Organisation for Economic Co-operation and Development

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Adverse Outcome Pathway External Review Report

AOP 21: Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2

Short Title: AhR mediated mortality

Enter any logistical information related to the meeting e.g. meeting date, time and location.

The title of the AOP was revised during the review.

Original title of the AOP: AhR Activation Leading to Early Life Stage Mortality

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1. Introduction and background to specific AOP

Background

The project for the development of AOP 21: *AhR Activation Leading to Early Life Stage Mortality* was included on the AOPs Development Programme Workplan in 2014 (project 1.27) and is led by USA and Canada.

Based on the internal review in early 2017 Internal review AOP 21, the Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) agreed at its June 2017 meeting, that the draft AOP [snapshot of 04-12-2017 PDF] was ready for external expert review. In addition, EAGMST recommended that AOP21 is reviewed in parallel with AOP 150: *Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty*, with which it shares several common elements.

A joint scientific review panel (Annex1) for both, AOP21 and AOP150, was selected by an independent review manager in accordance with the Standard Operation Procedure (SOP) for Adverse Outcome Pathway Scientific Review (v.7 December 2017).

The review panel was charged with reviewing the scientific content of the draft AOP based on four charge questions (CQ) previously agreed by the EAGMST and outlined in the SOPs:

CQ1. Scientific quality:

- Does the AOP incorporate the appropriate scientific literature?
- Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

CQ2. Weight of evidence:

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

CQ3. Regulatory applicability:

- Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

CQ4. Conclusion:

- What are your overall conclusions of the assessment of this AOP?

In addition, the joint panel was asked to particularly consider whether the content of the individual abstracts for each of these two similar AOPs represent a clear stand-alone guidance for the users.

The review was conducted during December 2017 and April 2018. Based on the initial responses to the charge questions (Annex 2) main issues (section 2) were discussed at a teleconference on 9 March 2018 (section 3). Based on the TC discussion (section 3.2), actions arising (section 3.3), and additional written discussion (Section 4), authors revised the AOP as outlined in section 3.3. Revisions were considered by reviewers before this report was finalised. As a result of the review the AOP was also renamed. The new title of AOP21 is: Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2

Introduction

This adverse outcome pathway AOP 21: *AhR Activation Leading to Early Life Stage Mortality* includes the description and assessment of the critical elements of the pathway initiated by activation of the aryl hydrocarbon receptor (AhR) leading to early life stage mortality in fish but also other oviparous vertebrate taxa.

The Molecular Initiating Event (MIE) of this AOP (Figure 1) is the activation of AhR by exogenous ligands/stressors leading to its nuclear translocation and interaction with the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT complex binds to dioxin-responsive elements on the DNA and modulates the expression of dioxin responsive genes. Specific to this AOP is the up-regulation of cyclooxygenase 2 (COX-2), inducible enzyme catalysing the synthesis of prostaglandins which have roles in cellular homeostasis and in promoting inflammatory responses.

Increased expression of COX-2 during the early stages of development is associated with perturbations in the development and function of the cardiovascular system, particularly evident in fish as reduced heart pumping efficiency, reduced blood flow, eventual cardiac collapse and death, ultimately leading to population decrease.

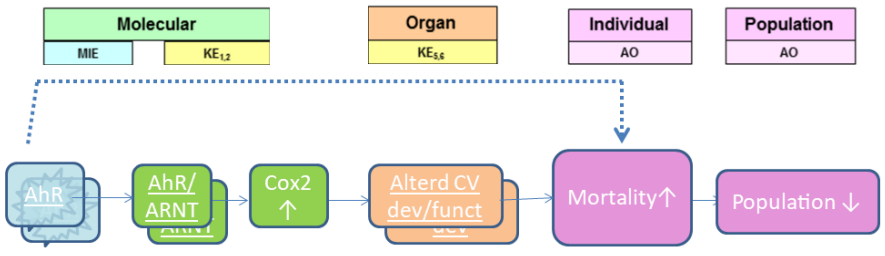

Figure1: Graphical representation of the components of AOP21 as submitted for review. Duplicated elements are shared by AOP150 and also relate to other components of AOP150. Dashed line represents non adjacent KER.

Another specificity of this pathway is that it is initiated via AhR isoforms (e.g. in fish) with low affinity for the prototypical AhR exogenous ligands, which may also be rapidly metabolised leading to rapid but transient AhR activation.

Notably, AhR isoforms in fishes show very low affinity for the most potent prototypical AhR activators (certain polychlorinated biphenyls (PCBs)), compared to AhRs from birds and mammals, and this may account for some of the observed interspecies differences.

Fishes represent the most sensitive taxa to adverse effects of stressors activating AOP21. In terms of life stage, fish embryos are more sensitive than juvenile or adults, based on endpoints that could be of significance to population trends. Hence, the development of this AOP draws heavily on evidence from zebrafish (*Danio rerio*). However, relevant information from studies showing comparable apical manifestations of activation of the AhR across other freshwater and marine teleost and non-teleost fishes, as well as birds has been included. In addition, relevant mechanistic data from other species (e.g. mice) have been considered for particular KEs and KERs which are shared with other related AOPs, such as AOP 150: *Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty*. However, at this stage of the pathway development, the applicability of AOP21 to mammals is uncertain.

Despite conservation of the AOP elements across taxa, great differences in sensitivity to their perturbation exist, both among and within taxonomic groups. Therefore, the AOP could help support the mechanistic exploration and understanding of these differences.

2. Synthesis of main issues of the review

Individual review comments are available in Annex 2 of this report.

The joint review of AOP 21 and AOP 150 presented a unique challenge for most reviewers. These two distinct adverse pathway share significant elements, some of which differ only in the life stage dimension (e.g. mortality at embryo or early adult stage), others in the taxonomic and even in the tissue applicability. All of this made the review demanding in terms of extracting specific issues for each particular pathway.

In this respect a main issue and recommendation for improvement was in the contextual presentation and better emphasis of the distinctive elements of the two pathways. Specifically it was suggested:

- Abstract and background should highlight the broader context of the interplay with AOP 150 and the distinct roles of COX2 and VEGF[1] in embryo and cardiovascular development and function.
- Abstract should better align with that of AOP150 where biological plausibility and WoE are briefly discussed.

[1] VEGF- Vascular endothelial growth factor

- Background section should include a reference to the specific element of this pathway, the Cox2 induction.

It was suggested that a natural progression over time could be to combine the two AOPs. However, there was also a view that at present they represent two distinct AOPs.

An issue was raised regarding the 'action' for the protein dimerization activity in KE944: dimerization, AHR/ARNT being designated "disrupted" for both AhR and ARNT. While this may be appropriate for ARNT in AOP150 where the normal ARNT/ HIF1 is disrupted, it is not so for the AhR, particularly in AOP21 and AOP 131. KE944 is shared in all these AOPs.

Summary of responses to CQ 1 - Scientific Quality

There was a general agreement that the AOP incorporates the most important scientific literature and current scientific knowledge in this field.

KER1492: activation of the AhR indirectly leads to early life stage mortality, was assessed as best supported by the evidence.

However some inconsistences and contradictions in the interpretation of the cited literature were noted throughout the document e.g. relative potency of the prototypical agonist TCDD[2] and taxonomic applicability.

Suggestion was made to include references to relevant (Q)SAR approaches for predictive assessment of the MIE and for other approaches for measurement of COX-2 induction, e.g. protein levels.

Additional discussion of scientific literature was requested to support KE 442: Population trajectory decrease and, KER1490: Altered, Cardiovascular development/function leads to Increased, Mortality.

Summary of responses to CQ 2 - Weight of Evidence

Reviewers generally agreed with the scoring of the WoE for the KEs and KERs. Some clarifications and additional considerations were requested for:

- KE 442: Population trajectory decrease
- the justification of the "strong" call for the WoE for KER1490 (also see above CQ1)
- clearer presentation of the relationship between the Cox- and Sox9- mediated pathways to cardiovascular alterations – issue of essentiality of Cox-2 activation
- the consistency of the stressor induced (particularly TCDD) effects across species in the overall assessment of the AOP – issue of the amphibians

[2] TCDD - Tetrachlorodibenzo-p-dioxin

In view of the joint review, the WoE for AOP21 was assessed as less convincing compared to that for AOP150.

Essentiality of Cox-2 activation for the downstream effects was assessed as the weakest link of this pathway. However reviewers agreed that the uncertainness of these aspects were well covered by the authors.

Summary of responses to CQ3 - Regulatory Applicability

Given the shared elements with AOP 150, it was remarked that, comprehensive set of AOPs covering cardiovascular (CV) alterations would be of great benefit for both, AOP21 and AOP150. In this context, additional consideration of the interconnectedness between COX-2 and VEGF mediated pathways, as well as the importance of other genes for cardiac development and function, would add value to the description of AOP21.

Reviewers view potential applicability of AOP21:

- for screening level hazard assessment and identification of COX-2 and AhR activating substances for further investigation
- to provide mechanistic information for development of testing strategies for AhR binding and activating substances

Summary of responses to CQ4 - Overall conclusions of the assessment

Overall this AOP provides a logical rationale for a possible mechanism by which dioxin-like compounds (DLCs) can lead to cardiotoxicity and ultimately early life stage mortality, while clearly identifying the uncertainties with the essentiality of Cox2 induction.
Opinions were split about the clarity of the discussion of the interspecies differences in sensitivity to this adverse pathway (e.g. comments 27 and 29).

Discussion of the links of this AOP to AOP150 and other CV toxicological relevant pathways was strongly encouraged. It was recommended to include it, either in the background or, in a different separate section(s), where WoE of relevant links is assessed in this pathway.

Additional Question: Is the Abstract Section clear enough to stand alone from the AOP page

All reviewers suggested that the Abstract section can benefit from the inclusion of considerations for the related pathways (AOP150 and even AOP 131) and increased emphasis on the role COX-2 as a specific element of this AOP.

3. Summary record of the teleconference

9 March 2018, 3pm Paris time

Joint end-of-review teleconference (TC) was held for AOP 21 and AOP150. It was attended by all reviewers, the authors of the two AOPs and the review manager (Annex 1).

Before the TC authors provided initial written responses to most of the comments (Annex 2). These provided the starting point for the discussion.

3.1. TC agenda

1. Introduction of participants

2. Short introduction by Review Manager (RM)

   - Context of the review process and the report content
   - Context of the guidance for development and assessment of AOPs
   - Joined overview of the AOP21 and AOP 150 with main issues

COMMON ISSUES for AOP21 and AOP150:

3. Need to **increase clarity** over the distinctive characters of the two AOPs (comm. no: 1, 2, 4 in both AOPs; comm. No: 25, 37 in AOP21; comm. No: 15a, 26, 27, 31, 32, 38 in AOP150)
   a) provide more context and discuss relevant aspects of the other AOP (comm. No: 31, 32 in AOP21; 15a, 16 in AOP150)
   b) consider the reference to early life mortality versus embryotoxicity in the AOP titles and as distinct aspects of the two AOPs (comm. No: 15b in AOP 150)
4. **KE944** – AHR/ARNT dimerization: is action "decreased" appropriate? (comm. no: 3 for both AOPs)
5. **KE18**: inclusion of QSAR methods for predicting MIE and corrections within the description of current methods (comm. no: 9 in AOP21; no: 12 in AOP150)
6. **KER972**- Quantitative understanding call: strong or weak with identical considerations? (comment introduced by RM)
7. Issues with **NCBI links** (comm. No 7 AOP150)

SPECIFIC SCIENTIFIC ISSUES AOP21

8. **Inconsistences in taxonomic applicability (TA) discussion** (com. no: 7, 12, 13, 17, 19b, 27, 29)

9. Support for **KER1351**: KE2 (Cox2 induction) to KE3 (CV development/function) moderate or weak? (comm. No: 11a, 15) where particular issue is dealing with **KE2 essentiality** (comm. No: 11a, 19c, 30)
10. Add info about **KE442** (Decreased, Population trajectory)**, KER1490** (Altered, Cardiovascular development/function leads to Increased, Mortality) to support "strong" call (comm. no: 10b, 11b, 16, 18)
11. **KE1269** (Increase, COX-2 expression) – Detection methods for of COX2 protein (comm. no: 10a)
12. Overall clarity of the **WoE discussion** (com. No: 20, 19a)

SPECIFIC SCIENTIFIC ISSUES AOP150

13. **WoE summary tables**– (comm. No: 21)
14. Description of the **stressors in the AOP summary**, including strength of evidence (comm. No: 11, 18)
15. **KE945** (reduced dimerization, ARNT/HIF1-alpha) – **ontology term** 'decreased' for both components (comm. No: 13a)
16. **KE948** (reduced production, VEGF): detection assays (comm. No: 13b)
17. **KE110** (impairment, endothelial network): in vitro-to-in vivo extrapolation of data (comm. No: 15c)

OTHER

18. Abstract changes/additions to "stand alone"

19. Regulatory applicability/significance discussion for both AOP (Comm. No: 21-25 in AOP21; 23-27 in AOP150)

20. Overall conclusion about the AOPs – open discussion guided by the initial written comments (AOP21- comm. No: 26-32; AOP150 – comm. No: 28-32; comm. introduced by RM)

3.2. Main issues and responses during the call

Agenda item 2: The review manager provided short overview of the OECD Review process and the expected outcomes. Shortlist of common and specific issues was also presented and agreed:

Common issues:

1. How to emphasise the distinctive character of each AOP up-front and clearly, while providing sufficient context.

2. Adding info to KEs and/or KERs

   - When not an original author
   - When obvious but authors are not experts

Specific scientific issues AOP21

3. Inconsistences in taxonomic applicability discussion

4. KER1351: KE2 (Cox induction) to KE3 (CV development/function) moderate or week?

    - particularly KE2 essentiality

5. Overall clarity of the WoE discussion

Specific scientific issues AOP150

6. Overall WoE summary tables versus narrative

The discussion followed the more detailed agenda (above) where individual comments (as numbered in Annex 2) were grouped around a common issue.

Agenda item 3: There was a general agreement that the scientific basis of both, AOP21 and AOP150 is solid. However, most find simultaneous navigation through the life stage and taxonomic applicability of the two AOPs sharing a number of KE and KERs, a unique challenge. In this context it was argued that there is a need to find a way to emphasise the **distinctive character of each AOP** up-front and clearly, **while** at the same **time providing a sufficient context** of the other AOP.

In addition, one reviewer commented that both, AOP21 and AOP150 focus on one single gene (Cox2 and VEGF, respectively) leading to the adverse outcome, without sufficient discussion of the wider context and links to other important phase I and phase II enzymes genes (e.g. Cyp1a1) and pathways.

In terms of the lack of clarity of **distinctive character/aspects** of the two AOPs, reviewers emphasised that the issue is not a matter of the content of the AOP elements or their particular sequence in each particular AOP, but that during the joint review it was hard to keep focus on what was the **key trigger**(s) for one or the other AOP while reading common elements.

It was recognised that these issues may have been augmented by the outline of the snapshot file provided for review. Many reviewers found the outline of the AOPs within the AOP-Wiki much more logical and appreciated the ease of navigating.

Response: Authors agree that AhR activation induces a number of genes. However it was pointed out that the evidence linking AhR activation to impairment of CV development/function via Cox2 was strong, and while other genes (e.g. Sox9b, Cyp1a) may play a role in modulating these AOPs, evidence from knock-out studies, does not link these genes to mortality as an endpoint.

To address the context issue, it was agreed to modify the Background to emphasise that the "low resolution" of the Cox2↑ to altered CV development/function is a consequence

of the limited supporting evidence and does not exclude refinement in the future (Action 1 below).

The proposed modification aims to help direct the users to consider other relevant pathways before the networks are developed which should address the issue of context in the future.

In terms of **key triggers** for AOP21 versus AOP150 following AhR induction, authors argued that currently, there is no information that would help distinguish them. It depends on time point observed, tissue, species etc. However it was agreed that the interaction/common elements of the two pathways should be discussed, even briefly, in each of the abstracts (Action 2).

Reviewers also brought up the possibility that the lack of **clarity/distinction between the AO** embryolethality in AOP150 and the AO mortality applicable to two life stages, embryo and early life in AOP21, may also be part of the confusion. It was agreed by the authors to develop a common AO at the individual level (Action 3).

Agenda item 4: A reviewer questioned the "decreased" action call for KE944: AHR/ARNT dimerization.

Response:
In their initial response the authors agreed that the action needs to be changed to "increased". There was also agreement on Action 4 by all at the TC (Action 4).

Agenda item 5: Modification required for KE18 (MIE) in terms of assays for detection were discussed and agreed at the TC leading to Action 5.

Agenda item 6: It was noted that the call for the quantitative understanding (QA) of KER972 differs for AOP21 and AOP150 even though the considerations in the text are the same.

KER972 in AOP	Directness	Weight of Evidence	Quantitative Understanding
150	Directly leads to	Strong	Strong
21	Directly leads to	Strong	Week

It became clear that calls can be made for KERs in an AOP specific manner where considerations can be added in the KER free text. In the case of AOP21 and AOP150, the considerations are the same, but AOP21 authors gave lower weight due to the mostly indirect evidence supporting this KER.

Response:
It was agreed that the strength of the indirect evidence warrants a moderate call for KER972 in both AOPs (Action 6).

It was also considered useful as a general practice to aim to present the argumentation for the WoE and QA calls in a manner more specifically referencing the guidance criteria (e.g. stating "based on….."), to avoid discrepancies resulting from inconsistent application of the criteria (Action 7).

Agenda item 7: It was noted that NCBI links sometime lead to blank pages and it was questioned whether that is a place holder for some information.

Response:
Authors think that NCBI links do not function when a general term is specified rather than a species (e.g. birds and not *Gallus galus*). However, everybody agreed that the option to have a general term for taxonomic applicability is useful, particularly for KE, KERs and AO at higher biological level. How to deal with NCBI links, applying and/or excluding taxonomic applicability for a KE, KER and overall AOP should be discussed further with the Wiki and KB developers' team.

Agenda item 8: A number of comments pointed out to inconsistences in the presentation of the taxonomic applicability (TA) discussion throughout AOP21. In their written responses authors indicated specific (e.g. comm. no 12) or general revisions (e.g. to comments 13, 17).

This agenda item also lead to a wider discussion about how determining applicability is approached for KE, KERs and overall AOP historically and practically in AOP21 and AOP150.

Response:
It was agreed that taxonomic applicability for KEs, KERs and overall for the AOPs will be reviewed (Action 8) in both AOPs following the principle:

- Call applicability for KE or KER is based on the species in which evidence is generated (including negative calls where evidence exists demonstrating NON-applicability)
- If applicability to more general taxonomic group is specified (particularly at higher organisational level and AOP level), a qualifier/justification would be included to indicate that TA is likely or uncertain considering (un)known structural and/or functional differences/similarities between the species in the group.

Agenda item 9: A reviewer noted that the evidence supporting KER 1351: KE2 (Cox2 induction) to KE3 (CV development/function) and essentiality of Cox2 for AOP21 is quite questionable and not compelling, while the pathway relays heavily on this specific element at molecular level.

In addition, it was questioned whether discussion of Sox9 and Cyp1a1 involvement should be included.

Response:
Authors and some reviewers, pointed out that the uncertainty relating to the relevant evidence have been addressed in the overall WoE assessment.

It was reiterated that the uncertainty about KER 1351 and Cox2 essentiality in AOP21 comes from the limited evidence and not from existence of conflicting evidence. Given this the call "moderate" for KER1351 was assessed as appropriate.

It was also reiterated that only Cox2 knock outs lead to AOP21 specific AO (i.e. mortality) and not Sox9 or Cyp1a1. The latter two play a role in other AhR induced AOPs that do not lead to mortality and may even have some role but not essential in modulating cardiovascular effects associated with mortality.

Some revisions were outlined in the initial authors' responses to emphasise the above point (e.g. response to comment 19c) and this was considered sufficient by the reviewers.

Agenda item 10: Reviewers note that there is no, or very little information, for KE442, KER1490 and yet they were given 'strong" WoE calls.

Response:
KER1490 will be modified as part of the revisions linking the KE317 (impaired CV development/function) to the new individual level AO (Action 3) in both AOPs.

For populating KE442 (decreased population trajectory which is a second AO at population level in AOP21), authors suggest that the effort is shared between the AOP authors for which this KE is used (AOPs 16, 218, 219). It was suggested that EAGMST may be able to identify experts to help with populating this KE, as AOP21 authors have no strong expertise in population measurements and modelling.

AOP21 already contains an AO at individual level and that may be sufficient for this stage of the AOP development.

Agenda item 11: A reviewer questioned why KE1269 (Cox2 induction) is not including protein detection based methods.

Response:
Authors indicated that in fish (relevant to AOP21) COX2 protein detection has not been possible as there are no appropriate reagents, but agreed to include methods useful in mouse as this KE is likely to be shared by other AOPs (Action 9).

Agenda item 12: Some reviewers found the overall WoE discussion in AOP21 not sufficiently clear, as suggested by their initial review comments.

However, following the discussion at the TC and considering the revisions committed to by the authors (see Action list) it was agreed that WoE discussion would become clearer as it is already robust. It was also mentioned that the new format of the AOPs (in the Wiki and also in the pdf documents available for endorsement most recently), which brings the overall WoE assessment at the start of the AOP, should significantly improve clarity.

Agenda item 13: Discussion applicable to AOP150 only

Agenda item 14: Discussion applicable to AOP150 only

Agenda item 15: Discussion applicable to AOP150 only

Agenda item 16: Discussion applicable to AOP150 only

Agenda item 17: Discussion applicable to AOP150 only

Agenda item 18: Reviewers provided some general and specific comments for revisions in the Abstract to shape them better to "stand alone" in describing the particular AOP.

a) Suggestions for AOP21:
   - highlight Cox2 more prominently (in Background as well, comm. No:28)
   - include reference/links to AOP150 (comm. No: 33-37)
   - add a few words on the biological plausibility and WoE result (comm. No: 34)

Response:
Authors agreed to make the suggested changes (Action 13)

Agenda item 19: In terms for regulatory utility, the group agreed that both AOPs provide a good scoping document for KEs and corresponding screening level assays of AhR inducing toxicants that could lead to impairment in CV development/function and mortality.

Furthermore, given that taxonomic applicability predominantly covers fish and avian species, both, AOP21 and AOP150, could have greater regulatory significance in the context of environmental safety assessment.

Agenda item 20: As indicated in the initial review comments, it was agreed that AOP21 is comprehensive but needs improvements in the discussion of interspecies differences to aid overall understanding.

It was also discussed whether the two AOPs, AOP21 and AOP150 could eventually be joined into one single branched AOP.

In relation to the above, authors agreed that it is difficult to ascertain the relative contribution of the Cox2 versus VEGF (AOP150) pathway to AhR-induced impairment in CV development/function in oviparous species. Furthermore, they argued that having two independent AOPs, linked through common elements rather than one branched AOP, is the best possible description considering the current knowledge.

Reviewers agreed that having the two AOPs separately is appropriate representation for these AOPs.

3.3. Action list with responses from authors

1. **Update Background in each corresponding AOP to provide a bit more context and emphasise that AhR activation as a pleotropic (network) effect activating a number of genes while the AOP focuses on data strongly supporting the role of a particular element (Cox2 or NIF1α/VEGF) for CV development/function and early stage mortality.**

Response

Background section now includes:

- High-throughput, next-generation 'OMICs' technologies have identified hundreds to thousands of different genes that are regulated, either directly or indirectly, by the AhR (Brinkmann et al 2016; Doering et al 2016; Huang et al 2014; Li et al 2013; Whitehead et al 2010).
- One gene which is regulated by AhR is cyclooxygenase-2 (COX-2) which is known to have roles in development of the heart in vertebrates (Dong et al 2010; Teraoka et al 2008; 2014). AhR-mediated dysregulation of COX-2 is associated with altered cardiovascular development, decreased blood flow, and cardiac failure causing mortality in early life stages of fish and birds (Dong et al 2010; Teraoka et al 2008; 2014).

2. Include brief discussion/reference in the abstract of each AOP to possible links and overlaps with the other AOP (150 or 21, as relevant).

Response

Reference to AOP 150 was added to the abstract. See response to item (13) for details. Title for AOP 21 was changed to "Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2" to better differentiate from the title of AOP 150.

3. Authors of both AOPs to work together to develop a single individual AO (e.g. early life stage mortality) for both AOP. Relevant life stage distinction for particular species may be added in the free text of the KE or the KER leading to the individual AO.

Response

Removed from AOP 21: KE 351 (increased, mortality), KE 442 (decreased, population trajectory), KER 428 (increased, mortality leads to decreased, population trajectory), KER 1490 (altered, cardiovascular development/function leads to increased, mortality), and KER 1492 (activation, AhR leads to increased, mortality).

Added to AOP 21: KE 947 (increase, embryotoxicity), KER 1567 (altered, cardiovascular development/function leads to increased, embryotoxicity), and KER 984 (activation, AhR leads to increase, embryotoxicity).

Moved applicable content for fishes from KE 351 to KE 947 and from KER 1492 to KER 984.

Changed "embryotoxicity" to "early life stage mortality" in KE 351 title to allow shared applicability across taxa. Definition of "early life stage mortality" for fishes is clarified as "In Fishes: Early Life Stage Mortality refers to death prior to yolk sac adsorption and swim-up.".

KE 351 is no longer used by AOP 21, but is still used by 10 other AOPs.

KER 1492 is no longer used by AOP 21, or any other AOP, and can be deleted as an extraneous page.

KE 442 and KER 428 are no longer used by AOP 21, but are still used by 3 and 1 other AOP, respectively.

These changes mean that AOP 21 and AOP 150 now share a single AO and associated KER. These changes also mean that AOP 21 no longer includes effects on population trajectory as sufficient information is not available to properly populate this page at this time.

4. Change action for KE944: dimerization, AHR/ARNT from "decreased" to "increased".

Response

Corrected by author of AOP 150.

5. Change content of "how is this event measured" for MIE (KE18)

Response

To be addressed by author of AOP150

6. Modify the quantitative understanding of KER972 to 'moderate' for both, AOP21 and AOP150.

Response

Corrected for AOP 21 as suggested.

7. Review WoE and quantitative understanding calls for all KE and KERs for consistency between the two AOPs, and if any revisions are necessary consider specifying the particular criteria aspects relevant to the call.

Response

The Quantitative Understanding section of the main AOP paper was modified to include "The majority of the quantitative understanding and the strongest quantitative understanding is for the indirect relationship between activation of the AhR and early life stage mortality."

All WoE and quantitative understanding calls were checked for consistency between AOP 21 and AOP 150 for shared KE/KERs and no differences were identified. Rationale for WoE calls in AOP 21 were added to the "Overall Assessment of the AOP" section as follows:

Activation, AhR leads to dimerization, AHR/ARNT: High

Rationale: The call of 'High' is based on overwhelming empirical evidence in numerous species of mammals, birds, amphibians, and fishes. Further, because of overwhelming evidence of essentiality based on targeted knockdown/knockout studies. No uncertainties or inconsistencies are known which affect the WoE call.

Dimerization, AHR/ARNT leads to increase, COX-2 expression: High

Rationale: The call of "High" is based on convincing empirical evidence in three species (two fish and one bird). Further, because of convincing biological plausibility based on identification of dioxin-response elements in the promoter region of COX-2. Uncertainties and inconsistencies are only related to lack of any information on species outside of the three model species that have been investigated.

Increase, COX-2 expression leads to altered, cardiovascular development/function: Moderate

Rationale: The call of "Moderate" is based on overwhelming empirical evidence and evidence of essentiality in three species (two fish and one bird) based on studies using targeted knockdown of genes and selective agonists/antagonists. However, a lack of information on the role of COX-2 in cardiovascular development/function makes biological plausibility questionable at this time. Further, there is some uncertainty associated with pleiotropic effects of AhR activation and the high probability of multiple mechanisms acting concurrently to cause altered cardiovascular development/function.

Altered, cardiovascular development/function leads to increase, early life stage mortality; High

Rationale: The call of "High" is based on overwhelming empirical evidence and biological plausibility in numerous species of mammals, birds, and fish. There are no known uncertainties or inconsistencies at this time.

Activation, AhR leads to increase, early life stage mortality: High

Rationale: The call of "High" is based on overwhelming empirical evidence and evidence of essentiality in numerous species of mammals, birds, amphibians, and fishes using regression analysis and targeted knockdown/knockout of AhR. There are no known uncertainties of inconsistences at this time.

8. Review all taxonomic applicability calls and the justifications for applicability to wider taxonomic groups following the principle discussed under agenda item 8.

Response

Completed for AOP 21. Each KE and KER page now only includes species for which data is available in the literature, except for broad pages, for example "increase, early life stage mortality" which states taxonomic applicability as "vertebrates". Taxonomic applicability of the main AOP page was limited to species for which data is available for all KEs using in AOP 21.

Taxonomic applicability calls were updated for KER 972 to include all species referenced in the "Key Event Relationship Description" text "AhRs can heterodimerize with ARNT1 and ARNT2 isoforms in order to activate reporter constructs in transfected cells and recognize response elements in gel shift assays in all investigated vertebrates, including birds, fishes, and reptiles (Abnet et al 1999; Andreasen et al 2002a; 2002b; Bak et al 2013; Doering et al 2014; Doering et al 2015; Farmahin et al 2012; 2013; Hansson & Hahn 2008; Karchner et al 1999; 2006; Lavine et al 2005; Shoots et al 2015; Tanguay et al 1999; 2000; Wirgin et al 2011)".

9. Add protein measurement methods for detection of cox2 induction.

Response

Text was added to KE 1269 page as "COX-2 could be measured by use of ELISA or Western Blot, but commercial kits are not currently available for fishes or birds."

10. Action applicable to AOP 150 only

11. Action applicable to AOP 150 only

12. Action applicable to AOP 150 only

13. Update Abstract to:

- *make reference to common elements with the other AOP*
- *include a conclusion about WoE now missing in AOP21*
- *reorganize AOP21 abstract to be more direct in getting to the specifics of this AOP. (e.g. cox2 role) before the general statements*

Response

The abstract for AOP 21 was modified to read:

This adverse outcome pathway details the linkage between activation of the aryl hydrocarbon receptor (AhR) and early life stage mortality in oviparous vertebrates. This AOP can be initiated by a range of planar aromatic hydrocarbons, but is best known as the target of dioxin-like compounds (DLCs). These planar compounds are able to bind to the AhR causing heterodimerization with the aryl hydrocarbon nuclear translocator (ARNT) and interaction with dioxin-responsive elements on the DNA causing an up-regulation in dioxin responsive genes. Hundreds to thousands of genes are regulated, either directly or indirectly, by the AhR. One dioxin-responsive gene is cyclooxygenase 2 (COX-2) which has roles in development of the cardiovascular system. Up-regulation in expression of COX-2 causes alteration in cardiovascular development and function which results in reduced heart pumping efficiency, reduced blood flow, and eventual cardiac collapse and death. Comparable apical manifestations of activation of the AhR have been recorded across freshwater and marine teleost and non-teleost fishes, as well as birds. Therefore, this AOP might be broadly applicable across oviparous vertebrate taxa. Despite conservation in the AOP across taxa, great differences in sensitivity to perturbation exist both among and within taxonomic groups. Therefore, this AOP has utility in support of application toward the mechanistic understanding of adverse effects of chemicals that act as agonists of the AhR, particularly with regard to cross-chemical, cross-species, and cross-taxa extrapolation.
In general, biological plausibility of this AOP is strong based heavily on evidence collected from zebrafish (*Danio rerio*) through mechanistic investigations by use of targeted knockdown of AhR, ARNT, or COX-2 and through use of selective agonists and antagonists of COX-2. However, uncertainties exist regarding the interaction of multiple potential targets of AhR activation, including CYP1A, Sox9b, and HIF1a/VEGF. Quantitative understanding is largely limited to the indirect KER between AhR activation and early life stage mortality.
Since activation of the AhR causes pleotropic responses, it is a challenge to elucidate the precise series of key events which link activation of the AhR to early life stage mortality. Because of this uncertainty, other possible AOPs (ex. AOP 150) have also been proposed and likely occur simultaneously with COX-2 to cause altered cardiovascular development and function leading to early life stage mortality.

ADDITIONAL COMMENTS FROM AUTHORS:
All revisions listed in Annex 2 of AOP 21 have been made to the associated wiki pages.

Additional revisions were included in KER1351 to clarify further aspects of WoE for essentiality of Cox2 (related to the discussion under Agenda item 9). The revised "Empirical evidence" for KER1351 now states:

"Blocking induction of COX-2 through knockdown of COX-2 or through selective antagonists of COX-2 in zebrafish, Japanese medaka (*Oryzias latipes*), and chicken (*Gallus gallus*) prevents alteration in cardiovascular development and function by 2,3,7,8-TCDD, including prevention of pericardial edema, changes in heart size, and reduction in blood blow (Dong et al 2010; Teraoka et al 2008; 2014).

Knockdown of and selective antagonists of thromboxane A synthase 1 (CYP5A), which is down-stream of COX-2 in the prostaglandin synthesis pathway, prevents alteration in cardiovascular development and function by 2,3,7,8-TCDD (Teraoka et al 2008)."

4. Further Discussion

Following the TC a written discussion was continued regarding the implications of Action 3, (the development of new and common AO for both AOPs) for the titles and the indirect KER leading from the MIE to AO.

Authors developed a new AO: Early life stage mortality. Reviewers agreed that this is an appropriate revision.

Given that the two AOPs now share the MIE and the AO, the title for AOP21 was changed to "Aryl hydrocarbon receptor activation leading to early life stage mortality, **via increased COX-2**"

Reviewers agreed that the new titles reflect well the AOP content and help distinguish clearly AOP21 from AOP150, whose title was changed to "Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF".

In addition, the indirect KER1492 that linked MIE to AO in AOP21 is now modified and merged with the similar KER984 in AOP150, by including the appropriate content (mostly related to fish) from KER1492 in KER 984.

During the TC the group also identified general AOP development points for further discussion by EAGMST, Wiki developers and the AOP training team:

- additional guidelines/considerations should be included in the User's handbook to facilitate necessary modifications by authors of new AOPs who use pre-existing KE and KER (discussion under agenda item 17).

- discuss and develop improvements for representing taxonomic applicability more clearly and consistently (see discussion under agenda item 7 and 8)

5. Outcome of the external review

Initial review found that AOP21 is comprehensive description of an adverse pathway activated by AhR ligands leading to Cox2 induction and early life mortality. However, clarification was suggested for the discussion of taxonomic applicability and particularly interspecies differences.

Taxonomic applicability was a major point of discussion at the teleconference following the initial review comments (see discussion under agenda item 8). Authors reviewed and revised taxonomic applicability of a number of AOP elements (see response to action 8).

Another critical point of discussion for AOP21 was the evidence supporting essentiality of Cox2 induction for this pathway (see discussion under agenda item 9). However it was agreed that the 'moderate' WoE call for KER 1351 (Increase, COX-2 expression leads to Altered, Cardiovascular development/function) is appropriate given the strong and clear evidence that blocking Cox2 (by knock-down or selective Cox2 antagonists) or its known downstream effects (knock down and selective agonists of thromboxane A synthase 1) also blocks AhR activation mediated alterations of the CV development/function, although in limited number of taxa (zebrafish, Japanese medaka and chicken).

Interconnectedness of AOP21 and AOP150 was discussed at all stages of the review. Related revisions lead to AOP21 and AOP150 sharing additional common elements (see figure 2 below).

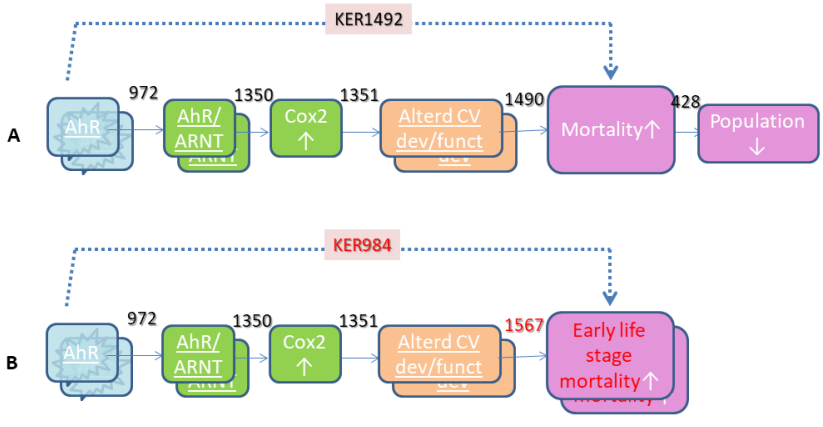

Figure2: Graphical representation of the components of AOP21 submitted for review (A) and after revision (B). Duplicated elements are shared with AOP150. Dashed line represents non adjacent KER. Numbers represent KER numerical identifier in the Wiki. KERs and KE in red represent elements modified as a result of the review that now contain merged AOP21 and AOP150 content.

As a result, only KER1350 and KER1351 are unique to the AOP21, together with KE Cox2 induction. KER972 was shared in the pre-review version.

In addition, the title of AOP21 was revised to *Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2 to* better reflect the key differences of AOP21 and AOP150.

Authors completed all revisions coming out of the review before the draft report was circulated to reviewers. Reviewers agreed that the revisions adequately addressed the issues identified during the review. However, one reviewer emphasised the significant gap of evidence supporting a direct role of Cox2 induction for altered early CV development (i.e. establishment of CV structure and anatomy), rather than CV function.

The revised AOP21, will be a valuable addition to the AOP-KB. Together with AO150 it provides a good scoping document for KEs and corresponding screening level assays for AhR inducing toxicants with potential impact on CV development/function and mortality, particularly in the context of environmental safety assessment. In addition, the uncertainties outlined in KER1351 provide a strong guide for future investigations to address the gap in the evidence linking Cox2 induction to CV development in particular.

Annex 1: List of Reviewers, Authors and Review manager

Reviewer	Affiliation	Representing country
Michael W. Hornung	U.S. Environmental Protection Agency National Health and Environmental Effects Research Laboratory Duluth, Minnesota , USA	USA
Helen Håkansson	Institute of Environmental Medicine Karolinska Institute Stockholm, Sweden	Sweden
Helmut Segner	Centre for Fish and Wildlife Health University of Bern Bern, Switzerland	Switzerland
Iva Sovadinova	Research Centre for Toxic Compounds in the Environment Faculty of Science Masaryk University Brno, Czech Republic	Czech Republic
Aude Kienzler	Joint Research Canter (JRC) Ispra, Italy	EC

Author	Affiliation
Jon Doering	National Research Council, US EPA Mid-Continent Ecology Division, Duluth, MN, USA
Markus Hecker	University of Saskatchewan, Saskatoon, Saskatchewan, Canada
Dan Villeneuve	US EPA Mid-Continent Ecology Division, Duluth, MN, USA
Xiaowei Zhang	Nanjing University, School of the Environment, Nanjing, China

Review Manager	Affiliation
Julija Filipovska	Independent Consultant

Annex 2: Individual reviewers' comments with initial responses from authors

Highlighted comments are common to both, review AOP21 and AOP150

General (relevant for both AOP 21 and AOP150)		Response	Comment NO
Reviewer 1	In general these did a good job of setting up the AOP and providing rationale and supporting evidence. I do think they stand alone as two distinct AOPs. I have suggested changes that can be made to the Abstracts and Background section to improve them.		1
	I did find this review a unique challenge. Because there were significant sections that were shared components, at times I lost track of which one I was looking at. So I spent some time initially to determine what parts were specific to these AOPs and which parts were shared components pulled from the AOP Wiki.		2
	Page 12 of both AOP21 and AOP150. 944: dimerization, AHR/ARNT (https://aopwiki.org/events/944); Short Name: dimerization, AHR/ARNT Why is the Action of the AHR/ARNT dimerization process classified as "disrupted"? For both the COX-2 and HIF1/VEGF pathways the dimerization is needed to get an adverse effect. It is required for the COX2 induction, and the AHR/ARNT dimerization is an essential step in removing ARNT from available cellular pools thereby reducing its availability to interact with HIF1a.	We agree with the reviewer and the authors of AOP150 and it looks like this has been corrected.	3
Reviewer 4	I think the scientific quality and analysis of the existing literature, the supporting evidence etc. is very well done in both AOPs – really a tremendous job. My main concern – and here I	We agree with the reviewer and the authors of AOP150 regarding complexities interpreting these two AOPs and the utility of "AOP Xplorer". The reality is that activation of the	4

	echo what the first reviewer said – is the distinction of the two AOPs. It is not only that certain parts of the text are identical in both AOPs – why re-writing how the AhR activation works, it is the same in both pathways - but t is that I am afraid of the users can deal with the two AOPs. Assume I am a regulator and have a compound form which I know that it binds and activates AHR. Now I go to AOP WIKI and I find 21 and 150 – how to decide which one I should use for my compound? Both, only 21, only 150, or 150 in case of hypoxia and 21 in case of normoxia, or… I feel we have to give the readers something at hand to find their way	AhR causes a diverse array of responses which most likely act in concert and cannot be captured within a single AOP.	
Charge Question 1: Scientific quality: Does the AOP incorporate the appropriate scientific literature? Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?			
Reviewer 1	Yes. The AOP includes appropriate and current scientific knowledge.		5
	Page 2. Summary of the AOP. In the Stressors Section, the statement "The prototypical and most potent DLC is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)" is not entirely true. This is contradicted by the statement on page 3 in the section "Evidence for Perturbation by Stressor" that indicates 23478-PCDF is more potent than TCDD in some avian species. Likewise, TCDF is also more potent than 2378-TCDD in three avian species (Cohen-Barnhouse 2011. ToxSci 119, 93-102). Suggest changing "most potent" to "highly potent" or "generally most potent".	This is correct and has been updated to "The prototypical and among the most potent of the DLCs is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)."	6
	Several places authors mention potential applicability of the AHR/ARNT/COX2 pathway to invertebrates. This would seem to contradict evidence presented on page 33 where it is stated that "Chemicals that activate the AhR of vertebrates are not known to bind AhRs of invertebrates and increased mortality in invertebrates has never been observed as a result of exposure to these agonists (Hahn 2002; Hahn et al 1994)." Also suggesting applicability to invertebrates goes against authors own	There is inconsistency in information regarding AhRs of invertebrates. Available information on invertebrates was included for each KE and KER in case these pages are incorporated into other AOPs in the future. Although some KE/KER pages are likely applicable to at least some invertebrates (despite inconsistent results in some cases), there is no evidence to suggest that the AOP as a whole (i.e. activation of the AhR leading to early life stage mortality through COX-2	7

	conclusions on page 38 where the second bullet under "This AOP is not applicable to:" is invertebrates.	mediated alteration in cardiovascular development) is applicable to any invertebrate. In order to clarify this, the domain of applicability statement was modified to "Invertebrates because AhRs of invertebrates have less diverse functionalities relative to vertebrates, AhRs of most invertebrates likely do not bind agonists that represent anthropogenic pollutants, and no AhR-mediated, critical adverse effects are known in invertebrates as a result of exposure to AhR agonists".	
Reviewer 2	To my knowledge the scientific content of the AOP reflect the current scientific knowledge on the topic and I am not aware of a particular paper/review of importance that would have been forgotten.		
Reviewer 3	The AOP is well developed incorporating the critical and recent scientific literature on the topic and reflecting current scientific knowledge on this specific topic. The abstract and background are well written and both give a reasonable overview of the AOP.		8
	The **MIE** description (KE18) is clear and biologically plausible and is shared by four other AOPs in the AOP wiki. **Specific comments:** In reporter gene assays, P-lactamase- or CAT-based assays are not the luminescent reporter gene assays. The expression of P-lactamase is commonly measured using the fluorogenic P-lactamase substrates and the expression of CAT is measured radioactively or using a fluorescing derivative of chloramphenicol. But, for sure, more recently developed models used luciferase as a reporter gene with luminescent end-point. This MIE can be predicted and supported by in silico studies (SAR and QSAR methods) and the authors can consider involving some information on this topic and some references – for example Hirano et al (2015) EST 49:3795; Bonati et al (2017) Curr Opin Toxicol 2: 42; Sovadinova et al (2006) ETC 25: 1291.	This content on reporter gene assays was added by another author (possibly an author of AOP 150?) and we are unsure about this comment.	9

	KEs and AOs are clearly described and informative. **Specific comments**: **KE1269** – Can be measured the gene expression of COX2 using ELISA or Western blot? **KE442** – This AO has very little information. Some basic descriptive text will be nice.	Although it is possible that COX-2 could be measured by ELISA or Western Blot, kits do not currently exist for fishes or birds and therefore measurement of protein content for COX-2 in these species is currently not practical for most investigators. The AO page is very basic and will be expanded in the future as time allows. However, it might be worth combining AOP150 and AOP21 for the AO to reduce duplicated effort. However, the current AO for AOP150 of "increase, embryolethality" is not entirely accurate to fishes as most mortality occurs between hatch and swim-up, except at extreme concentrations. Perhaps this title can be modified to something broadly applicable across taxa (i.e. birds, reptiles, amphibians, fishes) and equally applicable to both AOPs?	10 10a 10b
	KERs are well described and explained, but **less informative**. <u>The most solid</u> is the <u>link between activation of the AhR and early life stage mortality.</u> **Specific comments:** As the reviewers in the internal review, I think that the essentiality of COX2 and downstream events of COX2 is quite questionable and not compelling even through the authors quite adequately addressed this issue. This link is the weakness of this AOP.	We agree that uncertainties exist in this AOP, which is partially attributed to the fact that research into a COX-2 mediated pathway is relatively recent with the key papers having been published in 2008, 2010, and 2014, while research into a HIF1a/VEGF-mediated mechanism having begun more than 10 years prior. It is true that there are KEs that exist between up-regulation in COX-2 and altered cardiovascular development and function which are currently unknown and might be incorporated based on future research into this mechanism. However, based on strong mechanistic evidence with COX-2 knockdown, COX-2 inhibitors, and COX-2 inducers across two species of fish and one species of bird and a lack of any contradictory evidence at present (besides data gaps), we believe there is good evidence of essentially for a role of COX-2 in mediated toxicities with the understanding that activation of the AhR is inherently complex and many different mechanisms occur concurrently and likely each have some additive role in mediating the complete phenotype of toxicity.	11 11a
	KER1490 - this KER has very little information. Some basic descriptive text including evidence supporting this KER is warranted.	This KER page is very basic and will be expanded in the future as time allows. However, if a shared AO for AOP150 and AOP21 is incorporated, then need for added details on this page could be omitted.	11b
Reviewer	Yes.	Certain aspects of the AOP are applicable to mammals, however	12

4	One aspect that may be re-considered is the taxonomic applicability. Also here, the AOP incorporates the appropriate scientific literature, but I have some questions on this: In the table "taxonomic applicability" on page 38, a number of species – mainly fish, but also amphibians and birds – are listed for which the AOP application is indicated to be moderate to strong. In the subsequent text on taxonomic applicability, only 3 references are given (Antkiewicz for zebrafish, Jones and Kennedy for chicken – the reference is missing in the reference list- , and Thackaberry for murine species. However, a few lines down the page, it is said that the AOP cannot be applied to mammals, but here the paper of Thackaberry is cited which deals with mice !?).	to reduce confusion, the statement with the mouse refence was removed. References for the species listed in the "taxonomic applicability" are listed following the bullets under "This AOP is applicable to:"	
	What about the evidence of the AhR-COX-cardiovascular pathway for the other species listed in the table? The authors cite number of studies with fish, chicken, amphibians etc. that reported an adverse effect of AhR ligand on early life stage mortality (and partly on heart malformations), but did those studies indeed specify the linkage through the COX pathway? There might be species differences in the pathway leading from AhR activation via COX2 to altered cardiovascular function (see, for instance, the discussion in Teraoka et al., 2014, Aquat Toxicol), for instance, due to species/class differences in COX isoforms and the thromboxane pathway. Personally, I am convinced that the authors are right in their opinion that the taxonomic applicability of the AOP is broad, still I am not sure how strongly this is supported from the existing literature.	Species applicability for the AOP page and each KE/KER page were revised based on the guidance document.	13
Reviewer 5	Yes. The two AOPs #21 and #150 include appropriate information reflecting current knowledge on AhR activation, partnering with ARNT and other molecular events potentially preceeding functional cardiac consequences and lethality early in life. The selected focus on COX2 and VEGF, among the many genes involved in early cardiac development could be better motivated	The motivation for describing AOPs based on COX2 and VEGF is based purely on the availability of sufficient information to support an AOP and because other possible mechanisms have significant empirical evidence suggesting they do not mediate mortality in birds or fishes (i.e. SOX9b, CYP1A).	14

Charge Question 2: Weight of evidence: Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?			
Reviewer 1	Overall Assessment of AOP (beginning on page 37) does an excellent job of laying out the AOP and provides arguments for the strength of associations of the key events and key event relationships, including the experimental support for these steps. The temporal concordance of COX2 upregulation and onset of adverse effects is not a very strong association, and the authors do address that later in this assessment.		15
Reviewer 2	The overall assessment of AOP provides strong arguments for supporting the KE and KERs, and the addition of the KER "Activation AhR leads to increased mortality" after the internal review nicely completes the AOP. For the KER "Altered cardiovascular/ function lead to increased mortality" there is absolutely no data. Would it be possible to include some data? To be rated "Strong" a KER needs a minimum of underlying data and explanation.	Again, I would suggest that we alter the AO to something that is broadly applicable to both AOP150 and AOP21 so that duplicated effort isn't needed to fill in details on this or other comparable pages.	16
	Domain of applicability: There is a contradiction between the applicability domain (species) for the whole AOP which include Xenopus leavis, and the text in the "Taxonomic applicability: This tolerance is believed to result from AhRs of amphibians having very low affinity for agonists (Lavine et al 2005; Shoots et al 2015). Therefore, it is acknowledged that this AOP is likely to be applicable to reptiles. However, it might not be applicable to amphibians due to their extreme tolerance to activation of the AhR". The list of species for the applicability domains of the whole AOP is quite impressive, and much longer than the list of species for the applicability domain of the MIE or individual KEs/KERs…According to the OECD guidance document, the applicability domain will most often be defined based on the	Species applicability for the AOP page and each KE/KER page were revised based on the guidance document.	17

	most narrowly restricted of its KEs, which does not seems to be the case here? Has early life stage mortality linked to cardiotoxicity via the COX-2 pathway really been demonstrated in all those species?		
Reviewer 3	Inconsistencies, uncertainties and level of confidence are provided for all KEs, KERs and the overall AOP, except for KE442 and KER 1490. It is important to have limitation and weight of evidence captured under each KEs and KERs to understand the regulatory standpoint. The levels of support for essentiality of the KEs and for biological plausibility of the KERs are adequately described, justified and follow the Handbook guidance.	Regarding KER 1490, again, I would suggest that we alter the AO to something that is broadly applicable to both AOP150 and AOP21 so that duplicated effort isn't needed to fill in details on this or other comparable pages. For KE442, this page is sparse, however population related consequences of early life stage mortality is beyond the expertise of these authors. Perhaps someone with greater population level expertise can be found to complete this page appropriately and with the greatest comprehension as this page would be applicable to all AOPs. Alternatively, 9 KEs are available in the Wiki related to declining populations. Perhaps content on these pages can be combined into a single comprehensive population decline page.	18
Reviewer 4	The WOE judgement given in the AOP is very well balanced and generally excellent. I have only a few minor comments (all related to the overall assessment of the AOP): Population trajectory: It would be fair to indicate that the question of population relevance of AhR-induced early life stage mortality depends on the life history of the species. **Consistency:** The consistency in the TCDD-induced alterations in cardiovascular development between the two oviparous groups, fish and birds, is taken as one argument to support the BH criterion "consistency". But how to deal with the fact that TCDD does cause altered cardiovascular phenotypes in another oviparous group, the amphibians? **Essentiality:** As cited in the AOP, there are indeed several other pathways through which AhR activation may lead to altered cardiovascular development and function. Here, I am not sure	Re: population trajectory: Quantitatively there are many species and population specific factors beyond the scope of the current page, however, qualitatively, increased early life stage mortality can decrease population trajectory in any species. Re: consistency: It is true that there is a lack of consistency with amphibians (at least species studied to date). However, this is acknowledged to be dose-related and related to extreme tolerance of amphibians based on low binding affinity of amphibian AhRs (as discussed on the main AOP page) and at some hypothetical dose these cardiovascular effects would likely occur in amphibians (but this has not yet been demonstrated). Therefore, this isn't necessarily a lack of consistency. Further, this actually adds to essentially of altered cardiovascular development/function and has been added to the essentiality statement as "Concentrations of DLCs tested in amphibians studied to date were not sufficient to cause altered cardiovascular development or function and no increase in mortality was observed (Jung et al 1997)."	19 19a 19b 19c

	how this is deal with in an AOP context. If I think on the BH criterion of essentiality, i.e. the downstream event – here: altered cardiovascular development – is inhibited if the upstream event – here: COX activation - is blocked. Now, if there exist other mechanisms through which AhR activation links to altered heart development, could it be then be we block COX activation but still see cardiovascular impairment? If so, is then the BH criterion of essentiality fulfilled? The argument of the authors is – if I understood it correctly – that the Sox9 pathway produces another cardiovascular phenotype then the COX pathway. Here, it would be helpful to spell out a little more on the differences of the two phenotypes to support this statement.	Re: Essentiality: Sox9 alone does not mediate cardiovascular phenotypes which result in mortality, while COX-2 does. This refutes essentially of Sox9b (although Sox9b might additively contribute to the altered cardiovascular development and function in a small way), while COX-2 appears to have strong essentiality. The section on uncertainties associated with Sox9b was expanded to read "Investigations of knockdown and null strains for Sox9b in zebrafish do not result in the complete phenotype of altered cardiovascular development recorded in embryos following exposure to planar aromatic hydrocarbons (Hofsteen et al 2013). Specifically, knockdown or knockout of Sox9b is associated with mild pericardial edema, unlooping, loss of proepicardium, and failure to form epicardium and endocardial cushions, but does not result in typical TCDD-mediated effects of a compacted ventricle or an elongated string-like atrium (Hofsteen et al 2013). Altered cardiovascular development as a result of complete knockout of Sox9b is not severe enough to cause complete cardiac failure and early life stage mortality in zebrafish (Hofsteen et al 2013). Injection of TCDD exposed embryos with Sox9b mRNA was able to prevent the Sox9b phenotype of cardiovascular development, however it did not prevent altered cardiovascular development altogether (Hofsteen et al 2013). Considering, TCDD is only able to decrease expression of Sox9b in the heart by up to about 50% and complete knockout of Sox9b expression is not lethal suggests that Sox9b is not essential to TCDD-mediated alteration in cardiovascular development and function (Hofsteen et al 2013)."	
Reviewer 5	The WOE discussion in #150 was more convincing to me as I knew beforehand about VEGF and its role in CV development and maintenance, while COX-2 connection (#21) to me was not that clear, and still is not, even though I did some literature review to become more familiar.		20

Charge Question 3: Regulatory applicability: Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?			
Reviewer 1	Regulation of AhR agonists (DLC's) are based on current knowledge of their relative toxic potencies. This AOP will not change or modify this information, however, it can provide further mechanistic information on which to understand this toxicity. This AOP provides the basis upon which any new chemicals found to be COX2 inducers via an AHR/ARNT mechanism might flag them for further investigation.	Also, provides a foundation for an AOP for chemicals which induce COX-2 through mechanisms other than AHR/ARNT causing altered cardiovascular development and function leading to early life stage mortality.	21
Reviewer 2	This AOP can give insight into the toxicity of AhR agonist, and their relative toxic potency between species. It could help identifying chemicals that need to be further investigated in respect to their capacity to bind to the AHR receptor and to induce COX2.		22
Reviewer 3	It is difficult to see how a prediction based on this AOP would be applicable for regulatory purposes. I see a potential use this AOP in some integrative testing strategies or integrated approaches to testing assessment, to give mechanistic support to the adverse effects of chemicals binding to AhR and activating this receptor.		23
Reviewer 4	Most AhR ligands are already regulated, mainly based on their relative toxic potencies. I expect that this AOP will not basically change this but the information form this AOP can substantiate the existing regulatory classification and can help to classify (and/or screen) new chemicals.		24
Reviewer 5	From a regulators point of view I think these AOPs might become more helpful if they could include more information on links between the molecular and functional/organ/clinical levels. It is also important for regulators to understand relationship between COX2 and VEGF, as well as other genes of importance for cardiac development and function. What is each gene doing and when during embryo development. Meaning also, that for regulators it is likely difficult to decide on the use of #21 vs #150. Is the #21 meant for fish, birds and #150 meant for	We agree with the reviewer and the comments from the authors of AOP 150. These two AOPs could be applicable to both fishes and birds and could occur concurrently.	25

	mammals? Access to comprehensive AOPs on CV system is likely to be of high importance for regulators as well as other professionals as such information is largely missing.		
Conclusion: What are your overall conclusions of the assessment of this AOP?			
Reviewer 1	Overall the AOP provides a logical rationale for a possible mechanism by which DLCs can lead to cardiotoxicity and ultimately early life stage mortality.		26
	It is unclear what the recurring discussion of ranges of differences in sensitivity between species, contributes to making the argument that AhR/ARNT activation leads to early life stage mortality via a COX-2 mediated pathway. Although this is an interesting phenomenon, the amount of discussion of this in this AOP can be reduced to avoid it being distracting from the AOP.	Discussion of ranges of differences in sensitivity between species is an important aspect of this AOP and planned developments in this AOP because of the regulatory uncertainty associated with differences in sensitivity that can span multiple orders of magnitude. Ongoing work is focused on using this AOP to support methods for cross-species predictions of sensitivity as is briefly outlined in Relationship 1492.	27
	The "Background" section provides bulleted information about AhR and dioxin like chemicals and their effects in fish, but there is no discussion of COX-2. It would seem that the background section should not just include discussion of the MIE and the adverse outcomes, but the intermediate key event that is being proposed as integral to this AOP.	The background has been expanded by inclusion of "One gene which is regulated by AhR is cyclooxygenase-2 (COX-2) which is known to have roles in development of the heart (Dong et al 2010; Teraoka et al 2008; 2014). AhR-mediated dysregulation of COX-2 is associated with altered cardiovascular development, decreased blood flow, and cardiac failure causing mortality in developing embryos (Dong et al 2010; Teraoka et al 2008; 2014)."	28
Reviewer 2	This AOP bring together a big amount of information and addresses well the complex issue of potential interspecies sensitivity variability. Overall this AOP provide a clear potential mechanism by which AhR activation can lead to early life stage mortality, and a good understanding of the potential difference of sensitivities between species.		29
Reviewer 3	Even though the questionability of the role of COX-2 and COX-2 downstream events, the overall assessment of the AOP is solid, robust, and perfectly understood.		30
Reviewer 4	Overall this is a sound, useful. And well-founded AOP on a mechanism/toxicity pathway through which AhR ligands can impact (cardiac) development of oviparous vertebrates. The AOP is well underpinned with the existing knowledge and	We agree that it would be useful to add a discussion regarding overlap of the AOPs and this is something that could be worked out with the authors of AOP150 and added to each AOP page.	31

	personally I like very much that the AOP openly discusses uncertainties or gaps in the existing knowledge as well as possible alternative pathway. What I would consider to be helpful is to have a short section discussing possible links and overlaps with AOP 150, as both AOPs have a number of things in common.		
Reviewer 5	The two AOPs provide up-to-date and detailed information on AhR-mediated cardiac toxicity with focus on COX2 and VEGF, respectively; two selected genes that are important during heart development. A broader context for how these genes play roles during embryo-cardiac development would be welcome and could be part of the background section. How do COX2 and VEGF interact with other important genes during this time window, and how are these genes regulated not only through AhR but also through e.g. the retinoid system, which also is a well known vital regulator of the cardiac system during embryo development . A natural progression over time could be to combine the two AOPs, while at the current stage it would be helpful to explain and discuss possible links and overlaps between the two AOPs, as there are many commonalities.	We agree that it would be useful to add a discussion regarding overlap of the AOPs and this is something that could be worked out with the authors of AOP150 and added to each AOP page.	32
Additional question: consider whether all specific or important points for the AOP have been reflected in the Abstract so as to allow a user to decide on the suitability/applicability of one or the other (or both) AOP in their circumstances.			
Reviewer 1	This abstract provides general information on the fish early life stage toxicity by DLC's, but could be reorganized to be more direct in getting to the specifics of this AOP. Suggest moving these two sentences in the abstract toward the bottom of the abstract. "Fishes represent the most sensitive taxa to adverse effects resulting from activation of the AhR. Embryos are more sensitive than juvenile or adult fishes based on endpoints that could be of significance to population trends." These are more statements of general applied information than being integral to	The suggested statements were removed from the abstract.	33

	this AOP. They could also be removed from the AOP abstract. This would move the COX-2 part of the AOP earlier in the abstract and help in highlighting this.		
Reviewer 2	To complete the **abstract**, maybe add a few words on the biological plausibility and WoE result for the AOP, as this is done for the AOP 150. This would be necessary to have all the information in a stand-alone piece. The "**Background**" section could include some discussion on COX-2 to be more complete, as COX-2 is the particularity of this AOP (compared to other AOPs that share the same initial KE for example).	The abstract was expanded to include "In general, biological plausibility of this AOP is strong based heavily on evidence collected from zebrafish (*Danio rerio*) through mechanistic investigations by use of targeted knockdown of AhR, ARNT, or COX-2 and through use of selective agonists and antagonists of COX-2. However, uncertainties exist regarding the interaction of multiple potential targets of AhR activation, including CYP1A, Sox9b, and HIF1a/VEGF. Quantitative understanding is largely limited to the indirect KER between AhR activation and early life stage mortality."	34
Reviewer 3	As I mentioned in Question 1, the abstract is well written and gives a reasonable overview of the AOP. Both AOP21 and AOP150 are closely related and greatly overlapping through the same MIE and some KEs. In addition, the COX2 pathway (the KE in the AOP21) is mentioned as an alternative pathway in the AOP150. The AOP 21 developers mentioned in the responses to the internal reviewers that the different KEs which are proposing by the authors on AOP150 are weak candidates for KEs leading to mortality but they did not address that in their AOP. They questioned other pathways by which activation of the AhR could result in altered cardiovascular development and function in developing embryos such as down-regulation in Sox9b and oxidative stress. Why not the AhR cross-talk with HIF1α? I think that will be useful for potential users of these closely related and greatly overlapping AOPs. Is there any possibility that COX-2 and VEGFA signaling pathways can crosstalk? If yes, can be AhR involved?	We do believe, at least in zebrafish, that HIF1a is an unlikely mechanism for altered cardiovascular development because there is strong empirical evidence that refutes this mechanism and comparably strong studies have not been performed in any other species to our knowledge. This information is what led us to pursue COX-2 as a mechanism. Discussion of Hif1a/VEGF was left out of the uncertainties section of the AOP21 page because this discussion is most suitable for the AOP150 page and is discussed there, although briefly. The main concern is that knockdown of ARNT prevents all known effects of exposure to TCDD in zebrafish, including cardiovascular deformities (pericardial edema, peripheral ischemia, heart looping and morphology, decreased cardiomyocyte number, ventricle alteration, and decreased cardiac output), jaw malformations, and inhibition of regenerative growth (Antkiewicz et al 2006). This suggests that all critical effects of TCDD exposure in embryos of zebrafish are mediated by genes regulated by the AhR/ARNT heterodimer. If depletion of ARNT was essential for altered cardiovascular development and function then knockdown of ARNT should cause a TCDD-like phenotype, but it has no effect on cardiovascular function and protects against the TCDD phenotype following exposure (Antkiewicz et al 2006). Further, when co-exposed to hypoxia and TCDD, HIF1a outcompetes	35

		AhR for ARNT and actually protects against the TCDD phenotype – which does suggest possibilities for cross-talk between AhR/HIF1a (Prasch et al 2004). However, HIF1a is rapidly and massively up-regulated during hypoxia so it is able to deplete ARNT, but AhR is only up-regulated by a small amount, if at all, and therefore is unlikely to deplete ARNT which is expressed in massive excess. This is supported by the fact that ARNT is typically not up-regulated by TCDD and if it was being depleted you would expect ARNT to be up-regulated as compensation. Considering these studies in zebrafish represent in vivo conditions, in our opinion they represent very strong evidence. That being said, zebrafish is only one species and these studies were all performed in one lab, but similar studies have not been performed in any other species to our knowledge. Further, the authors of AOP150 might have explanations for the results of these studies in zebrafish?	
Reviewer 4	In my opinion, the current Abstract does not allow the reader to decide on the suitability/applicability of AOP 21 vs AOP 150. I suggest to remove general sentences like "Embryos are more sensitive than juveniles…" what – in this generality – is anyway not true) and instead to spell out more on when to use 21 or 150 or both.	The suggested statement was removed from the abstract, also we agree that it would be useful to add a discussion regarding overlap of the AOPs and this is something that could be worked out with the authors of AOP150 and added to each AOP page.	36
Reviewer 5	In my opinion, the current Abstracts does not allow the reader to decide on the suitability/applicability of AOP 21 vs AOP 150. There is not enough contextual information, in my opinion, in the current Abstract to allow the reader to decide on the suitability/applicability of AOP 21 vs AOP 150.	We agree that it would be useful to add a discussion regarding overlap of the AOPs and this is something that could be worked out with the authors of AOP150 and added to each AOP page.	37