinsic factors (e.g., circadian rhythms, stress, food intake, housing, noise) (see for example, Döhler et al., 1979).

Domain of applicability

Life stage: 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. In zebrafish, Opitz et al. (2011) showed the formation of a first thyroid follicle at 55 hours post fertilization (hpf), Chang et al. (2012) showed a first significant TH increase at 120 hpf and Walter et al. (2019) showed clear TH production already at 72 hpf but did not analyse time points between 24 and 72 hpf. In fathead minnows, a significant increase of whole body thyroid hormone levels was already observed between 1 and 2 dpf, which corresponds to the appearance of the thyroid anlage at 35 hpf prior to the first observation of thyroid follicles at 58 hpf (Wabuke-Bunoti and Firling, 1983). It is still uncertain when exactly embryonic TH synthesis is activated and how this determines sensitivity to TH disruptors.

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- Changes made to KE 1003

Key Event Description

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

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).

Domain of applicability

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.

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- Changes made to KE 1002

How it is Measured or Detected

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

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):29422952.

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.

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

- Changes made to KE 1009

How it is Measured or Detected

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 decreased DIO1 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

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):29422952.

Houbrechts AM, Vergauwen L, Bagci E, Van Houcke J, Heijlen M, Kulemeka B, Hyde DR, Knapen D, Darras VM. 2016. Deiodinase knockdown affects zebrafish eye development at the level of gene expression, morphology and function. Molecular and Cellular Endocrinology. 424(C):81-93.

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

- Changes made to KER 1026

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 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).

3. Sex applicability

Refine sex applicability	We will re-evaluate the sex applicability descriptions in all AOPs and refine where possible.
Add comment on sex ·entiation in fathead minnow	We will add a comment for fathead minnow and species with sex markers.
Sex differences are investigated in species with sex markers	
Change evidence for unspecific sex to moderate	We will change the WoE call for unspecific sex from high to moderate.

- Changes made to AOP 155 and AOP 157

Domain of applicability

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.

- Changes made to KER 1027, KE 1004, KER 1028, KER 1044, KER 1042

Domain of applicability

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.

- Changes made to AOP 156, AOP 158 and AOP 159

Domain of applicability

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

- Changes made to KER 1035, KE 1007, KER 1034, KER 1039

Domain of applicability

Sex: This KE/KER 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). For zebrafish and fathead minnow, it is currently unclear whether sex-related differences are important in determining the magnitude of the changes in this KE/KER. Different fish species have different sex determination and differentiation strategies. Zebrafish do not have identifiable heteromorphic sex chromosomes and sex is determined by multiple genes and influenced by the environment (Nagabhushana and Mishra, 2016). Zebrafish are undifferentiated gonochorists since both sexes initially develop an immature ovary (Maack and Segner, 2003). Immature ovary development progresses until approximately the onset of the third week. Later, in female fish immature ovaries continue to develop further, while male fish undergo transformation of ovaries into testes. Final

transformation into testes varies among male individuals, however finishes usually around 6 weeks post fertilization. Since the anterior chamber inflates around 21 days post fertilization in zebrafish, sex differences are expected to play a minor role. Fathead minnow gonad differentiation also occurs during larval development. Fathead minnows utilize a XY sex determination strategy and markers can be used to genotype sex in life stages where the sex is not yet clearly defined morphologically (Olmstead et al., 2011). Ovarian differentiation starts at 10 dph followed by rapid development (Van Aerle et al., 2004). At 25 dph germ cells of all stages up to the primary oocytes stage were present and at 120 dph, vitellogenic oocytes were present. The germ cells (spermatogonia) of the developing testes only entered meiosis around 90–120 dph. Mature testes with spermatozoa are present around 150 dph. Since the anterior chamber inflates around 14 days post fertilization (9 dph) in fathead minnows, sex differences are expected to play a minor role in the current AOP.

References added to AOPs 155-159, KER 1027, KE 1004, KER 1028, KER 1035, KE 1007, KER 1034

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):12151223.

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.

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.

- Changes made to KE 279

Domain of applicability

Sex: This KE is plausibly applicable to both sexes. The molecular components responsible for thyroid hormone synthesis, including thyroperoxidase, are identical in both sexes. Therefore inhibition of deiodinases is not expected to be sex-specific.

- Changes made to KE 1002, KE 1009

Domain of applicability

Sex: This KE is plausibly applicable to both sexes. Deiodinases are important for TH homeostasis and identical in both sexes. ~~Sex-specific differences in this KE have not been described in fish.~~Therefore inhibition of deiodinases is not expected to be sex-specific.

- Changes made to KER 309, KE 277, KER 305, KE 281, KER 366, KER 2038, KER 1026, KER 1037, KE 1003

Domain of applicability

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.

References added

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.

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.

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.

- Changes made to WoE calls

The WoE call for unspecific sex was changed from high to moderate in

AOPs 155-159

KE 1003, KE 1009, KE 1002, KE 1007, KE 1004, KE 1005, KE 351

KER 2038, KER 1026, KER 1037, KER 1035, KER 1027, KER 1042, KER 1039, KER 1044, KER 1028, KER 1034, KER 2013, KER 2213

In cases where a WoE call of high had been attributed to Male and Female previously in the context of endorsed AOPs, these calls were left unchanged.

4. Life stage applicability

Refine life stage applicability: Swimming performance is applicable to post-embryonic life stages	We will refine the life stage applicability in KE1005.

- Changes made to KE 1005

Domain of applicability

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

The life stage term was changed from ‘all life stages’ to ‘larvae’, ‘juvenile’ and ‘adult’ with WoE call moderate.

5. Description of mortality

Refine KE351: "How it is measured section" seems to be related to fish only. As this KE is applicable to any living being, an alternative strategy would be to use a general biological definition of death plus a general definition of mortality rate. (114)	We will add a more general definition of mortality to KE351.

Changes made to KE 351

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.

~~Lack of any heart beat, gill movement, and body movement are typical signs of death used in the evaluation of mortality of animals.~~

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

in the lab by recording mortality during ~~prolonged~~ exposure experiments ○ in dedicated setups simulating a realistic situation such as mesocosms~~,~~ or ~~in~~ drainable ponds for aquatic species
in the field, for example by determining age structure after one capture, or by capturemark~~tag~~-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.

5. Uncertainties and inconsistencies

Refine "uncertainties and inconsistencies") Oxidative stress among other mechanisms may contribute to impaired swim bladder inflation KER2038 T4→T3: TPO inhibition may not necessarily result in reduced T3 levels, even though T4 levels were decreased	We will add other contributing mechanisms where relevant The fact that reduced T4 levels do not always result in reduced T3 levels is mentioned with a few examples (KER2038 – Uncertainties and inconsistencies)

Changes made to KER 2038

Uncertainties and inconsistencies

It seems that the following statement caused some confusion:

This relationship depends on the MIE that is causing the decrease in T3. For example, deiodinase inhibition results in reduced activation of T4 to T3 and thus in reduced T3 levels; increased T4 levels have been observed, probably as a compensatory mechanism in response to the lower T3 levels. For example, Cavallin et al. (2017) exposed fathead minnows to iopanoic acid, a deiodinase inhibitor, and observed T4 increases together with T3 decreases.

Since this statement is not directly relevant to this KER, it was removed.

- Changes made to KER 1026

Uncertainties and inconsistencies

A sentence was added:

Deiodinase 2 inhibition may not always directly lead to decreased T3 levels as there may be agespecific, exposure window-specific, and exposure duration-specific effects that may deviate from that dynamic. Differences in feedback mechanisms may be an important contributor.

- Changes made to KER 1035

Uncertainties and inconsistencies

○ Reduced anterior chamber inflation upon disruption of the thyroid hormone system is in most cases, but not always, accompanied by reduced whole body T3 levels. Stinckens et al. (2016) found a consistent relationship between reduced whole body T4 levels, but not

T3 levels, and reduced anterior chamber inflation after exposure to 2-mercaptobenzothiazole (MBT). Possibly, local T4 levels in the swim bladder tissue were too low to allow for enough local activation to T3. This relates to the general uncertainty on serum versus tissue TH levels. Alternatively, differences in timing between T3/T4 measurements (at 120hpf and 32dpf), the moment when there is a need for T3 to inflate the swim bladder (unknown but probably in between 120hpf and 32dpf) and the observation of the phenotype (32dpf), could lead to the hypothesis that T3 concentration was reduced in between the two measurements. There is also a possibility that the effect of MBT on anterior chamber inflation is not directly caused by decreased thyroid hormone levels, but rather by another mechanism such as oxidative stress. MBT is known to elevate the production of reactive oxygen species (ROS) levels in fish cells (Zeng et al., 2016). In general, chemicals may have multiple modes of action and effects on autophagy, ROS, cardiac function may impact swim bladder inflation.

Reference

Zeng FX, Sherry JP, Bols NC. 2016. Evaluating the toxic potential of benzothiazoles with the rainbow trout cell lines, rtgill-w1 and rtl-w1. Chemosphere. 155:308-318.

The above revised statement was moved to 'Uncertainties and inconsistencies' in KER 1039 (decreased T4 → reduced AC inflation) since absence of reduced T3 levels makes it less applicable to KER 1035 (decreased T3 → reduced AC inflation) where it was originally written.

○ The mechanism underlying the link between reduced T3 and reduced anterior chamber inflation remains unclear, but several hypotheses exist (Stinckens et al., 2020). For example, altered gas distribution between chambers could be the result of impaired development of smooth muscle fibers, delayed and/or impaired evagination of the anterior chamber, impaired anterior budding through altered

Wnt and hedgehog signalling, etc. Reinwald et al. (2021) showed that T3 and propylthiouracil treatment of zebrafish embryos altered expression of genes involved in muscle contraction and functioning in an opposing fashion. The authors suggested impaired muscle function as an additional key event between decreased T3 levels and reduced swim bladder inflation.

Reference

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

- Changes made to KER 1039

Empirical evidence

Chopra et al. (2019) found that a nonsense mutation of the duox gene, coding for the enzyme dual oxidase involved in thyroid hormone synthesis, resulted in decreased intrafollicular T4 levels and impaired anterior chamber inflation until at least 54 dpf in zebrafish. It should be noted that dual oxidase is not only involved in thyroid hormone synthesis, but also in the production of reactive oxygen species (ROS) (Flores et al. 2010; Niethammer et al. 2009). In zebrafish, ROS can also be induced e.g. by copper (Zhou et al. 2016), which has also been shown to impair swim bladder development (Xu et al. 2017). Impaired production of ROS after dual oxidase knockdown may contribute to an impairment of swim bladder development.

References

Flores MV, Crawford KC, Pullin LM, Hall CJ, Crosier KE, Crosier PS. 2010. Dual oxidase in the intestinal epithelium of zebrafish larvae has anti-bacterial properties. Biochemical and Biophysical Research Communications. 400(1):164-168.

Niethammer P, Grabher C, Look AT, Mitchison TJ. 2009. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature. 459(7249):996-U123.

Xu JP, Zhang RT, Zhang T, Zhao G, Huang Y, Wang HL, Liu JX. 2017. Copper impairs zebrafish swimbladder development by down-regulating wnt signaling. Aquatic Toxicology. 192:155-164.

Zhou XY, Zhang T, Ren L, Wu JJ, Wang WM, Liu JX. 2016. Copper elevated embryonic hemoglobin through reactive oxygen species during zebrafish erythrogenesis. Aquatic Toxicology. 175:1-11.

- Changes made to AOP 159

WoE evaluation: Essentiality of the Key Events

Overall, the confidence in the supporting data for essentiality of KEs within the AOP is moderate. There is indirect evidence that reduced thyroid hormone synthesis causes reduced anterior swim bladder inflation from a study where a similar MIE was targeted: Chopra et al. (2019) showed that knockdown of dual oxidase, important for thyroid hormone synthesis, reduced anterior swim bladder inflation. Additionally, there is indirect evidence from deiodinase knockdowns supporting the downstream part of

the AOP linking decreased T3 levels to reduced swim bladder inflation (targeted at posterior chamber inflation, not specifically at anterior chamber inflation). There is also evidence that alleviation of the effect on anterior chamber inflation reduces the effect on swimming performance.

It should be noted that dual oxidase is not only involved in thyroid hormone synthesis, but also in the production of reactive oxygen species (ROS) (Flores et al. 2010; Niethammer et al. 2009). In zebrafish, ROS can also be induced e.g. by copper (Zhou et al. 2016), which has also been shown to impair swim bladder development (Xu et al. 2017). Impaired production of ROS after dual oxidase knockdown may contribute to an impairment of swim bladder development.

References

Flores MV, Crawford KC, Pullin LM, Hall CJ, Crosier KE, Crosier PS. 2010. Dual oxidase in the intestinal epithelium of zebrafish larvae has anti-bacterial properties. Biochemical and Biophysical Research Communications. 400(1):164-168.

Niethammer P, Grabher C, Look AT, Mitchison TJ. 2009. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature. 459(7249):996-U123.

Xu JP, Zhang RT, Zhang T, Zhao G, Huang Y, Wang HL, Liu JX. 2017. Copper impairs zebrafish swimbladder development by down-regulating wnt signaling. Aquatic Toxicology. 192:155-164.

Zhou XY, Zhang T, Ren L, Wu JJ, Wang WM, Liu JX. 2016. Copper elevated embryonic hemoglobin through reactive oxygen species during zebrafish erythrogenesis. Aquatic Toxicology. 175:1-11.

- Changes made to KER 309

Uncertainties and inconsistencies

~~It is important to note that d~~Data from studies on genistein highlight this uncertainty. Doerge and colleagues have demonstrated that for this compound up to 80% TPO inhibition did not result in decreased serum T4 in rats (Doerge and Chang, 2002). This is not consistent with other prototypical TPO inhibitors (e.g., PTU, MMI). ~~It remains to be determined if, for some presently unknown reason, genistein is an outlier or not. This again points to the need for quantitative modeling of the relationship between TPO inhibtion and downstream KEs.~~Genistein is however a well-known phytoestrogen and the observed inconsistency may be the result of feedback mechanisms resulting from its estrogenic effect.

The same nuance was added to the section 'Response-response relationship'.

- Changes made to KER 2213

Uncertainties and inconsistencies

Some studies showed an absence of increased mortality after impaied 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

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.

7. Improve descriptions

☐	Improve description	☐	We will improve the descriptions of KE277, KE351, KE1003, KER2038

☐ Changes made to KE351

Rather than listing all signs of death (comment 93), we opted to add a more general description of how mortality is observed in line with what was suggested in comment 114, see response 6.

☐ Changes made to KE 277, KE 281

Pig was replaced by Sus scrofa as taxonomic applicability term. (Comment 103, 105)

☐ Changes made to KE 281

Evidence for perturbation by stressor (comment 105)

Methimazole

Methimazole is a c~~C~~lassic positive control for inhibition of TPO.

Domain of applicability

As such extrapolation regarding TH action across species and developmental stages should be done with caution.

The paragraph on TH transport was added by another author. In the spirit of collaborative AOP development we opted not to remove this explanation.

☐ Changes made to KER 1035

Key Event Relationship Description (Comment 122)

Thyroid hormones are known to be involved in development, especially in metamorphosis in amphibians and in embryonic-to-larval transition and larval-to-juvenile transition, including anterior chamber inflation in fish. Reduced T3 levels 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 anterior chamber inflation.~~Inflation of the anterior swim bladder chamber is part of the larval-to-juvenile transition in fish, together with the development of adult fins and fin rays, ossification of the axial skeleton, formation of an adult pigmentation pattern, scale formation, maturation and remodeling of organs including the lateral line, nervous system, gut and kidneys.~~

8. Swimming performance

☐	KE1005 swimming performance: isn't this an AO already?	☐	We agree that the level at which an AO is defined can be subject to discussion. We chose to limit AOs to outcomes that are considered of direct regulatory relevance in ecotoxicology.

No changes were made.

9. References

☐	Add references	☐	We will include the most important/relevant suggested references.
		☐	We will add a brief "Methods" section to all AOP pages describing the overall rationale and approach for developing these AOPs, including the approach for citing literature. Specifically, we will clarify that 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. We aimed at including as much literature as needed to support these particular AOPs. Updates to the AOP-wiki and the Users Handbook to support the description of the AOP development methodology are currently under development. We will update the AOP-wiki as soon as the relevant formal procedures become available.

A large number of references has been added as indicated throughout this document.

☐ Changes made to AOPs 155-159

Background

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.

10. Small modifications

☐	Suggestions for small modifications	☐	We will implement these small modifications as suggested.

Some of these comments have been addressed in other responses. Bellow any changes in response to the remainder of the comments are listed. Minor changes (e.g., explaining an abbreviation) are not mentioned here.

☐ Changes made to AOP 155

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 (https://aopwiki.org/aops/159).

☐ Changes made to AOP 156

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 anterior swim bladder inflation (https://aopwiki.org/aops/158). 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 may be of higher biological relevance compared to AOP 158. Starting from reduced serum T3 levels, this AOP is also identical to the AOP leading from thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation (https://aopwiki.org/aops/159).

- Changes made to KER 1042, KER 1044

Empirical evidence

~~Exposure to PTU, a very potent DIO1 inhibitor, caused thyroid hypertrophy in X. leavis because of the inhibition of the peripheral conversion of T4 to T3 (Degitz et al., 2005).~~

This sentence was removed because Xenopus DIO1 has been shown to be insensitive to PTU (Kuiper GG et al. 2006 Endocrinology) and the effect seen by Degitz et al. is likely due to the inhibition of TPO by PTU.

- Changes made to KER 2213

Empirical evidence

MeHg and HgCl2 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)

References

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.

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.

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.

- Changes made to KE 1002, 1009

Key event description

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

The part on TH synthesis was removed from the page describing DIO inhibition. (Comment 14)

11. Response A19: WoE call KER 309

☐	KER309 TPO→TH: change evidence from moderate to high	☐	KER309: We agree to change evidence from moderate to high

The overall WoE call for KER 309 in AOP 159 was changed from moderate to high.

12. Response A20: quantitative understanding

☐	Quantitative understanding KERs1027 (T3→PC: low)1035(T3→AC: moderate): Is there an actual difference between evidence?	☐	KERs1027-1037: The difference in the level of quantitative understanding is based on the lower availability of quantitative data for T3-PC compared to T3-AC. The general weight of evidence calls are moderate for both KERs.

No changes were made.

13. Response A21: uncertainties

☐	Refine KER1027 T3→PC: link uncertain, add impaired muscle development	☐	KER1027: We will add impaired muscle development

☐ Changes made to KER 1027

Uncertainties and inconsistencies

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

Reference added

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