OECD EXTERNAL REVIEW REPORT, September 2015

AOP-23

Title: Androgen receptor agonism leading to reproductive dysfunction

Authors: Dan Villeneuve

Reviewers:

Ramachandran Balasubramanian
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Content

1. Introduction

1.1 Material for the review:

- Snapshot for OECD Review (September 2015): File:Aop23-Snapshot-REV-September2015.pdf
- Associated wiki page (link) to Aop:23

1.2 Background of the AOP

This adverse outcome pathway details the linkage between binding and activation of androgen receptor as a nuclear transcription factor in females and reproductive dysfunction as evidenced through reductions cumulative fecundity and spawning in repeat-spawning fish species. Cumulative fecundity is the most apical endpoint considered in the OECD 229 Fish Short Term Reproduction Assay. The OECD 229 assay serves as screening assay for endocrine disruption and associated reproductive impairment (OECD 2012). Cumulative fecundity is one of several variables known to be of demographic significance in forecasting fish population trends. Therefore, this AOP has utility in supporting the application of measures of androgen receptor binding and activation as a nuclear transcription factor as a means to identify chemicals with known potential to adversely affect fish populations.

2. Synthesis of the main issues on reviewers comments

All the reviewers considered the AOP well written and scientifically correct. However, there was often a wish to have the limitations and the strength of weight of evidence captured under each key events to understand the regulatory standpoints. The AOP is based mainly on the fathead minnow data on limited chemicals tested, and hence some reviewers considered that it should take into account also data on other species (e.g. zebra fish, medaka and posibly stickleback) and chemicals.

However, all the reviewers recommend this to be submitted to the WNT and TFHA.

The reviwers comments are organized below according to the charge questions given to them.

Charge question 1: *Check if the AOP incorporates the critical scientific literature and if the scientific content of the AOP reflects the current scientific knowledge on this specific topic*

Reviewer's main comments:

- The current AOP was consider well written with current scientific knowledge. However, it would be important to have limitations and the strength of weight of evidence captured under each key events to understand the regulatory standpoints.
- The considerations for the applicability of the current AOP to evaluate different taxonomic groups and the range chemicals would add more clarity.
- More fish data on other AR agonists e.g. 17α-methyltestosterone, androstenedione, diazinon, and methyldihydrotestosterone should be included with relevant references to increase understanding and application of this AOP in the regulatory field more. Also, an explanation is needed whether or not these chemicals produce similar effects as described in the AOP.
- In some KEs, fathead minnow and medaka have been listed but information on the other fish like zebrafish, sheepshead minnow etc has not yet been included. Some KEs may be applicable to vertebrates, including fish. It may be important to include some results of e.g. AR and Aromatase knockout mice, and transgenic fish if possible. Information on species similarity and difference in ARs has not yet been included in the AOP. Such information may be of use for the extrapolation among species at least in future versions of this AOP.

- The current description may be only applied to the genomic action. Similar to ERs, both genomic and non-genomic mechanisms are applied to ARs too. It was suggested that this issue should be mentioned too.
- Different names for the same assays, e.g. radioimmunoassay and RIA; enzyme immunoassay and ELISA, have been used in KEs. It is important to consistently use the same expressions for these methods in the whole AOP.
- In the methods described, no current OECD activities on ARTA were included. In fact, several ARTA assays have been validated and will be developed into the test guideline. This development should be included in this section. For the in vivo fish tests, secondary sex characteristics in fathead minnow and western mosquitofish were described. Furthermore, information on medaka should be included too. Fecundity is an important apical endpoint for fish full/partial life cycle tests, and for TG240 (MEOGT).
- In addition, the other patterns of spawning, more common amongst fish should be presented as a reference at least. Fish are a highly diverse group encompassing more than 30,000 species of which very few share the spawning pattern observed in continuous spawners such as the fathead minnow and other regulatory species. As far as the androgen axis goes, the literature doesn't include traits such as the anal fin elongation in the medaka and the spiggin regulation in the stickleback, both of which can provide very insightful information on xenoandrogens.
- As a detailed observation “interrenal”, instead of “adrenal”, is used in fish.
- More MIEs e.g. receptor binding types should be included and also androgen agonist on gonadotrophins- different fish and different mechanisms of feedback.
- It was noted that the AOP is not necessarily relevant to invertebrates.

Charge question 2: *Verify the weight of evidence judgement/scoring provided by AOP developers for KEs, KERs and the overall AOP*

Reviewer’s main comments

- The weight of evidence should be more clear regarding the regulatory requirements.
- According to the description of the KE "Gonadotropins, circulating concentrations, reduction", it seems that there is a problem to measure LH and FSH in small model fish. If so, this may have a conflict with the notion that KEs must be measurable.
- In the section of Empirical Support for Linkage, results of DEHP/MEHP in mammals have been included. It was questionned if these compounds have similar mechanisms to those of fadrozole and prochloraz in fish
- In the section of Empirical support for linkage, some lines of evidence were based on an increase in 17β-estradiol concentration resulting in an enhancement in VTG in male fish.
- A weight of evidence table is needed in the annex and the tables should be presented according to the order of KEs.
- It should be noted that, if AR agonist is not aromatisable and reduces LH, then the AOP is OK, but the ecological importance of this was questionned.

Charge question 3: *What would be the regulatory applicability of this AOP in your opinion?*

Reviewer’s answers and comment

- Globally across different geographies identification and evaluation of EDCs are recommended by various regulatory agencies. This AOP covers the existing and additional events of evaluation. The set of regulatory specification/ data requirement for the AOP could help broader usage for different chemicals types.
- Identification of Endocrine Disrupting Chemicals (EDCs) is needed under several pieces of European Union (EU) legislation. Currently, the regulatory identification of EDCs is mainly based on the general consensus on the WHO definition, which consists of three essential elements, i.e.

chemical-induced adverse effects (adversity), chemical specific endocrine modes/mechanisms of action (MOAs) and the causal relationship (causality) between adverse effects and endocrine MOAs. AOPs cover all essential elements for identification of EDCs and show the complex biology of adversity and MOAs. These will help regulators understand the complexity of identification of EDCs. This AOPs includes not only EATS pathways but also other pathways, e.g. PPARs, RXR, that are essential to development, growth, and reproduction.

- This AOP is restricted clearly to female fish only as adversity is linked to reduced oestrogen synthesis (via reduced androgen synthesis); it is also limited to fully reproductive mature fish (not fish entering puberty or juvenile fish) and importantly is limited to fish that once they reach sexual maturity they spawn constantly. The latter is a reproductive strategy employed by fish that tend to occupy tropical areas (around the equator). Unfortunately, most fish species have different reproductive strategies (annual life cycle) hence the level of gonadotropin expression (and consequently steroid production) is regulated by photoperiodic and temperature changes throughout the year.

Charge question 4: *Overall Assessment of the AOP - Would you recommend this AOP to be submitted to the Working group of the National Coordinators for the Test Guidelines Programme (WNT) and the Task Force on Hazard Assessment (TFHA) for endorsement?*

Reviewer's main comments

- All the reviewers recommend this to be submitted to the WNT and TFHA. This will set the right direction for regulatory application sooner. Additionally, the strengths and weakness of this AOP should be clearly stated for continuous usage and updation.
- However all the limitations should be clearly stated in order to have transparent regulatory use.

3. Summary of the teleconference

A teleconference was organized on the AOP23 reviews back to back with AOP25 teleconference 23rd October 2015. At the time of this there were no any written responses by the author to reviewers' comments. Some general issues and some more detailed comments were discussed at the teleconference. There was a general agreement on the value of the AOP23 and that it should proceed after EAGMST review and discussion to the WNT and TFHA. It was also noted that a single AOP should be simple and robust and it should be then well interinked to other possible close AOPs, as in the case of AOP23 and AOP24, which describe linking aromatase inhibition, androgen receptor agonism, estrogen receptor antagonism, or steroidogenesis inhibition, to impaired reproduction in small repeat-spawning fish species.

The notes of the teleconference are in Annex 2.

4. Outcome of the external review

Most of the reviews (3) were uploaded in due time (end of September or early October 2015).

There were no general comments on the AOP23 by the reviewers. The detailed comments related to charge questions are available in Annex 3. It was clear during the teleconference that this AOP 23 is welcomed by all the reviewers with suggested revisions.

The author's responses were not available at the time of this report (14th December 2015).

They have been provided afterwards by the author and have been incorporated by the Secretarat in Annex 3 of this report.

Annex 1. Table of the reviewers who gave responses on AOP23

Name	Affiliation	representing	e-mail
Ramachandran Balasubramanian	ICAPO	research/academia	rbalasubramanian@hsi.org
Ioanna Katsiadaki	Centre for Environment Fisheries and Aquaculture Science (Cefas), UK	research/academia	ionna.katsiadaki@cefas.co.uk
ZhiChao Dang	Rijksinstituut voor Volksgezondheid en Milieu, Research, NL	regulator	zhichao.dang@rivm.nl

Annex 2.

Notes of the 23rd October 2015 joint teleconference on AOP 23 and AOP 25

Participants:

Author of both AOPs: Dan Villeneuve (villeneuve.dan@epa.com)
Review manager: Jukka Ahtiainen (mikris.ahtiainen@gmail.com)

- Christopher Fassbender (ICAPO, ChristopherF@peta.de)
- Ionna Katsiadaki (UK, ionna.katsiadaki@cefas.co.uk)
- François Busquet (DE, caat-eu-policy@uni-konstanz.de)
- ZhiChao Dang (NL, zhicao.dang@rivm.nl)
- Elke Muth-Koehne (DE, elke.muth-koehne@ime.fraunhofer.de)
- Helmut Segner (Switzerland, helmut.segner@vetsuisse.unibe.ch)

Project 1.12: AOP linking aromatase inhibition, androgen receptor agonism, estrogen receptor antagonism, or steroidogenesis inhibition, to impaired reproduction in small repeat-spawning fish species

As the AOP 23 Androgen receptor agonism leading to reproductive dysfunction and AOP25 Aromatase inhibition leading to reproductive dysfunction (in fish) have interlinkages and the reviewer groups have a certain overlap it was decided to have these teleconferences back to back.

- At the time of the teleconference there were no any written responses to the reviews available.
- There was a general agreement on the value of the AOP23 and that it should proceed after EAGMST review and discussion to the WNT and TFHA.
- It was also noted that a single AOP should be simple and robust and it should be then well interinked to other possible close AOPs, as in the case of AOP23 and AOP24, which describe linking aromatase inhibition, androgen receptor agonism, estrogen receptor antagonism, or steroidogenesis inhibition, to impaired reproduction in small repeat-spawning fish species.

Annex 3. Detailed comments by the reviewers and authors responses

Charge question 1: ***Check if the AOP incorporates the critical scientific literature and if the scientific content of the AOP reflects the current scientific knowledge on this specific topic***

Responses

Reviewer 1:

The current AOP is well written with current scientific knowledge. It would be important to have limitation and weight of evidence captured under each key event to understand the regulatory standpoint. The wide applicability of the current AOP to evaluate different taxonomic groups and chemicals would add more clarity.

Response from author: Weight of evidence and major uncertainties or inconsistencies are covered on the KER pages. The taxonomic domain of applicability of the AOP is described on p.35 of the pdf snapshot.

Reviewer 2:

The AOP is well described and included the essential literature, which reflects the current understanding in reproductive impairments resulted from the exposure to AR agonists in small model fish. To increase understanding and application of this AOP in the regulatory field, following points need to be clarified and further elucidated. Two chemicals, 17β-trenbolone and spironolactone, were listed. There are, however, fish data on other AR agonists e.g. 17α-methyltestosterone, androstenedione, diazinon, and methyldihydrotestosterone, which should be included too. Explanation is need whether or not these chemicals produce similar effects as described in the AOP.

Response from author: Literature was searched for reproduction studies conducted with the compounds mentioned. Two additional relevant studies with 5α-dihydrotestosterone (a non-aromatizable androgen) and six with 17α-methyltestosterone (MT) were identified and incorporated into the concordance table and weight of evidence analysis. However, an important point to emphasize is that while some of the responses to MT are consistent with the proposed AOP, it has been shown that MT can be converted to 17α-methylestradiol in vivo and elicit estrogenic responses as well. Thus, while in vitro MT would be classified as an androgen, in vivo some effects of MT may not be consistent with the present AOP depending on the degree of aromatization that occurs. Additional notes regarding this potential limitation of the AOP relative to domain of chemical applicability were added to the "uncertainties and inconsistencies" section of the AOP evaluation. This caveat regarding applicability to non-aromatizable estrogens only was already noted in the "Applicability of the AOP" section of the AOP page. The description for Event:25 also notes that "structures subject to aromatization may behave in vivo as estrogens despite exhibiting potent androgen receptor agonism in vitro."

Information has been filled in the sections of some, but not all of KEs. In some KEs, fathead minnow and medaka have been listed. But information on the other fish like zebrafish, sheepshead minnow etc has not yet been included.

Response from author: On the KE pages, only species for which a particular KE measurement has been made are identified using taxa id ontology terms. Potential applicability to other species is discussed in the "evidence supporting taxonomic applicability section". All KE pages were reviewed to make sure that entries were made wherever feasible.

Some KEs may be applicable to vertebrates including fish. It may be important to include some results of e.g. AR and Aromatase knockout mice, and transgenic fish if possible.

Response from author: Data from AR knockout studies are not necessarily relevant as support for the present AOP which deals with strong AR agonism (i.e., excess signaling through the AR), rather than a lack of AR signaling. Likewise, given that the effects described in this AOP are thought to be mediated by endocrine feedback, one cannot necessarily assume that responses to AR over-expression would behave in a manner consistent with that described for this pathway.

Information on species similarity and difference in ARs has not yet been included in the AOP. Such information may be of use for the extrapolation among species.

Response from author: Some additional information regarding nuclear AR paralogs in fish has been added the key event description for Event 25.

The current description may be only applied to the genomic action. Similar to ERs, both genomic and non-genomic mechanisms are applied to ARs too. It is suggested that this issue should be mentioned too.

Response from author: A sentence indicating that the key event is applicable to nuclear ARs, but not necessarily membrane ARs was added to the description of Event: 25.

Different names for the same assays, e.g. radioimmunoassay and RIA; enzyme immunoassay and ELISA, have been used in KEs. It is important to consistently use the same expressions for these methods in the whole AOP.

Response from author: The "How it is Measured or Detected" text was examined for each key event and radioimmunoassay or enzyme-linked immunosorbent assay were used consistently throughout as relevant.

In the methods described, no current OECD activities on ARTA were included. In fact, several ARTA assays have been validated and will be developed into the test guideline. This development should be included in this section.

Response from author: OECD Test No. 458 was added to the listing of methods for measuring or detecting the molecular initiating event of androgen receptor agonism (Event 25). Both the citation for the method and a hot link to the URL was added to the key event description.

For the in vivo fish tests, secondary sex characteristics in fathead minnow and western mosquitofish were described. How about medaka? Information on medaka should be included too.

Response from author: Information on the use of papillary process formation in female medaka as an indicator of androgen agonism in the species was added to the methods description for Event 25 along with citation and link to TG229 which provides details on how this secondary sex characteristic is measured.

Fecundity is an important apical endpoint for fish full/partial life cycle tests, and for TG240 (MEOGT). In addition to TG229, it is important to add these long term toxicity tests.

Response from author: Reference to TG240 MEOGRT and fish life cycle tests was added to the "Regulatory Examples Using this Adverse Outcome" section Event 78.

Interrenal, instead of adrenal, is used in fish. Should the term interrenal be included in the AOP?

Response from author: Adrenal changed to interrenal where cited in reference to fish.

Reviewer 3:

In general the AOP incorporates relevant current knowledge, although some aspects are restricted to one publication only; for example the process of vitellogenesis is solely references as the Tyler and Sumpter paper although it was Wallace and Selman (1981) that first (and in more detail) described the cellular and dynamic aspects of oocyte growth in teleosts.

Response from author: Reference to Wallace and Selman 1981 was added to the KER description for Event 255.

Also I think, that other patterns of spawning, more common amongst fish should be presented as a reference at least. Fish are a highly diverse group encompassing more than 30,000 species of which very few share the spawning pattern observed in continuous spawners such as the fathead minnow and other regulatory species.

Response from author: Agreed. Additional text was added to the "Domain of Applicability" section of the "Overall Assessment of the AOP" to clarify that significant uncertainty remains regarding the applicability of this AOP to fish species synchronous and group-synchronous patterns of oocyte development.

As far as the androgen axis goes, the literature doesn't include traits such as the anal fin elongation in the medaka and the spiggin regulation in the stickleback, both of which can provide very insightful information on xenoandrogens.

Response from author: Additional information on secondary sex characteristics in medaka and spiggin production by female 3-spined sticklebacks has been added as additional methods for indirectly measuring the MIE of androgen receptor agonism.

Importantly the AOP doesn't touch at all in the number and type of receptors present in different species; this is very important at binding to the receptor is key for the MIE and fish with only one type of AR are compromised by nature in their ability to detect a wide range of xenobiotics.

Response from author: Additional information concerning AR paralogs in fish has been added to the key event description for Event 25.

The weakest by far link in this AOP is the effect of androgen agonists on gonadotrophins; if we assume there is a negative feedback then the AOP stands strong all the way from the MIE to the KE, the KER and even the population trajectory besides some knowledge gaps there. However, this is not the case; many (if not most) fish species employ different reproductive strategies to the FHM or the medaka and as such they have developed feedback mechanisms that suit them best. During puberty for example many species have a positive feedback mechanism (see review by Trudeau, 1997; Antonopoulou et al, 1999 and many many more!!).

Response from author: We agree with the reviewer on this point and have added additional caveats to the taxonomic applicability section of the overall assessment of the AOP as well as uncertainties and inconsistencies section of the weight of evidence summary. Additional text regarding these uncertainties was also added to Relationship 31.

I wonder since the AOP relies on this reduction of endogenous androgens leading to reduction of endogenous oestrogens to materialise all downstream KE why there is practically no literature cited here other than a single paper on eels that if I am correct is not relevant as it is an in vitro study. In fact this reference (Huang et al, 1997) is missing from the list but is cited on page 35.

Response from author: Evidence supporting the idea that reductions in endogenous androgen production leads to reductions in endogenous estrogen production is provided as part of the key event relationship description for Relationship 302. The Huang et al. 1997 paper (cited on the AOP page) was only cited to point out that there is at least some evidence that androgens may not always elicit negative feedback on gonadotropins at the level of the pituitary. Additional sentences have been added to clarify that the KER linking androgen receptor agonism to reduced gonadotropin secretion has significant uncertainties, particularly with regard to fish employing different reproductive strategies.

In terms of taxonomic applicability, none of this is relevant to invertebrates as they do not use vertebrate steroids to regulate their reproduction. I suggest we clearly state this rather than say ...not necessarily relevant to invertebrates (i.e. page 3) it's definitely not relevant to them.

Response from author: The taxonomic applicability of Event 25 was updated to indicate that key event of androgen receptor agonism is not relevant to invertebrates. Taxonomic applicability text was also added to events 274 and 3, indicating that those events are relevant to vertebrates and amphioxus only, not invertebrates.

A minor point, I am not sure the term lutenising hormone is used in fish; instead we recognize two FSH; trivial perhaps but worth considering

Response from author: There is an extensive literature referring to luteinizing hormone in fish and at least some fish appear to conform with the two cell-two gonadotropin model for regulation of steroid biosynthesis (e.g., Senthilkumaran et al. 2004). No changes were made in response to this comment.

Charge question 2: ***Verify the weight of evidence judgment/scoring provided by AOP developers for KEs, KERs and the overall AOP***

Responses

Reviewer 1:

The weight of evidence summary in page 30 of this AOP lists that the WoE for androgen receptor, agonism as 'weak', while the other events are moderate to strong. This is may need further detailing to address the regulatory requirements and ways by which the limitation could be addressed.

Response from author: Note, the weight of evidence linking AR agonism to reduced circulating gonadotropins was judged to be weak, in part because gonadotropins cannot be readily measured in most fish species and in part because there are life-stage and reproductive strategy-dependent differences in endocrine feedback regulation which may make it difficult to generalize this KER across a broad range of fish species. While this represents a weakness in the mechanistic understanding of the association between AR agonism and the other downstream KEs, there is strong empirical support for the association between exposure to AR agonists and reductions in T and E2 production, decreased circulating E2 and VTG, etc. In order to better capture the weight of evidence that bridges over this portion of the AOP for which there is currently weaker support, we have added some KERs linking KEs that are non-adjacent in the AOP. Relationships 31 and 143 attempt to lay out the mechanistically-plausible connection, while the new relationships added (Relationships 32; Relationship 1384; Relationship 1385; Relationship 1386 lay out empirical support for the association of AR agonism with KEs further downstream.

Reviewer 2:

According to the description of the KE of Gonadotropins, circulating concentrations, Reduction, it seems that there is a problem to measure LH and FSH in small model fish. If so, this may have a conflict with the notion that KEs must be measurable.

Response from author: While we have focused on assembling evidence for fish, aspects of this AOP may apply to other vertebrates as well and in other vertebrates gonadotropins are measureable. Thus, we felt it was reasonable to include this as a KE, despite the general inability to measure it in fish. It also serves the important role of establishing the mechanistic plausibility of a relationship between AR agonism and decreased production of E2 and T in female fish, which is observed despite the fact that the AR agonists that act as MIEs in this AOP do not directly inhibit steroid biosynthesis. While the empirical support for the gonadotropin-related KERs is weak, we feel the plausibility strengthens the overall AOP.

In addition, KEs have been numbered in the concordance table, in which the KE of Gonadotropin reduction was not included. Explanation is needed why this KE is not included.

Response from author: The concordance table has been revised to list gonadotropin reduction as a KE.

Over the relationship: Androgen receptor, Agonism Directly Leads to Gonadotropins, Circulating Concentrations, Reduction, the term “directly leads to” seems confusing because the direct target of AR agonists may not be pituitary and a decrease in gonadotropin levels results from a negative feedback mechanism in which neurotransmitters are involved. This relationship can be understood as indirect process too.

Response from author: This is a common point of misunderstanding with regard to what's intended by the terms "directly leads to" versus "indirectly leads to". Relative to AOP development, direct versus indirect pertains only to whether KEs being linked together are adjacent to one another in the sequence defined for the AOP (directly linked), or whether they are non-adjacent (indirectly link). It is not intended to convey whether the biological interaction is direct or mediated through additional steps. Arguably, steps in the pathway could nearly always be broken down to finer and finer levels of resolution with regard to defining what is direct. However, the goal of the AOP is to provide enough biological detail to support extrapolation along the pathway and regulatory use, but not to get lost in the weeds of every detail of the biology. From the standpoint of androgen receptor agonism and circulating gonadotropins reduction those event are adjacent in the sequence defined, and thus the term "directly leads to is applied" and they are represented with a solid arrow in the network diagram.

In the section of Empirical Support for Linkage, results of DEHP/MEHP in mammals have been included. Did they have similar mechanisms to those of fadrozole, prochloraz in fish?

Response from author: This evidence was added by another contributor. Nonetheless, it shows that exposure to DEHP/MEHP in mammals results in both reduced production of E2 and reduced circulating E2 concentrations. Whether it acts through mechanisms similar to those of fadrozole and prochloraz or not, it provides evidence that changes in E2 production by ovary tissue can lead to changes in circulating concentrations, which is what this KER is concerned with.

In the section of Empirical support for linkage, some lines of evidence were based on an increase in 17β-estradiol concentration resulting in an enhancement in VTG in male fish. Such evidence is different from the title of reduction and female of AOPs.

Response from author: While induction of VTG in male fish exposed to estrogens is not synonymous with the current KER of reduced 17β-estradiol leading to reduced VTG production, what those data help establish is that VTG production is estrogen dependent. To make this distinction clearer, data for male fish were organized under the heading "support for estrogen-dependent regulation of vitellogenin production".

A weight of evidence table is needed in the annex.

Response from author: A weight of evidence table was added.

The table of the KEs was not presented according to the orders of KEs, which may cause some confusing. It is suggested that the table of KEs should be organized according to the order of KEs.

Response from author: Following the update to AOP-Wiki 2.0 the order of the KEs and KERs within the tables on the AOP page was corrected.

Reviewer 3:

As expanded above, once we accept that an AR agonist is not aromatisable (species differ enormously on their ability to aromatase or not specific androgens) and has a negative feedback for LH, then the weight of evidence is moderate to strong and the AOP stands. However, my concerns are entirely on how relevant is this for ecologically important species that ultimately we are trying to protect via this exercise. If we are interested in the adverse outcome for a laboratory strain of FHM, then by all means the evidence is there in most relationships including the adversity. However, I do not believe this is how most species regulate their reproduction and more information should be made available to regulators on these critical assumptions.

Response from author: Agreed. We have added additional caveats concerning the uncertainties regarding the relevance of this AOP to fish species employing reproductive strategies different from those of fathead minnow. Additional research is underway to examine the validity of these relationships for fish species with synchronous oocyte development. Nonetheless, examining the applicability of this AOP for a broader range of fish and other vertebrate species should be a priority to enhance regulatory application.

Charge question 3: ***What would be the regulatory applicability of this AOP in your opinion?***

Responses

Reviewer 1:

Globally across different geographies identification and evaluation of EDCs are recommended by various regulatory agencies. This AOP covers the existing and additional events of evaluation. The set of regulatory specification/ data requirement for the AOP could help broader usage for different chemicals types

Reviewer 2:

Identification of Endocrine Disrupting Chemicals (EDCs) is needed under several pieces of European Union (EU) legislation, including the Regulation on industrial chemicals (Registration, Evaluation, Authorization and restriction of Chemicals, EC 1907/2006, REACH), the Plant Protection Products Regulation (EC 1107/2009, PPPR), and the Biocides Products Regulation (528/2012, BPR). Currently, the regulatory identification of EDCs is mainly based on the general consensus on the WHO definition, which consists of three essential elements, i.e. chemical-induced adverse effects (adversity), chemical specific endocrine modes/mechanisms of action (MOAs) and the causal relationship (causality) between adverse effects and endocrine MOAs. AOPs cover all essential elements for identification of EDCs and show the complex biology of adversity and MOAs. These will help regulators understand the complexity of identification of EDCs. Besides, current regulatory tests focus on EATS pathways. In contrast, AOPs include not only EATS pathways but also other pathways, e.g. PPARs, RXR, that are essential to development, growth, and reproduction. Within each AOP, different targets at molecular, cellular, organ/tissue and individual levels could be identified and the adverse outcome would be predicated. Such information would be of help for prioritizing chemicals, for grouping chemicals and for developing an integrated testing strategy. It is important to indicate that the AOP needs extensive amount of data which might be possible for a few chemicals but will not be possible for a majority of chemicals. Current data requirements under REACH, PPPR, BPR, etc. do not cover all key events of the AOP.

Reviewer 3:

This is restricted clearly to female fish only as adversity is linked to reduced oestrogen synthesis (via reduced androgen synthesis); it is also limited to fully reproductive mature fish (not fish entering puberty or juvenile fish) and importantly is limited to fish that once they reach sexual maturity they spawn constantly. The latter is a reproductive strategy employed by fish that tend to occupy tropical areas (around the equator). Unfortunately most fish species have different reproductive strategies (annual life cycle) hence the level of gonadotropin expression (and consequently steroid production) is regulated by photoperiodic and temperature changes throughout the year. Even if a negative feedback mechanism operates in all of these species and in all life stages (which is certainly not the case) we still need to establish what is the relative strength of the AR agonist induced negative feedback to the environment-induced stimulation of gonadotropins! This link has never been studied and is critical if we really mean to protect wildlife.

Response from author: Agreed. I believe the reviewer provides an excellent critical assessment of the domain of applicability of this AOP. Consequently, I have copied this comment, verbatim, into the "domain of applicability" section of the overall assessment of the AOP, crediting the statement to reviewer 3.

Charge question 4: ***Overall Assessment of the AOP - Would you recommend this AOP to be submitted to the Working group of the National Coordinators for the Test Guidelines Programme (WNT) and the Task Force on Hazard Assessment (TFHA) for endorsement?***

Responses

Reviewer 1:

YES, recommend this to be submitted to the WNT. This will set the right direction for regulatory application sooner. Additionally, the strengths and weakness of this AOP should be clearly stated for continuous usage and updating

Reviewer 2:

Yes. It is important to get the official stamp for publishing the AOP. To increase the regulatory applications of the AOP, following points are suggested: The current AOP focuses only on female fish. As the majority of test guidelines include both males and females, it is important to include certain information/statement over male fish so that regulators can get an overall picture. It is also important to indicate that the major androgen in male teleost fish is 11-ketotestosterone. In addition, it is important to specify the uncertainties or inconsistencies that are related to chemicals. This would be of great help for non-experts and risk assessors to understand confounding factors.

Reviewer 3:

Yes as long as the limitations based on feedback mechanisms and reproductive strategies are made even clearer than currently are. This is relevant ONLY for small repeat-spawning fish species but this is mentioned only once in the title throughout the AOP. Assuming this is presented as a warning on the first paragraph, then my recommendation would be to submit to WNT.

Response from author: A prominent statement regarding the potential limits of the domain of applicability of this AOP was added to the abstract.