

**USERS' HANDBOOK SUPPLEMENT TO THE GUIDANCE DOCUMENT FOR DEVELOPING
AND ASSESSING AOPs**

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ABOUT THIS DOCUMENT

This document is a supplement to the Guidance Document for developing and assessing Adverse Outcome Pathways (AOPs) [ENV/JM/MONO(2013)6] (AOP guidance hereafter).

The AOP Guidance, published in 2013, only one year after the OECD programme on the development of AOPs was initiated, was considered a first version which would be revised as expert groups and member countries gained experience in developing and assessing AOPs. The AOP guidance consists of two main parts: (1) advice on the development of AOPs including early assessment of their relevance and relative uncertainties and (2) a template intended to assist developers in assembling and organising information supporting an AOP in a consistent manner that would facilitate transparent, fit-for-purpose use by different stakeholders.

Soon after publication, the OECD sought feedback from users on their experiences with the AOP guidance. Whilst feedback from the limited number of initial users was generally favourable, a number of shortcomings were identified. Specifically, refinements to the template were proposed, mainly to avoid redundancy, to streamline its completion and to ensure consistency with the format of the AOP-Wiki being developed as a platform for aggregating and disseminating AOP knowledge. In addition, the need for more focused and practical instructions was also identified and as a result, it was concluded that a user's manual/handbook would be beneficial. As a result, a drafting group was established in June 2013. This comprised members from the Extended Advisory Group on Molecular Screening and Toxicogenomics which itself included experts in AOP development who were tasked with developing this *Users' Handbook* as a supplement to the first AOP guidance document ([ENV/JM/MONO(2013)6]).

This handbook contains an updated template for AOP development and provides focused and practical instructions for both AOP developers and reviewers and is intended to assist in identifying, organising and evaluating critical information on key events (KE) as well as linkages between KEs within the AOP (i.e., AOP development). It also provides more evolved and explicit guidance on how to assess the weight of evidence (WOE) (degree of confidence) supporting the overall AOP and its relevance for life stage, sex and taxonomy (i.e., AOP evaluation).

The handbook is intended to be used as a supplement to the AOP guidance and a replacement for the AOP Template (Part II of the guidance) and evaluation for which improvement was needed. As with the AOP guidance document itself, this handbook is not intended to provide a review or summary of the literature informing the AOP concept. Instead, it focuses on practical aspects of AOP development and assessment.

The present supplement is not intended to provide guidance on determining the appropriate or inappropriate regulatory application of AOPs. However, by following the template and practices outlined in the *Users' Handbook*, AOP developers should be in a position to systematically and efficiently assemble information pertinent to their AOP (the focus of sections 1-6), and evaluate the underlying WOE (the focus of section 7). This should provide transparent assessment of the level of confidence in the overall AOP as well as critical gaps and uncertainties, relevant to decisions regarding appropriate regulatory applications as addressed in Section 8, which itself is considered optional (e.g., developing test guidelines, forming categories, informing integrated approaches to testing and assessment (IATA), or risk assessments within different regulatory contexts).

AOPs also provide a relevant construct to promote collaboration between experts in various areas of research and the regulatory risk assessment community as a basis to better coordinate and tailor research to practical application. Collaboration between a range of experts with expertise in these different areas in the development and assessment of AOPs is therefore strongly encouraged.

It is recognised that the literature related to the AOP concept is evolving rapidly and that the growing number of AOPs being developed under the OECD AOP Programme will contribute to experience that will additionally inform revision of both the AOP guidance and the present supplement. However, early provision of clearer guidance on the practical aspects of AOP development and evaluation is anticipated to facilitate their evolution and should ultimately lead to a better understanding of the potential utility of AOPs for different purposes. The template and evaluation of the KERs and overall AOP replaces that which appeared in the earlier guidance.

BACKGROUND

Conceptually, an AOP can be viewed as a sequence of events commencing with initial interactions of a stressor with a biomolecule in a target cell or tissue (i.e., molecular initiating event), progressing through a dependent series of intermediate events and culminating with an adverse outcome. AOPs are typically represented sequentially, moving from one key event to another, as compensatory mechanisms and feedback loops are overcome. Definitions of key terms used in this guidance are provided in Table 1.

Table 1: Definitions

Molecular initiating event	MIE	A specialised type of key event that represents the initial point of chemical interaction on molecular level within the organism that results in a perturbation that starts the AOP.
Key event	KE	A change in biological state that is both measurable and essential to the progression of a defined biological perturbation leading to a specific adverse outcome.
Key event relationship	KER	A scientifically-based relationship that connects one key event to another, defines a directed relationship between the two (i.e., identifies one as upstream and the other as downstream), and facilitates inference or extrapolation of the state of the downstream key event from the known, measured, or predicted state of the upstream key event.
Adverse Outcome	AO	A specialised type of key event that is generally accepted as being of regulatory significance on the basis of correspondence to an established protection goal or equivalence to an apical endpoint in an accepted regulatory guideline toxicity test.

Key events (KEs) are those that are essential to the progression of the toxicological response as hypothesised in the AOP. An important feature of KEs is that they must be measurable. KEs are connected to one another (i.e., linked); this linkage is termed a KE relationship (KER). For some AOPs, KERs may be described quantitatively, while for others, our current level of understanding is such that only qualitative or semi-quantitative descriptions may be possible. Regardless, the AOP concept provides a transparent and scientifically-based frame of reference to organise and present current knowledge of predictable relationships between molecular initiating events (MIEs), subsequent KEs and adverse outcomes (AOs). The objective underlying AOP development is to ultimately support inference or extrapolation from one KE to another. Most notably, consistent with the proposed vision for regulatory toxicology in the 21st century, there is considerable interest in extrapolating from KE measurements that may be made efficiently and cost-effectively, typically at low levels of biological organisation proximal to the initiation or early progression of a toxic response, to adverse effects that are relevant to regulatory protection goals and decision-making (Krewski *et al.* 2010). The overall weight of evidence and level of certainty underlying the inference and extrapolation will in turn dictate the most suitable application of the AOP.

Assessment of AOPs and evaluation of their suitability for application in different regulatory contexts relies in part on (1) the confidence and precision with which the KEs can be measured, (2) the level of confidence in the relationships between the KEs linked in an AOP based on biological plausibility, empirical support for the KER and consistency of supporting data and among different biological contexts, and (3) weight of evidence for the overall hypothesised pathway, taking into account a number of additional considerations. Therefore, overall assessment of AOPs is best supported by providing thorough descriptions of the KEs [Section 5], relationships between those KEs [i.e., KERs, Section 6] and robust

consideration of weight of evidence for the essentiality of KEs and their interrelationships [Section 7]. Consequently, the template and AOP-Wiki are structured in a manner that prompts AOP developers to provide relevant types of supporting information. However, it is worth noting that AOP descriptions should be regarded as “living documents”. Not all sections of the template or wiki pages need be completed immediately. It is expected that early in development, AOPs may have many gaps in completeness, which may be addressed over time as the science progresses or as other researchers contribute. Rather than representing a daunting compilation of information that must be assembled to adequately describe an AOP, the template should be viewed as a transparent record of an AOP’s stage of development and level of support, and a basis for clear delineation of current gaps in our knowledge. As such, even development of “incomplete” AOP descriptions represents a potentially useful contribution to the scientific and regulatory community, though necessarily for different applications than those for which there is better understanding and greater confidence.

As a pragmatic convention, AOPs are conceptualised as a single sequence of events proceeding from the MIE to the AO via a series of intermediate KEs. However, it is recognised that MIEs, KEs, and AOs may be shared by more than one AOP. Consequently, from a practical standpoint, particularly with regard to development of an AOP knowledgebase such as the AOP-Wiki, it is desirable to describe KEs as discrete units without reference to a specific MIE or AO or other KEs. Likewise, it is useful to describe relationships between discrete pairs of KEs (KERs), without reference to other elements of the AOP. This facilitates generation of generic KE or KER descriptions that can be linked to multiple other AOPs. Such an approach will create both consistency and efficiencies in the AOP development process by eliminating the need to generate distinct KE or KER descriptions for all AOPs that share common KEs or KERs as these could be readily imported from or linked to existing KE or KER descriptions. Maintaining KE and KER descriptions as discrete units that avoid reference to other elements of the AOP also facilitates the updating of KE and KER descriptions as new methods for measuring KEs or new evidence supporting KERs are developed. Finally, it lends to the construction and conceptualisation of AOP networks, which is critical for addressing exposures to multiple stressors or to individual stressors that perturb multiple MIEs.

Recognising that each component of an AOP may itself be influenced by other pathways ongoing within the biological system, consideration of AOPs as networks of intersecting and interacting KEs and KERs may ultimately prove critical for prediction. Additionally, to support application of AOP knowledge in quantitative risk assessment, there may be a need, in some cases, to incorporate the description of known factors that modulate various KEs and may alter the probability or magnitude of the AO (modulating factors (ModFs)). Likewise, there is recognised utility in describing markers, which may, in and of themselves, not be causally linked to the progression of an AO but may serve as useful surrogates for a KE in an AOP evaluation. Such considerations are not explicitly addressed in the context of the present supplement. However, information regarding known ModFs or associated events (AEs) can be incorporated in the KE or KER descriptions. For example, a biomarker response that is tightly correlated with a specific KE, but is not, itself, essential to the progression of the AO (i.e., causally-linked) may be included in a KE description as a suitable indirect measure of a change in that KE.

In this handbook, particular emphasis is placed on sections of the template related to the description of the MIE, KEs and AO in an AOP (i.e., section 5), as well as an assembly (section 6) and evaluation (section 7) of available scientific evidence supporting the KERs individually and the essentiality of the KEs in the context of the AOP as a whole.

Sections 5 and 6 outline the types of information that should be included in KE and KER descriptions, respectively. Delineation of the information outlined below for each KE and KER in an AOP allows for overall assessment of the AOP (as described in section 7) as a basis to consider its appropriate application (as described in Section 8). Each field of the KE or KER description should be completed as thoroughly as is feasible, and supported by references to appropriate published literature and guidelines, to the extent

possible. In developing the KE and KER descriptions, it is recognised that AOPs are descriptions reflecting the current knowledge and will need to change, as additional information becomes available. Consequently, it is recommended that descriptions are structured in a way that facilitates addition and revision of information as it is developed; for example, through the use of bullets or tables. For examples of KE and KER descriptions, see aopwiki.org.

SECTION 1 – AOP IDENTIFIER/TITLE

Each AOP should be given a descriptive title that takes the form “MIE *leading to* AO”. For example, “Aromatase inhibition [MIE] leading to reproductive dysfunction [AO]” or “Thyropoxidase inhibition [MIE] leading to altered neurodevelopment [AO]”. In the cases where the MIE is unknown or undefined, the earliest known KE in the chain (i.e., furthest upstream) should be used in lieu of the MIE. For AOPs under development as part of OECD’s “Workplan for the AOP Development Programme”, please include the project number assigned by the Secretariat after the descriptive title for the AOP.

Implementation in the AOP-Wiki (see Screen Shot 1)

Upon selecting the action “Create New AOP”, the user will see a form for the entry of two names. The long title should be of the form described above. The short title should be a reasonable abbreviation of the long name and will be used in labelling this object throughout the wiki. Upon entering the titles and clicking on “Create AOP” a new AOP page is created in the wiki and the user is redirected to a second form for entering the summary data about the AOP as described in the sections 5 & 6 below. If the user wishes to enter the information for sections 2-4 first, there is a link at the top of the page that will open the newly created wiki page. The wiki page name will include a unique numeric identifier (this is not the same as the project number which should be manually entered by the user in the long name if applicable). The Title section of the AOP page will contain the longer descriptive title and the short name.

SECTION 2 – AUTHORS OF AOP

List the name and contact information of the individual(s)/organization(s) that developed the AOP. In the context of the OECD AOP Development Workplan, this would typically be the individuals and organisation that submitted an AOP development proposal to the EAGMST. Indicate the communicating author, to whom correspondence should be sent.

Implementation in the AOP Wiki (see Screen Shots 2&3)

Each AOP page includes a free-text section where the authors of the AOP along with their affiliation and contact information can be provided (screen shot 2). Contributors that have not met the criteria for authorship such as wiki reviewers can also be listed in that section. In addition to the user-entered information, user names of all authors contributing to or revising pages in the AOP wiki are automatically tracked under the View History tab (screen shot 3).

SECTION 3 – DATE OF UPDATING THE AOP

Indicate the date (day/month/year) of any update of the AOP.

Implementation in the AOP Wiki (see Screen Shots 2&3)

The date and time of all entries and revisions to the wiki are tracked automatically in the View History tab. The last modification date for the AOP will show under the Status heading.

SECTION 4A – ABSTRACT

Provide a concise and informative summation of the AOP under development that can stand-alone from the AOP page. Abstracts should typically be 200-400 words in length (similar to an abstract for a journal article). Suggested content for the abstract includes the following: (1) the background/purpose for initiation the of the AOP's development (if there was a specific intent); (2) a brief description of the MIE, AO, and/or major KEs that define the pathway; (3) a short summation of the overall weight of evidence supporting the AOP and identification of major knowledge gaps (if any); (4) if section 8 was addressed, a brief statement about how the AOP may be applied. The aim is to capture the highlights of the AOP and its potential scientific and regulatory relevance.

Implementation in the AOP-Wiki (See Screen Shot 2)

Abstract is a free text section found on AOP pages within the AOP wiki.

SECTION 4B – BACKGROUND (OPTIONAL).

This optional section should be used to provide background information for AOP reviewers and users that is considered helpful in understanding the biology underlying the AOP and the motivation for its development. The background should NOT provide an overview of the AOP, its KEs or KERs, which are captured in more detail below.

Implementation in the AOP-Wiki (see Wiki screen shot 2)

The AOP page contains a background section suitable for up to one paragraph of text. If more extensive background is needed, a new page (or series of pages) should be created in the wiki and referenced in the one paragraph summary found on the AOP page. This not only keeps the AOP page focused, but it also allows the reuse of general biological information that would be contained in the background for other AOPs. While this process is not currently supported by widgets, the Help section includes detailed instructions on how to efficiently create and reference new pages in the AOP-Wiki as well as referencing additional resources on the internet. When a report is created for the AOP, the one paragraph summary will be included in the main text, the referenced AOP-Wiki pages will be included as an Appendix, and external internet references will reference the external URL for that resource.

SECTION 5 - SUMMARY OF THE AOP AND KEY EVENT DESCRIPTIONS

A. Summary of the AOP in Figure or Table Format

The summary of the AOP should include a listing of all the KEs, including the MIE (if known) and AO, and the pair-wise relationships (links or KERs) between those KEs. This is easily achieved using either the standard box and arrow AOP diagram (Figure 1) or table listing each pair-wise relationship (e.g. Table 2). Starting with the summary provides a useful overview of the KE descriptions that follow (i.e., are described on linked KE pages).

Figure 1, Example of a generic AOP diagram (see also wiki screen shot 4)



Table 2. Example of a generic AOP summary table (see also wiki *Screen shot 5*).

KE (upstream)	KE (downstream)	Level of confidence in the KER*
MIE	KE ₁	
KE ₁	KE ₂	
KE _{n-1...}	KE _n	
KE _n	AO	

*To be filled out after developing KER descriptions (see section 7).

Determining the number of KEs to include in an AOP and the specificity with which they are defined is one of the more challenging aspects of AOP development. In describing KEs within an AOP, it is important to recognise their distinction with “mechanism of action”. AOPs provide a description of a limited number of critical, measurable events leading to induction of the relevant end-point of toxicity. They do not necessarily provide a comprehensive molecular description of every aspect of the biology involved. Rather, a limited number of KEs should be selected; these are normally those for which there is the most information to support assessment of weight of evidence in a regulatory context. While it is difficult to propose “universal rules” for KE selection and description, consideration of the intended purpose of AOPs and how that differs from detailed elucidation of mechanism of action lends itself to development of best practices recommendations or “rules of thumb” that can help guide the process of KE definition (*Villeneuve et al. 2014a, b; https://aopkb.org/saop/workshops/somma.html*).

Recommendations - number of KEs to include:

- *Where possible and appropriate for application, try to include one KE at each major level of biological organisation (molecular, cellular, tissue, organ, organ system, individual).*
- *Focus on KEs that could be measured in a relatively routine manner over those that would require highly specialised expertise, equipment, or supplies to measure. These will tend to be the KEs for which essential evidence to support KERs is more likely to be available.*
- *It is not necessary to provide a comprehensive molecular description of every aspect of the biology involved (i.e., mechanism of action). Rather, select a limited number of key events*

which are measurable and for which evidence supports plausibility and relevance in a regulatory context. Where relevant, such details can generally be incorporated into the descriptions of the biological plausibility linking two KEs (see section 6).

B. KE Descriptions

Following the summary, each KE (including MIE and AOs) should be described in detail. Each KE description should address the following items, to the extent feasible:

1. Description

A description of the biological state being observed or measured, the biological compartment in which it is measured, and its general role in the biology. For example, the biological state being measured could be the activity of an enzyme, the expression of a gene or abundance of an mRNA transcript, the concentration of a hormone or protein, neuronal activity, heart rate, etc. The biological compartment may be a particular cell type, tissue, organ, fluid (e.g., plasma, cerebrospinal fluid), etc. The role in the biology could describe the reaction that an enzyme catalyses and the role of that reaction within a given metabolic pathway; the protein that a gene or mRNA transcript codes for and the function of that protein; the function of a hormone in a given target tissue, physiological function of an organ, etc. The following are some general recommendations and “rules of thumb” concerning how specifically to define a KE (see also Villeneuve et al. 2014a, b; <https://aopkb.org/saop/workshops/somma.html>):

- a. The biological context of the KE (e.g., the tissue type/ taxa/ life stage / sex/ etc.) should only be restricted to the extent that function changes with context. If the function is equivalent in both sexes, do not restrict the context by sex. If the function is equivalent in all cell types, do not restrict to a specific cell type. This facilitates generalisation which will allow the KE to be linked to multiple AOPs while preserving adequate specificity to define function.
- b. Define the KE with enough specificity that one would know what to measure to determine the state of the KE. For example “histological changes” is too broad; “oocyte atresia” or “hyperplasia” would be better.
- c. KEs should generally refer to/focus on a single measurable event within a specific biological level of organisation, rather than compounding events together. For example, it would be better to define a KE as increased enzyme activity (if that can be measured), rather than increased transcription and translation leading to increased enzyme activity. Such compounding essentially embeds KERs into KE descriptions. This can limit the ability to link shared KEs to other AOP descriptions in the AOP-Wiki.

2. Measurement/detection

Methods that can be used to detect or measure the biological state represented in the KE should be briefly described and/or cited. These can range from citation of specific validated test guidelines, citation of specific methods published in the peer reviewed literature, or outlines of a general protocol or approach (for example – a protein may be measured by ELISA). One of the primary considerations in evaluating AOPs is the confidence and precision with which the KEs can be measured. The aim of this section of the KE description is not to provide detailed protocols, but rather to capture, in a sentence or two, per method, the type(s) of measurements that can be employed to evaluate the KE and the relative level of scientific confidence in those measurements. As suggested in the guidance document (ENV/JM/MONO(2013)6) key considerations regarding

scientific confidence in the measurement approach include whether the assay is fit for purpose, whether it provides a direct or indirect measure of the biological state in question, whether it is repeatable and reproducible, and the extent to which is accepted in the scientific and/or regulatory community.

3. Taxonomic applicability/Species Concordance

The KE description should also include an indication of the general taxonomic relevance of the biology and the rationale or scientific basis for that assessment. For example, for a KE that is described as a measurable enzyme activity, the taxonomic relevance of that KE may be defined by the phylogenetic conservation of an orthologous protein. In the case of a KE related to the function of a specific organ, that KE would only be relevant to taxa that possess that organ. For example, a measure of lung capacity would have little relevance to a fish. Likewise, a measure of gill damage would have little relevance to terrestrial vertebrates. Defining the taxonomic relevance of each KE helps to bound the taxonomic relevance of the AOP as a whole and provides an understanding of how broadly data represented by a KE measurement may be extrapolated. In practice, specific taxa in which the KE has been measured can be identified using drop-down taxonomic relevance tables found on the KE description pages within the AOP-Wiki. More general, biological plausibility-based rationale for the probable taxonomic applicability of the KE should be defined in the corresponding free text section on the KE description page.

C. Molecular Initiating Events and Adverse Outcome Pathway Descriptions

The MIE and AO represent specialised types of KEs that bound the beginning (point of interaction between a chemical and the biological system) and end (an AO of regulatory significance) of an AOP, respectively. Descriptions of the MIE and AO should include all the information listed above for KEs. In addition, where feasible, further considerations that can enhance application of the AOP knowledge should be included:

- a. **Molecular Initiating Event Descriptions** – The MIE is the direct site of interaction with a chemical. The MIE involves a chemical interaction (e.g., a reaction, covalent binding, hydrogen bonding, electrostatic interaction, etc.) between a chemical stressor and chemically defined biomolecules within an organism. In some cases, this may be a highly specific interaction, for example between an exogenous ligand and a specific receptor. In other cases, it may be non-specific, as in the case of a reactive chemical that can covalently modify a wide array of proteins. Either can be described as an MIE, provided that the general nature of the stressor-biomolecule interaction is understood. Therefore, when a specific MIE can be defined (i.e., the molecular target and nature of interaction is known), in addition to describing the biological state associated with the MIE, how it can be measured, and its taxonomic applicability it is useful to list known **chemical initiators** (or other stressors known to trigger the MIE) and provide evidence supporting that initiation. This will often be a list of prototypical compounds demonstrated to interact with the target molecule in the manner detailed in the MIE description to initiate a given pathway (e.g., 2,3,7,8-TCDD as a prototypical AhR agonist; 17 α -ethynyl estradiol as a prototypical ER agonist). However, depending on the information available, this could also refer to chemical categories (i.e., groups of chemicals with defined structural features known to trigger the MIE). The evidence supporting the chemical initiation will typically consist of a brief description and citation of literature showing that particular chemicals, or classes of chemicals, can trigger the MIE.

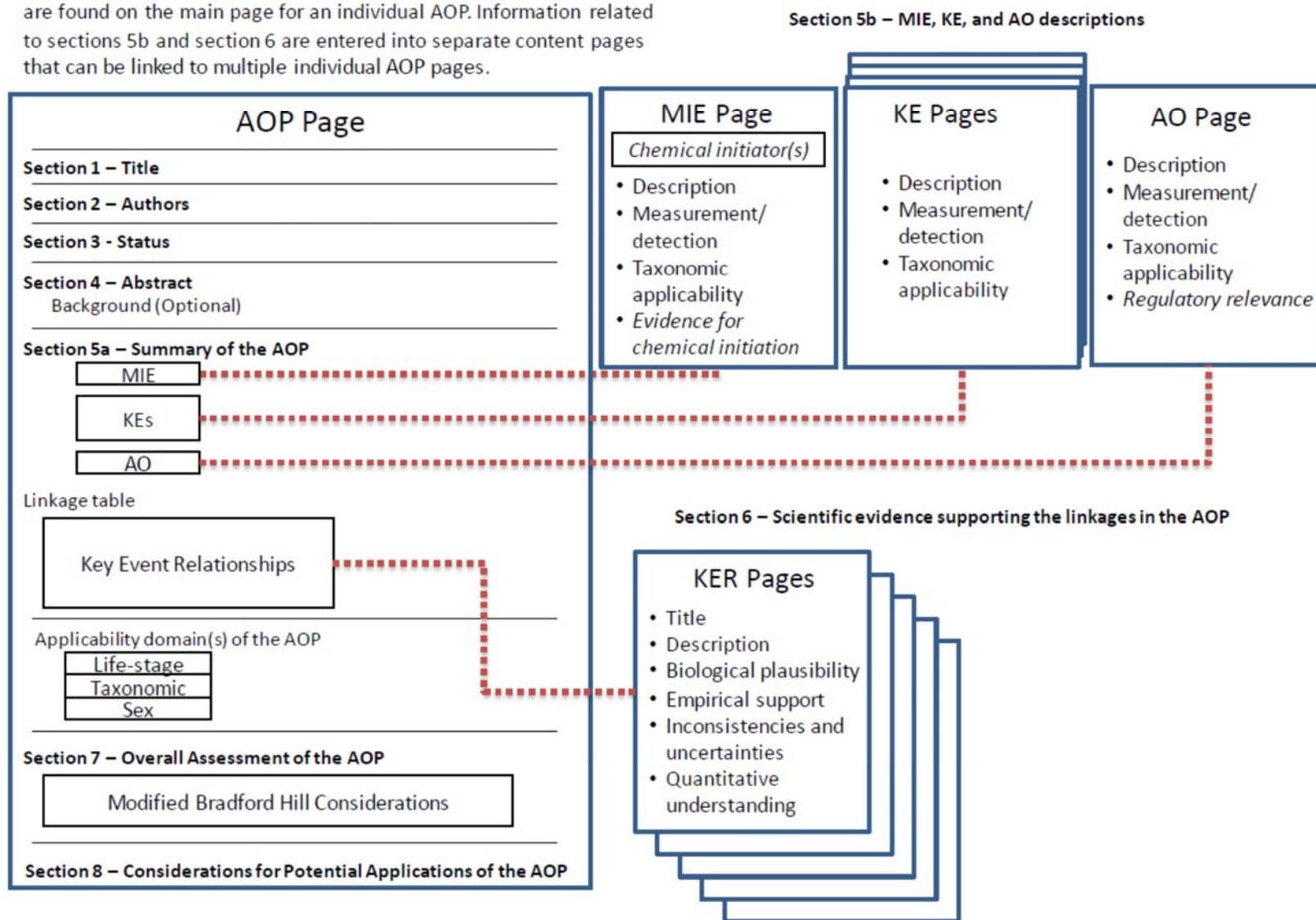
- b. Adverse Outcome Description – A key criterion of defining the terminal end of an AOP is that it represents an outcome that is considered relevant to regulatory decision-making (i.e., it corresponds to an accepted protection goal or common apical endpoint in an established regulatory guideline study). For example in humans, this may constitute increased risk of disease in a particular organ or organ system in an individual or in either the entire or a specified subset of the population. In wildlife, this will most often be an outcome of demographic significance that has meaning in terms of estimates of population sustainability. Given this consideration, in addition to describing the biological state associated with the AO, how it can be measured, and its taxonomic applicability, it is useful to describe the established **regulatory relevance of the AO**.

Implementation in the AOP-Wiki (see Figure 2)

Section 5A is implemented in the AOP-Wiki in two steps. To add events to an AOP, the user will click the link for the “widget page” at the top of the “Summary of the AOP” section. This will bring up a separate window for adding the new components (see screen shot 5). Each component is added by clicking the button over the table corresponding to the event type: “Add Molecular Initiating Event to Table”, “Add Key Event to Table”, “Add Adverse Outcome to Table”. As the titles would suggest, when these buttons are selected a MIE, KE, or AO either defined by the user or selected from a drop-down list of previously defined MIEs, KEs, or AOs is added to a table on the main AOP page (see screen shot 6). Once the user has entered the desired information, they can return to the wiki via the link at the top of the page (see screen shot 5). The summary section of the wiki page will now contain tables matching the ones shown on the data entry form (see screen shot 7). The names in each table are hyperlinks directing the user to a MIE, KE, or AO page, which is now linked to the AOP page (see Figure 2; screen shots 8, 9, 10). If the MIE, KE, or AO was selected from the drop-down menu of previously defined events, the MIE, KE, or AO description will already be populated with content – which the user can now add to, if appropriate. If the user created a new MIE, KE, or AO, a new MIE, KE, or AO page is automatically created and the user can then fill in the KE description information (section 5B) as outlined above. In this manner, either newly defined or existing MIEs, KEs, or AOs from the AOP wiki can be linked to the AOP under development.

In the case of MIEs and KEs, when the event populates into the table, the user will also be prompted to enter an evaluation of the support for the essentiality of the KE (i.e., weak, moderate, strong). This evaluation is part of the overall assessment of the AOP (detailed in section 7). It does not need to be entered at the time the KE is created and described; rather it should be filled in at the time the overall AOP is evaluated. It is included in the KE summary table as a convenience to readers/users of the AOP-KB who may want a quick overview when viewing and using the information after it has been entered.

Figure 2. Overview of the organization of content pages in the AOP-wiki relative to sections of the AOP template. Sections 1, 4, 5a, and 7 are found on the main page for an individual AOP. Information related to sections 5b and section 6 are entered into separate content pages that can be linked to multiple individual AOP pages.



SECTION 6 – KER DESCRIPTIONS

The utility of AOPs for regulatory application is defined to a large extent by the confidence and precision with which they facilitate extrapolation of data measured at low levels of biological organization to predicted outcomes at higher levels of organization and the extent to which they can link biological effect measurements to their specific causes. Within the AOP framework, the predictive relationships that facilitate extrapolation are represented by the KERs. Consequently, the overall weight of evidence for an AOP is a reflection in part, of the level of confidence in the underlying series of KERs it encompasses. Therefore, describing the KERs in an AOP involves assembling and organising the types of information and evidence that defines the scientific basis for inferring the probable change in or state of a downstream KE from the known or measured state of an upstream KE.

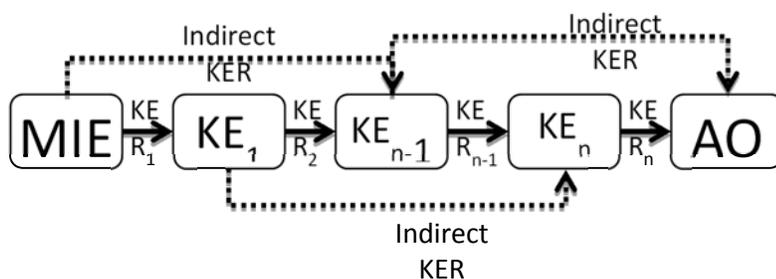
Description of the scientific evidence supporting KERs in an AOP is an important step in the AOP development process that sets the stage for assessment of the AOP (section 7). The modified Bradford Hill considerations of biological plausibility and empirical support can be evaluated with regard to the predictive relationships/associations between pairs of KEs as a basis for considering weight of evidence of KERs (Section 7). The plausibility of the relationship between two KEs with respect to current understanding of normal (i.e., unperturbed biology) can be evaluated. Concordance of empirical evidence (i.e., dose-response, temporal and incidence concordance) can also be assessed and is usually based on consideration of these relationships following exposure to specific stressors that are believed to initiate the pathway. For example, temporal concordance can be evaluated by considering whether each “upstream” KE precedes the next “downstream” KE in the series. For empirical evidence derived for a specific stressor, dose-response and incidence concordance can also be evaluated to determine whether the pattern of results supports the hypothesized KER – i.e., does KE_{upstream} occur at equivalent or lower doses and/or with less frequency than $KE_{\text{downstream}}$.

Consistencies or inconsistencies in supporting data across different biological contexts and/or multiple studies can also help define confidence in the KER. Therefore, the suggested subsections of the KER description included in the current template are intended to aid the user in collecting relevant information that will support evaluation of the level of confidence in each KER, which in turn contributes to the assessment of the weight of evidence of the AOP, overall (section 7).

By convention, KERs may take one of two forms. They may refer specifically to direct linkage between a pair of KEs that are adjacent in an AOP. Alternatively, a KER may refer to indirect linkages between a pair of KEs for which the relationship is thought to run through another KE or a gap in current understanding (i.e., non-adjacent KEs in an AOP; represented as dashed arrows in Figure 3). It is not necessary to describe a KER for every possible binary pair of KEs that could be indirectly linked. However, the option to provide KER descriptions for indirect KERs is particularly useful within the AOP-Wiki, because empirical evidence supporting the linkages among KEs in an AOP (see below) may often skip steps. For example, some KE measurements may be fairly difficult to make, such that they are rarely made in routine studies. While there may be sufficient data to establish the KE as part of the AOP, much of the available weight of evidence may ignore or “leap over” that particular KE. Including indirect KER descriptions allows the weight of evidence for these indirect relationships to be readily described and linked to other AOPs. Additionally, it can aid the process of developing and expanding putative AOPs where initial

linkages may span significant knowledge-gaps which are later filled in with additional KEs as more information becomes available and/or targeted research is completed.

Figure 3. Generic AOP diagram illustrating the two general types of key event relationships [KERs] that an AOP developer may want to describe. Both represent a predictive relationship between a pair of key events and can be supported by weight of evidence. However, direct KERs typically represent direct connections while indirect KERs may represent correlation or indirect connections mediated by another key event (or a gap in current understanding).



To support evaluation of the scientific evidence supporting the AOP, each KER description should address the following topics, to the extent feasible:

A. Title of KER

The title of the KER should clearly define the two KEs being considered, the sequential relationship between them (i.e., which is upstream and which is downstream), and whether the KEs are adjacent (directly leads to) or non-adjacent (indirectly leads to) in an AOP.

- a. Direct KER titles take the form: “KE_i directly leading to KE_{i+1}”.
- b. Indirect KER titles typically take the form: “KE_i indirectly leading to KE_{i>(i+1)}”

B. Description of the KER

Provide a brief, descriptive summation of the KER. While the title itself is fairly descriptive, this section can contain details that aren’t inherent in the description of the KEs themselves (see section 5, recommendations regarding number of KEs to include). For example, if the upstream KE was binding to a specific receptor, the description could stipulate that “persistent binding to the receptor for a period of days” will trigger the downstream KE. Shorter term binding to the same receptor (i.e., same upstream KE) may trigger a different downstream KE, and thus would be described as a different KER. This description section can be viewed as providing the increased specificity in the nature of upstream perturbation (KE_{upstream}) that leads to a particular downstream perturbation (KE_{downstream}), while allowing the KE descriptions to remain generalised so they can be linked to different AOPs. The description is also intended to provide a concise overview for readers who may want a brief summation, without needing to read through the detailed support for the relationship (covered below).

C. Weight of Evidence for the KER

1. Biological Plausibility

Define the biological rationale for a connection between the pair of KEs in question. What are the structural or functional relationships between the KEs? In the case of indirect KERs, this may

entail briefly describing an intermediate KE or a gap in knowledge. Supporting references should be included. However, it is recognised that there may be cases where the biological relationship between two KEs may be very well established, to the extent that it is dogma. In such cases, it may be impractical to exhaustively cite the relevant primary literature. Citation of review articles or other secondary sources like text books, etc. may be reasonable in such cases. The primary intent is to provide scientifically credible support for the structural and/or functional relationship between the pair of KEs if one is known. In general, the structural and/or functional relationship supporting plausibility is based on understanding of normal biological function, rather than response to a specific stressor. The description of biological plausibility can also incorporate additional mechanistic detail that helps inform the relationship between KEs, but is not critical to represent as separate KEs due to the difficulty or relative infrequency with which it's likely to be measured. For example, in the case of G protein coupled receptor activation ($KE_{upstream}$) leading to increased activity of a specific enzyme ($KE_{downstream}$), there may be numerous mechanistic steps in between those KEs (e.g., alterations in signal transduction pathways, transcriptional regulation, post-translational modifications, etc). These underlying details, if known, can be captured in the description of biological plausibility (if desired) rather than represented as independent key events (see section 5a). The KER descriptions are an appropriate place for “compounding” or “embedding” that type of biological detail without compromising the reusability of KE descriptions within the AOP-Wiki.

2. Empirical support

Cite specific evidence that supports the idea that a change in the upstream KE ($KE_{upstream}$) will lead to, or is associated with, a subsequent change in the downstream KE ($KE_{downstream}$), assuming the perturbation of $KE_{upstream}$ is sufficient. In particular, it is useful to cite evidence showing that stressors that perturb $KE_{upstream}$ also perturb $KE_{downstream}$. Like-wise, specific evidence showing the temporal concordance of the KEs (i.e., $KE_{upstream}$ precedes $KE_{downstream}$) should be included, where possible. Evidence of dose response and/or response-response relationships (later KEs) and dose-dependent- and time-dependent transitions from $KE_{upstream}$ to $KE_{downstream}$ should be presented as should those related to dose-specific incidence – i.e., incidence of $KE_{downstream}$ versus $KE_{upstream}$ induced by a similar dose of a stressor. Given the likelihood that new empirical support will be developed over time, particularly as various AOPs are tested and applied, it is most practical to provide empirical support in the form of a bulleted list or table that includes a short description of the nature of the empirical support along with the corresponding reference(s). Because this section of the KER description cites evidence from specific studies, when describing the empirical evidence, it is also helpful to provide as much detail about the toxicological and biological context in which the measurements were made, as is feasible, including the stressor(s) tested, the effective doses at each KE, etc. While the KER itself is not intended to be stressor-specific, those details can aid the overall assessment of the individual AOPs that include that KER and help inform the question of consistency of supporting data and across different biological contexts for which the KER is relevant. In this context, consideration of the information in tabular format of one of the columns in Figure 4 (Section 7) may be helpful in identifying extent of empirical support or inconsistencies.

3. Uncertainties or Inconsistencies

In addition to outlining the evidence supporting a particular linkage, it is also important to identify inconsistencies or uncertainties in the relationship. This could include, for example, empirical evidence showing changes in $KE_{upstream}$ that did not elicit alterations in $KE_{downstream}$. It could also include description of gaps in biological understanding that lead to uncertainties in understanding of the exact nature of the structural or functional relationship between the two KEs. Identification

of uncertainties and inconsistencies serves to contribute to evaluation of the overall weight of evidence supporting the AOPs that contain a given KER (see Section 7) and to the identification of research gaps that may warrant ongoing or future investigations. Given that AOPs are intended to support regulatory applications, AOP developers should focus on those inconsistencies or gaps that would have a direct bearing or impact on the confidence in the KER and its use as a basis for inference or extrapolation in a regulatory setting. Uncertainties that may be of academic interest but would have little impact on regulatory application need not be described. This section essentially details evidence that may raise questions regarding the overall validity (including consideration of both biological plausibility and empirical support) to support application of the KERs. It also contributes along with several other elements to the overall evaluation of the weight of evidence for the KER (see, Section 7).

D. Quantitative Understanding

Finally, while qualitative relationships between KEs may be adequate for some regulatory applications, others will require that quantitative relationships between KEs be defined. Therefore, to the extent possible, KER descriptions should provide an overall characterisation of the degree of quantitative understanding of the relationship between the two KEs. These quantitative relationships may be defined in terms of correlations, response-response relationships, dose-dependent transitions or points of departure (i.e., a threshold of change in KE_{upstream} needed to elicit a change in $KE_{\text{downstream}}$), etc. They may take the form of simple mathematical equations or sophisticated biologically-based computational models that consider other modulating factors such as compensatory responses, or interactions with other biological or environmental variables. Regardless of form, the idea is to briefly describe what is known regarding the quantitative relationship between the KEs and cite appropriate literature that defines those relationships and/or provides support for them. In most cases, quantitative understanding of the KER will also serve as empirical support for the KER. This section is not intended to be redundant with section 3b. Rather, it is intended to aid application of the AOP by allowing a reader to rapidly identify the relationships that would support quantitative prediction of the probability or magnitude of change in $KE_{\text{downstream}}$ based on a known state of KE_{upstream} . For transparency, the toxicological and biological context in which the quantitative relationships were defined should be indicated within the description. However, the ultimate goal is to identify quantitative relationships that generalise across the entire applicability domain of the two KEs being linked via the KER.

Implementation in the AOP-Wiki (see Figure 2 & Screen Shots 11, 12)

KERs that make up an AOP are tracked via a KER table (currently titled “Relationships among key events and the adverse outcome”) that is included on the AOP page within the AOP-Wiki (see Figure 2; see wiki screen shot 11). New KERs are added to the table by following the link to the “widget page” (see wiki screen shot 5) and clicking on the “Add record to table” button (see wiki screen shot 11a). This brings up a dialogue box allowing the user to select a pair of KEs (including MIE or AO) and the type of relationship between them (i.e., “directly leads to” or “indirectly leads to”). The KE names link to the corresponding pages (see wiki screen shot 11b), but the type of relationship listed in the Description column is hyperlinked to a KER page where the AOP developer can enter a KER description, support for the KER, consisting of biological plausibility, empirical support, uncertainties and inconsistencies, and the quantitative understanding of the KER (see wiki screen shot 12). The KER table includes a column for Weight of Evidence and one for Quantitative Understanding of the KER. As with the Support for Essentiality in the KE tables, it is not necessary to specify these values initially as they follow from the evidence accumulated on the KER page and the evaluation of that evidence in Section 7.

Distinguishing indirect from direct KERs has several useful functions in the AOP-Wiki. Firstly, it facilitates the entry of KERs in an AOP (e.g., MIE and AO), but for which a number of important KERs are missing (i.e., significant gaps in the AOP remain). Capability to enter KERs for incomplete AOPs containing gaps facilitates the use of the wiki as a collaborative/crowd-sourced platform for AOP development. A second key function is that it allows for entry of weight of evidence that skips over KERs in an AOP without requiring reference to other KERs in the pathway (other than the two being associated). This facilitates construction of “stand alone” KER descriptions that can be linked to multiple AOPs for which they may be relevant.

SECTION 7. ASSESSMENT OF THE AOP

This section addresses the relevant domain of applicability (i.e., in terms of taxa, sex, life stage, etc.) and weight of evidence for the overall hypothesised AOP (i.e., including the MIE, KEs and AO) as a basis to consider appropriate regulatory application (e.g., priority setting, testing strategies or risk assessment). It draws upon the evidence assembled for each KER in section 6 as one of several components which contribute to relative confidence in supporting information for the entire hypothesised pathway.

An important component in assessing confidence in supporting information as a basis to consider regulatory application of AOPs beyond that described in Section 6 is the essentiality of each of the key events as a component of the entire pathway. This is normally investigated in specifically-designed stop/reversibility studies or knockout models (i.e., those where a key event can be blocked or prevented).

Assessment of the overall AOP also contributes to the identification of KEs for which confidence in the quantitative relationship with the AO is greatest (i.e., to facilitate determining the most sensitive predictor of the AO).

The evaluation process can be organised into a number of steps, for which guidance on the extent or weight of evidence depending on the nature of supporting data is provided in Annexes 1 and 2.

A. Define Domain of Applicability of the AOP

The relevant domain(s) of applicability in terms of sex, life-stage, taxa, and other aspects of biological context are defined in this section. Domain of applicability is informed by the “Description” and “Taxonomic Relevance” section of each KE description and the “Description of the KER” section of each KER description. The relevant domain of applicability of the AOP as a whole will most often be defined based on the most narrowly restricted of its KEs. For example, if most of the KEs apply to either sex, but one is relevant to females only, the domain of applicability of the AOP as a whole would generally be limited to females. While much of the detail defining the domain of applicability may be found in the individual KE descriptions, the rationale for defining the relevant domain of applicability of the overall AOP should be briefly summarised on the AOP page.

B. Assess Relative Level of Confidence in the AOP Based on Rank Ordered Elements and Quantitation

This involves evaluation of the Overall AOP based on Relative Level of Confidence in the KERs, Essentiality of the KEs and Degree of Quantitative Understanding based on Annexes 1 and 2.

Annex 1 (“Guidance for assessing relative level of confidence in the Overall AOP”) guides consideration of the weight of evidence or degree of confidence in the predictive relationship between pairs of KEs based on KER descriptions and support for essentiality of KEs. It is designed to facilitate assignment of categories of high, moderate or low against specific considerations for each a series of defined element based on current experience in assessing MOAs/AOPs. In addition to increasing consistency through delineation of defining questions for the elements and the nature of evidence associated with assignment to each of the categories, importantly, the objective of completion of Annex 1 is to transparently delineate the rationales for the assignment based on the specified considerations. While it is not necessary to repeat lengthy text which appears in earlier parts of the document, the entries for the rationales should explicitly express the reasoning for assignment to the categories, based on the considerations for high, moderate or low weight of evidence included in the columns for each of the relevant elements.

While the elements can be addressed separately for each of the KERs, the essentiality of the KEs within the AOP is considered collectively since their interdependence is often illustrated through prevention or augmentation of an earlier or later key event. Where it is not possible to experimentally assess the essentiality of the KEs within the AOP (i.e., there is no experimental model to prevent or augment the key events in the pathway), this should be noted.

Identified limitations of the database to address the biological plausibility of the KERs, the essentiality of the KEs and empirical support for the KERs are influential in assigning the categories for degree of confidence (i.e., high, moderate or low).

Consideration of the confidence in the overall AOP is based, then, on the extent of available experimental data on the essentiality of KEs and the collective consideration of the qualitative weight of evidence for each of the KERs, in the context of their interdependence leading to adverse effect in the overall AOP. Assessment of the overall AOP is summarized in the Network View, which represents the degree of confidence in the weight of evidence both for the rank ordered elements of essentiality of the key events and biological plausibility and empirical support for the interrelationships between KEs. The AOP-Wiki provides such a network graphic based on the information provided in the MIE, KE, AO, and KER tables. The Key Event Essentiality calls are used to determine the size of each key event node with larger sizes representing higher confidence for essentiality. The Weight of Evidence summary in the KER table is used to determine the width of the lines connecting the key events with thicker lines representing higher confidence.

Additional detail on consideration of each of the rank ordered elements and degree of quantitation as a basis to assess confidence in supporting information for the overall AOP is presented below.

1. Consider Extent of Support for the Biological plausibility of each of the KERs

: Biological plausibility of each of the KERs in the AOP is the most influential consideration in assessing weight of evidence or degree of confidence in an overall hypothesised AOP for potential regulatory application (Meek et al., 2014; 2014a). The defining question for biological plausibility (Annex 1) is: Is there a mechanistic (i.e., structural or functional) relationship between KE_{upstream} and KE_{downstream} consistent with established biological knowledge? Weight of evidence for the biological plausibility of the KERs would be considered high if it is well understood based on extensive previous documentation, has an established mechanistic basis and broad acceptance (e.g., mutation leading to tumours), moderate if the KER is plausible based on analogy to accepted biological relationships but scientific understanding is not completely established and low if there is empirical support for a statistical association between KEs but structural or functional relationship between them is not understood.

2. Consider Extent of Support for the Essentiality of each of the KEs in the AOP

The essentiality of various of the KEs is influential in considering confidence in an overall hypothesised AOP for potential regulatory application being secondary only to biological plausibility of KERs (Meek et al., 2014; 2014a). The defining question for determining essentiality (included in Annex 1) relates to whether or not downstream KEs and/or the AO is prevented if an upstream event is experimentally blocked. It is assessed, generally, then, on the basis of direct experimental evidence of the absence/reduction of downstream KEs when an upstream KE is blocked or diminished (e.g., in null animal models or reversibility studies). Weight of evidence for essentiality of KEs would be considered high if there is direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important key events [e.g., stop/reversibility studies, antagonism, knock out models, etc.) moderate if there is indirect

evidence that experimentally induced change of an expected modulating factor attenuates or augments a key event (e.g., augmentation of proliferative response (KE_{upstream}) leading to increase in tumour formation ($KE_{\text{downstream}}$ or AO)) and weak if there is no or contradictory experimental evidence of the essentiality of any of the KEs (Annex 1).

3. *Consider Extent of Empirical Support for each of the KERs and the Overall AOP*

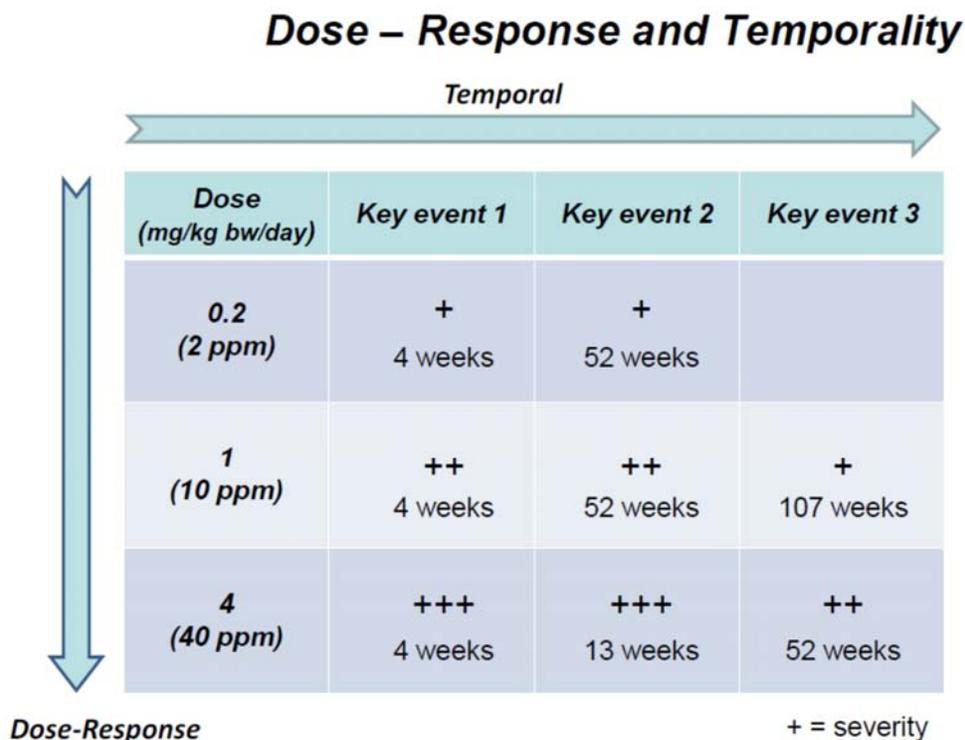
While it is important supporting information, the least influential element in considering confidence in an overall hypothesized AOP for potential regulatory application is the extent of empirical support (Meek et al., 2014; 2014a). This is seemingly not well understood with many of the analyses to support hypothesised chemical specific MOAs being restricted solely to empirical analysis based on existing data rather than identification of critical data gaps from a regulatory perspective. The defining question for empirical support (Annex 1) is: Does the empirical evidence support that a change in KE_{upstream} leads to an appropriate change in $KE_{\text{downstream}}$? This requires consideration of dose-response concordance, temporality (i.e. Does KE_{upstream} occurs at lower doses and earlier time point than $KE_{\text{downstream}}$) and incidence concordance (i.e., is the incidence of $KE_{\text{upstream}} >$ than that for $KE_{\text{downstream}}$?). Inconsistencies in empirical support across taxa, species and stressors that don't align with the expected pattern for the hypothesised AOP as described in Section 6 should be identified.

Empirical support for each of the KERs would be considered high if there is dependent change in both events following exposure to a wide range of specific stressors (extensive evidence for temporal, dose-response and incidence concordance) and no or few data gaps or conflicting data. Demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with expected pattern which can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc. would be considered moderate support. Limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all) and/or lacking evidence of temporal or dose-response concordance or identification of significant inconsistencies in empirical support across taxa and species which don't align with the expected pattern for the hypothesised AOP would be considered low.

It's important to recognize that empirical support relates to “concordance” of dose response, temporal and incidence relationships for KERs rather than the KEs; the defining question is not whether or not there is a dose response relationship for an associated KE but rather, whether there is expected concordance with the dose-response relationships for earlier and later KEs. This is normally demonstrated in studies with different types of stressors. Empirical support for the entire AOP is normally evaluated based on a template such as that presented below for tested stressors. If the hypothesised linkages in the AOP are supported by empirical data, the table completes from the top left hand corner to the bottom right hand corner. Presentation in this manner readily identifies any exceptions to the expected pattern which are considered as inconsistencies and detract from the overall weight of evidence (see Figure 4).

Figure 4. Consideration of dose-response, temporal and incidence concordance for tested stressors

From: Meek & Klaunig, 2010



4. Assess Degree of Quantitative Understanding for Each KER

The extent of quantitative understanding of the various KERs in the overall hypothesised AOP is also critical in consideration of potential regulatory application. For some applications (e.g. dose-response analysis in in depth risk assessment), quantitative characterisation of downstream KERs may be essential while for others, quantitative understanding of upstream KERs may be important (e.g., QSAR modelling for category formation for testing). Because evidence that contributes to quantitative understanding of the KER is generally not mutually exclusive with the empirical support for the KER, evidence that contributes to quantitative understanding should generally be considered as part of the evaluation of the weight of evidence supporting the KER (see Annex 1, footnote b). General guidance on the degree of quantitative understanding that would be characterised as weak, moderate, or strong is provided in Annex 2.

Implementation in the AOP Wiki (see Figure 2; Screen Shots 13a-d))

A series of widgets for defining the applicability domain of the AOP with regard to sex, life-stage, and taxa are included on “widget page” associated with the AOP page in the AOP-wiki (see wiki screen shot 5). Clicking on the respective “add life stage/species/sex to table” button brings up a

drop-down list of possible controlled vocabulary that can be used to define the applicability domain (see wiki screen shot 13a). In addition to the widgets for creating tables defining the applicability domain, a free text section is provided where the rationale for the applicability domain of the overall AOP can be defined (see wiki screen shot 13b).

Based on the evidence assembled for the AOP, the user can then make a call for each KE on whether the evidence supports an essential role for that KE and therefore whether it actually represents a KE in the AOP. These assessments are captured in the Molecular Initiating Event and KE tables under the AOP Summary section with a text description under the Overall Assessment of the AOP section (see wiki screen shot 14a). Completion of the text description should be guided by section 2 of Annex 1. The next step is the consideration of weight of evidence for the AOP. The AOP-Wiki currently combines the biological plausibility and empirical support into a single Weight of Evidence evaluation. Based on the evidence assembled on the KER pages (see wiki screen shot 12b), the user has the information required to make a weight of evidence call within the KER table (see wiki screen shot 14b). This describes the relative level of confidence in the predictive relationship between the two KEs as evaluated based on Annex 1. A corresponding free text section, “Weight of Evidence Summary,” should include the short justification information as outlined in Annex 1. The KER table also has a field for evaluation of the general level of quantitative understanding of the relationship. The description of the quantitative understanding of the KER from the linked KER page is intended to support an AOP-specific “quantitative understanding call” in the KER table on the AOP page, as evaluated based on Annex 2. A free text section under the Overall Assessment of the AOP allows the user to provide a brief justification of the quantitative understanding call based on the guidance in Annexes 1 and 2 (see wiki screen shot 14b).

SECTION 8. POTENTIAL APPLICATIONS OF THE AOP (OPTIONAL):

At their discretion, the developer may include in this section discussion of the potential applications of an AOP to support regulatory decision-making. This may include, for example, possible utility for test guideline development or refinement, development of integrated testing and assessment approaches, development of (Q)SARs / or chemical profilers to facilitate the grouping of chemicals for subsequent read-across, screening level hazard assessments or even risk assessment.

While it is challenging to foresee all potential regulatory application of AOPs and any application will ultimately lie within the purview of regulatory agencies, potential applications may be apparent as the AOP is being developed, particularly if it was initiated with a particular application in mind. This optional section is intended to provide the developer with an opportunity to suggest potential regulatory applications and describe his or her rationale. Detailing such considerations can aid the process of transforming narrative descriptions of AOPs into practical tools. In this context, it is necessarily beneficial to involve members of the regulatory risk assessment community on the development and assessment team.

The Network view which is generated based on assessment of weight of evidence/degree of confidence in the hypothesized AOP taking into account the elements described in Section 7 provides a useful summary of relevant information as a basis to consider appropriate application in a regulatory context. Consideration of application needs then, to take into consideration the following rank ordered qualitative elements:

Confidence in biological plausibility for each of the KERs

Confidence in essentiality of the KEs

Empirical support for each of the KERs and overall AOP

The extent of weight of evidence/confidence in both these qualitative elements and that of the quantitative understanding for each of the KERs (e.g., is the MIE known, is quantitative understanding restricted to early or late key events) is also critical in determining appropriate application.

For example, if the confidence and quantitative understanding of each KER in a hypothesised AOP are low and or low/moderate and the evidence for essentiality of KEs weak (Section 7), it might be considered as appropriate only for applications with less potential for impact (e.g., prioritisation, category formation for testing) versus those that have immediate implications potentially for risk management (e.g., in depth assessment). If confidence in quantitative understanding of late key events is high, this might be sufficient for an in depth assessment.

The analysis supporting the Network view is also essential in identifying critical data gaps based on envisaged regulatory application.

Implementation in the AOP-Wiki.

A free text section for describing potential applications of the AOP is included at the bottom of the AOP page within the AOP-Wiki (see wiki screen shot 14b).

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Annex 1: Guidance for assessing relative level of confidence in the overall AOP based on rank ordered elements

1. Support for Biological Plausibility of KERS ¹	Defining Question	High (Strong) ^{2,3}	Moderate	Low (Weak)
	a) Is there a mechanistic (i.e., structural or functional) relationship between KE _{up} and KE _{down} consistent with established biological knowledge?	Extensive understanding of the KER based on extensive previous documentation and broad acceptance (e.g., mutation leading to tumours) -Established mechanistic basis	The KER is plausible based on analogy to accepted biological relationships but scientific understanding is not completely established.	There is empirical support for a statistical association between KEs (See 3.), but the structural or functional relationship between them is not understood.
⁴ MIE => KE1: (cut and paste the KER description into this cell)	Biological Plausibility of the MIE => KE1 is xxx . Rationale:			
KE1 => KE2 : (cut and paste the KER description into this cell)	Biological Plausibility of KE1 => KE2 is xxx Rationale:			
KE2 => KE3 ((cut and paste the KER description into this cell)	Biological Plausibility of KE1 => KE2 is xxx . Rationale:			
2. Support for Essentiality of KEs⁵				
	Defining Question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs (e.g., stop/reversibility studies, antagonism, knock out models, etc.)	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE (e.g., augmentation of proliferative response (KE _{up}) leading to increase in KE _{down} or AO).	No or contradictory experimental evidence of the essentiality of any of the KEs.
MIE: (cut and paste the MIE description into this cell)	Essentiality of the MIE is xxx .			
KE1: (cut and paste the KE1 description into this cell)	Essentiality of the KE1 is xxx .			
KE2: (cut and paste the KE2 description into this cell)	Essentiality of the KE2 is xxx . Rationale for Essentiality of KEs in the AOP:			

¹ Rank ordered elements adapted from Meek et al. (2014b)

² The guidance for “high”, “moderate” and “low” draws on limited current experience. Additional delineation of the nature of relevant evidence in these broadly defined categories requires more experience with larger numbers of documented AOPs.

³“Direct evidence” implies specifically designed experiments to consider the relevant element. “Indirect evidence” normally relates to empirical support and is largely duplicative of Element 3.

⁴ To the extent possible, each of the relevant Bradford Hill considerations is addressed for each of the KERs (biological plausibility and empirical support) and KEs (essentiality) and separate rationales provided.

⁵ While the essentiality of each of the KEs is addressed separately, delineation of the degree of confidence is based on consideration of evidence for all of the KEs within the AOP and therefore, only one rationale is required.

3. Empirical Support ^b for KERs	Defining Questions	High (Strong)	Moderate	Low (Weak)
	<p>Does the empirical evidence support that a change in KE_{up} leads to an appropriate change in KE_{down}? Does KE_{up} occur at lower doses and earlier time points than KE_{down} and is the incidence of KE_{up} > than that for KE_{down}?⁶⁷.</p> <p>Are there inconsistencies in empirical support across taxa, species and stressors that don't align with expected pattern for hypothesized AOP?</p>	<p>Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. (Extensive evidence for temporal, dose-response and incidence concordance) and no or few critical data gaps or conflicting data</p>	<p>Demonstrated dependent change in both events following exposure to a small number of specific stressors and some evidence inconsistent with expected pattern that can be explained by factors such as experimental design, technical considerations, differences among laboratories, etc..</p>	<p>Limited or no studies reporting dependent change in both events following exposure to a specific stressor (i.e., endpoints never measured in the same study or not at all); and/or significant inconsistencies in empirical support across taxa and species that don't align with expected pattern for hypothesized AOP</p> <p>-</p>
MIE => KE1: (cut and paste the KER description into this cell) ³	<p>Empirical Support of the MIE => KE1 is. xxx.</p> <p>Rationale:</p> <ul style="list-style-type: none"> • 			
KE1 => KE2 : (cut and paste the KER description into this cell)	<p>Empirical Support of the KE1 => KE2 is xxx.</p> <p>Rationale:</p>			
KE2 => KE3 (cut and paste the KER description into this cell)	<p>Empirical Support of the KE1 => KE2 is xxx. .</p> <p>Rationale:</p>			
<p>^b In many cases, evidence that contributes to quantitative understanding (section 4 of a KER description) will also provide empirical support for the relationship. Consequently, relevant information from the "Quantitative Understanding" section of the KER description should be considered as part of the overall weight of evidence evaluation of the concordance of empirical observations and consistency for the KER.</p>				

⁶ This is normally considered on the basis of tabular presentation of available data on temporal and dose-response aspects, in a template that documents the extent of support. See, for example, Meek and Klaunig (2010).

⁷ Note that this relates to concordance of dose response, temporal and incidence relationships for KERs rather than the KEs; the defining question is not whether or not there is a dose response relationship for the KE but rather there is concordance with that for earlier and later KEs. This is normally demonstrated in studies with different types of stressors.

Annex 2. General guidance for characterizing the level of quantitative understanding of a KER as weak, moderate, or strong

Extent of Quantitative Understanding⁸	Characteristics
Strong	<ul style="list-style-type: none"> • Change in KE_{down} can be precisely predicted based on a relevant measure of KE_{up}. • Uncertainty in the quantitative prediction can be precisely estimated from the variability in the relevant measure of KE_{up}. • Known modulating factors are accounted for in the quantitative description. • There is evidence that the quantitative relationship between the KEs generalizes across the relevant applicability domain of the AOP.
Moderate	<ul style="list-style-type: none"> • Change in KE_{down} can be precisely predicted based on a relevant measure of KE_{up}. • Uncertainty in the quantitative prediction is influenced by factors other than the variability in the relevant measure of KE_{up}. • Quantitative description does not account for all known modulating factors. • The quantitative relationship has only been demonstrated for a subset of the overall applicability domain of the AOP (e.g., based on a single species).
Weak	<ul style="list-style-type: none"> • Only a qualitative or semi-quantitative prediction of the change in KE_{down} can be determined from a measure of KE_{up}. • Known modulating factors are not accounted for. • The quantitative relationship has only been demonstrated for a narrow subset of the overall applicability domain of the AOP (e.g., based on a single species).

⁸ The guidance for “high”, “moderate” and “low” draws on limited current experience. Additional delineation of the nature of relevant evidence in these broadly defined categories requires experience with larger numbers of documented AOPs.

Annex 3

AOP wiki screen shots

These screen shorts will be updated as the wiki platform evolves.

AOP Wiki – Screen Shot 1
Section 1 – AOP Identifier/Title

Create New AOP

Short Title:

Long Title:

[Back](#)

Page [Discussion](#)

Aop:3
Template:Adverse Outcome Pathway > Event:4 > Aop:2 > Aop:3 > MediaWiki:Common.css

AOP Title [\[edit\]](#)

Aromatase inhibition leading to reproductive dysfunction

Short name:Aromatase and reproduction

AOP Wiki – Screen Shot 2

Section 2,3,4 – Authors, Status, Abstract, Background

AOP Title [\[edit\]](#)

Aromatase inhibition leading to reproductive dysfunction (in fish)

Short name: Aromatase inhibition leading to reproductive dysfunction (in fish)

Authors [\[edit\]](#)

Dan Villeneuve, US EPA Mid-Continent Ecology Division (villeneuve.dan@epa.gov)

Status [\[edit\]](#)

Alert: The Weight of Evidence column in the Molecular Initiating Event and Key Event tables has changed to Essentiality. Consider re-evaluating the columns in these tables.

Open for comment

Abstract [\[edit\]](#)

This adverse outcome pathway details the linkage between inhibition of gonadal aromatase activity in females and the adverse effect of reduced cumulative fecundity. Initial development of this AOP draws heavily on evidence collected using 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 aromatase, or in silico predictions of the ability to inhibit aromatase, as a means to identify chemicals with known potential to adversely affect fish populations.

Background (optional) [\[edit\]](#)

AOP Wiki – Screen Shot 3

Section 2, 3 – Authors of AOP; Date of Updating of AOP

Revision history of "Aromatase inhibition leading to reproductive dysfunction (in fish)"

[View logs for this page](#)

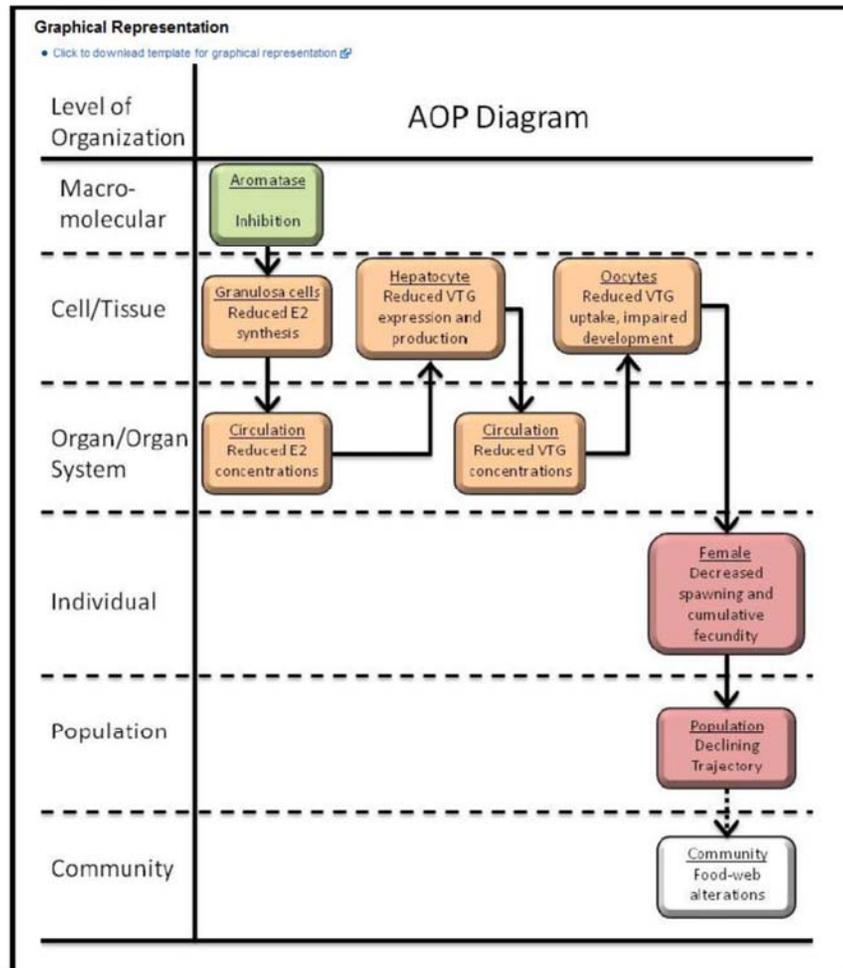
Browse history

From year (and earlier): From month (and earlier): Deleted only

Diff selection: Mark the radio boxes of the revisions to compare and hit enter or the button at the bottom.
Legend: **(cur)** = difference with latest revision, **(prev)** = difference with preceding revision, **m** = minor edit.

- (cur | prev) 09:39, June 28, 2014 [Wikibot](#) (Talk | contribs | block) . . (44,577 bytes) (+67) . . (rollback 2 edits | undo)
- (cur | prev) 09:38, June 28, 2014 [Wikibot](#) (Talk | contribs | block) . . (44,510 bytes) (+206) . . (undo)
- (cur | prev) 10:16, May 16, 2014 [Dvillene](#) (Talk | contribs | block) . . (44,304 bytes) (+1) . . (→Essentiality of the Key Events) (undo)
- (cur | prev) 10:15, May 16, 2014 [Dvillene](#) (Talk | contribs | block) . . (44,303 bytes) (+1,388) . . (→Essentiality of the Key Events) (undo)
- (cur | prev) 09:44, May 16, 2014 [Dvillene](#) (Talk | contribs | block) . . (42,915 bytes) (-154) . . (→Weight of Evidence Summary) (undo)
- (cur | prev) 09:43, May 16, 2014 [Dvillene](#) (Talk | contribs | block) . . (43,069 bytes) (-478) . . (→Weight of Evidence Summary) (undo)
- (cur | prev) 09:35, May 16, 2014 [Dvillene](#) (Talk | contribs | block) . . (43,547 bytes) (+79) . . (→Authors) (undo)
- (cur | prev) 15:30, May 15, 2014 [Dvillene](#) (Talk | contribs | block) . . (43,468 bytes) (+72) . . (→Abstract) (undo)
- (cur | prev) 11:45, May 12, 2014 [Sedwar02](#) (Talk | contribs | block) . . (43,396 bytes) (+1,936) . . (→Weight of Evidence Summary) (undo)
- (cur | prev) 11:24, May 12, 2014 [Sedwar02](#) (Talk | contribs | block) . . (41,460 bytes) (-2,065) . . (→Overall Assessment of the AOP) (undo)
- (cur | prev) 11:17, May 12, 2014 [Sedwar02](#) (Talk | contribs | block) . . (43,525 bytes) (+3) . . (→Domain(s) of Applicability) (undo)

AOP Wiki – Screen Shot 4
 Section 5a – Summary of the
 AOP as Figure/AOP Diagram



AOP Wiki – Screen Shot 5
Section 5a – Summary of the AOP
Table of Key Events and Key Event
Relationships

Summary of the AOP

Please follow link to [widget page](#) to edit this

Molecular Initiating Event

[Reference web](#)

Aromatase inhibition leading to reproductive dysfunction (in fish)

NOI WAS SUCCESSFULLY CREATED

Molecular Initiating Event

[Add Molecular Initiating Event](#)

Molecular Initiating Event	Support for Essentiality

Key Events

[Add Key Event](#)

Event	Support for Essentiality

Adverse Outcome

[Add Adverse Outcome](#)

Adverse Outcome

Relationships Among Key Events and the Adverse Outcome

[Add Relationships](#)

Event	Description	Triggers	Weight of Evidence	Qualitative Understanding

Life Stage Applicability

[Add Life Stage](#)

Life Stage	Evidence	Links

Taxonomic Applicability

[Add Species](#)

Name	Scientific Name	Evidence	Links

Sex Applicability

[Add a Sex](#)

AOP Wiki – Screen Shot 6
 Section 5a – Summary of the AOP Table of Key Events
 and Key Event Relationships

Molecular Initiating Event

[Add Molecular Initiating Event](#)

Molecular Initiating Event	Support for Essentiality
Aromatase, Inhibition	<input type="checkbox"/> <input type="checkbox"/>

Key Events

[Add Key Event](#)

Event	Support for Essentiality
Plasma 17beta-estradiol concentrations, Reduction	<input type="checkbox"/> <input type="checkbox"/>
Transcription and translation of vitellogenin in liver, Reduction	<input type="checkbox"/> <input type="checkbox"/>
Plasma vitellogenin concentrations, Reduction	<input type="checkbox"/> <input type="checkbox"/>
Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	<input type="checkbox"/> <input type="checkbox"/>
Cumulative fecundity and spawning, Reduction	<input type="checkbox"/> <input type="checkbox"/>
17beta-estradiol synthesis by ovarian granulosa cells, Reduction	<input type="checkbox"/> <input type="checkbox"/>

Adverse Outcome

[Add Adverse Outcome](#)

Adverse Outcome
Population trajectory, Decrease

Add a Record x

Action [?]

Biological Process/Object [?]

Evidence supporting essentiality [?]

Add a Record x

Action [?]

Biological Process/Object [?]

AOP Wiki – Screen Shot 7
Section 5a – Summary of the AOP Table of Key Events
and Key Event Relationships

Summary of the AOP

Please follow link to [widget page](#) to edit this section.

Molecular Initiating Event

Molecular Initiating Event ↕	Support for Essentiality ↕
Aromatase, Inhibition	

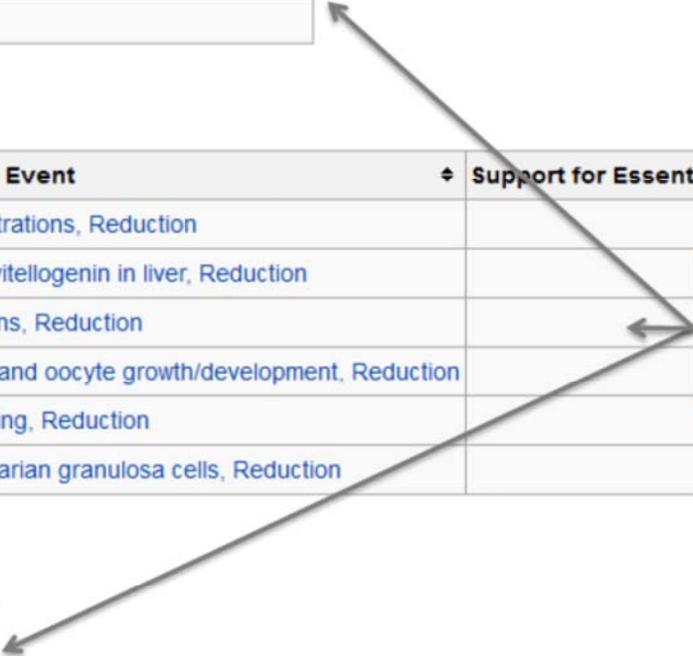
Key Events

Event ↕	Support for Essentiality ↕
Plasma 17beta-estradiol concentrations, Reduction	
Transcription and translation of vitellogenin in liver, Reduction	
Plasma vitellogenin concentrations, Reduction	
Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	
Cumulative fecundity and spawning, Reduction	
17beta-estradiol synthesis by ovarian granulosa cells, Reduction	

List of Key Events,
including the MIE and
AO

Adverse Outcome

Adverse Outcome ↕
Population trajectory, Decrease



AOP Wiki – Screen Shot 8a

Section 5b – Key Event Description Page (Structured content)

Event Title

Plasma 17beta-estradiol concentrations, Reduction

Short name: Plasma 17beta-estradiol concentrations, Reduction

Key Event Overview

Please follow link to [widget page](#) to edit this section.

AOPs Including This Key Event

Note – Key event description pages may link to more than one AOP

AOP Name	Event Type	Essentiality
Androgen receptor agonism leading to reproductive dysfunction	KE	
Aromatase inhibition leading to reproductive dysfunction (in fish)	KE	

Taxonomic Applicability

Name	Scientific Name	Evidence	Links
------	-----------------	----------	-------

Level of Biological Organization

Biological Organization

AOP Wiki – Screen Shot 8b

Section 5b – Key Event Description Page (Textual content)

Level of Biological Organization [\[edit\]](#)

organ system

How this Key Event works [\[edit\]](#) Description of the biology

Estradiol synthesized by the gonads and other steroidogenic tissues (e.g., brain, adipose) is transported to other tissues via blood circulation.

How it is Measured or Detected [\[edit\]](#) Measurement and detection

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Total concentrations of 17 β -estradiol in plasma can be measured by radioimmunoassay (e.g., (Jensen et al. 2001)), enzyme-linked immunosorbent assay (available through many commercial vendors), or by analytical chemistry (e.g., LC/MS). Total steroid hormones are typically extracted from plasma or serum via liquid-liquid or solid phase extraction prior to analysis.

Evidence Supporting Taxonomic Applicability [\[edit\]](#) Taxonomic applicability

Key enzymes needed to synthesize 17 β -estradiol first appear in the common ancestor of amphioxus and vertebrates (Baker 2011). Consequently, this key event is applicable to most vertebrates.

References [\[edit\]](#)

- Jensen K, Korte J, Kahl M, Pasha M, Ankley G. 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comparative Biochemistry and Physiology Part C* 128: 127-141.
- Baker ME. 2011. Origin and diversification of steroids: co-evolution of enzymes and nuclear receptors. *Molecular and cellular endocrinology* 334(1-2): 14-20.

AOP Wiki – Screen Shot 9a

Section 5b – Molecular Initiating Event Description Page (structured content)

Aromatase, Inhibition

[Template:Adverse Outcome](#) > [Special:Export](#) > [45681Aromatase and reproduction](#) > [MediaWiki:Common.css](#) > [Plasma 17beta-estradiol concentrations, Reduction](#)

Contents [\[show\]](#)

Key Event Overview [\[edit\]](#)

AOPs Including This Key Event [\[edit\]](#)

AOP Name	Molecular Initiating Event?	Support for Essentiality
Aromatase inhibition leading to reproductive dysfunction (in fish)	Yes	
Aromatase, Inhibition to Curved body axis, Increase	Yes	
Aromatase, Inhibition to Body length, Decreased	Yes	

Note – MIE description pages may link to more than one AOP

Chemical Initiators [\[edit\]](#)

The following are chemical initiators that operate through this AOP:

[Add Chemical Initiator from list](#)

- [Fadrozole](#)
- [Letrozole](#)

List of chemical initiators – links to structure information and chemical initiator pages

Taxonomic Applicability [\[edit\]](#)

[Add Species from list](#)

Name	Scientific Name	Evidence	Links	
Medaka	<i>Oryzias latipes</i>	Moderate	NCBI	✎ 🗑
Zebrafish	<i>Danio rerio</i>	Moderate	NCBI	✎ 🗑
Fathead minnow	<i>Pimephales promelas</i>	Strong	NCBI	✎ 🗑

Taxonomic applicability

AOP Wiki – Screen Shot 9b

Section 5b – Molecular Initiating Event Description Page (textual content)

Level of Biological Organization [\[edit\]](#)

How this Key Event works [\[edit\]](#) Description of the biology

Inhibition of cytochrome P450 aromatase (CYP19; specifically *cyp19a1a* in fish). 6.1.1. Site of action: The site of action for the molecular initiating event is the ovarian granulosa cells. 6.1.2. Responses at the macromolecular level: Aromatase catalyzes three sequential oxidation steps (i.e., KEGG reactions R02501, R04761, R03087 or R01840, R04759, R02351) involved in the conversion of C-19 androgens (e.g., testosterone, androstenedione) to C-18 estrogens (e.g., 17 β -estradiol, estrone). Aromatase inhibitors interfere with one or more of these reactions, leading to reduced efficiency in converting C-19 androgens into C-18 estrogens. Therefore, inhibition of aromatase activity results in decreased rate of 17 β -estradiol (and presumably estrone) production by the ovary.

How it is Measured or Detected [\[edit\]](#) Measurement and detection

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible? 6.1.4. Measurement/detection: Aromatase activity is typically measured by evaluating the production of tritiated water released upon the aromatase catalyzed conversion of radio-labeled androstenedione to estrone (Lephart and Simpson 1991). Aromatase activity can be measured in cell lines exposed in vitro (e.g., human placental JEG-3 cells and JAR choriocarcinoma cells, (Letcher et al. 1999); H295R human adrenocortical carcinoma cells (Sanderson et al. 2000)). Aromatase activity can also be quantified in tissue (i.e., ovary or brain) from vertebrates exposed in vivo (e.g., (Villeneuve et al. 2006; Ankley et al. 2002)). In vitro aromatase assays are amenable to high throughput and have been included in nascent high throughput screening programs like the US EPA ToxCastTM program.

Evidence Supporting Taxonomic Applicability [\[edit\]](#) Taxonomic applicability

6.1.5. Taxonomic applicability: Aromatase (CYP19) orthologs are known to be present among most of the vertebrate lineage, at least down to the cartilaginous fishes. Orthologs have generally not been found in invertebrates, however, CYP19 was detected in the invertebrate chordate, amphioxus and analysis of conservation of gene order and content suggests a possible origin among primitive chordates (Castro et al. 2005). Fishes generally have two aromatase isoforms, *cyp19a1a* which is predominantly expressed in ovary and *cyp19b*, predominantly expressed in brain.

Evidence for Chemical Initiation of this Molecular Initiating Event [\[edit\]](#) Evidence for chemical initiation of the MIE

6.1.3. Characterization of chemical properties: Chemicals are known to inhibit aromatase activity through two primary molecular mechanisms. Steroid-like structures can inhibit the enzyme at its active site, with structures having $\Delta 4$ positioned double bonds generally acting as stronger inhibitors than those with $\Delta 5$ positioned double bonds (Petkov et al. 2009). Non-steroidal aromatase inhibitors generally act by interfering with electron transfer via the cytochrome P450 heme group of the aromatase enzyme, with greater nucleophilicity of the heteroatom contributing to greater potency as an inhibitor (Petkov et al. 2009). Petkov et al. (Petkov et al. 2009) have provided a detailed analysis of structural categorization of chemicals as potential steroidal or non-steroidal aromatase inhibitors.

References [\[edit\]](#)

AOP Wiki – Screen Shot 10a

Section 5b – Adverse Outcome Description Page

Event Title

Population trajectory, Decrease

Short name: Population trajectory, Decrease

Key Event Overview

Please follow link to [widget page #6](#) to edit this section.

Note – AO description pages may link to more than one AOP

AOPs Including This Key Event

AOP Name	Event Type	Essentiality
Neurotoxicity induced by competitive antagonists of ionotropic GABA receptors	AO	
Androgen receptor agonism leading to reproductive dysfunction	AO	
Aromatase inhibition leading to reproductive dysfunction (in fish)	AO	
Estrogen receptor agonism leading to reproductive dysfunction	AO	
Estrogen receptor antagonism leading to reproductive dysfunction	AO	

Taxonomic Applicability

Name	Scientific Name	Evidence	Links
------	-----------------	----------	-------

Affected Organs

Synonym	Scientific Name	Evidence	Links
---------	-----------------	----------	-------

Level of Biological Organization

Biological Organization

AOP Wiki – Screen Shot 10b
Section 5b – Adverse Outcome Description Page

How this Key Event works Description of the biology

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

How it is Measured or Detected Measurement and detection

Fish population trajectories, either hypothetical or site specific, can be estimated via population modeling based on measurements of vital rates or reasonable surrogates measured in laboratory studies (e.g., cumulative fecundity (Miller and Ankley 2004)).

Evidence Supporting Taxonomic Applicability

Regulatory Examples Using This Adverse Outcome Regulatory relevance

References

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

AOP Wiki – Screen Shot 11a
 Section 6 – Key Event Relationship Table

Relationships Among Key Events and the Adverse Outcome

Add Relationship →

Event	Description	Triggers	Weight of Evidence	Quantitative Understanding	
Aromatase, Inhibition	Directly Leads to	17beta-estradiol synthesis by ovarian granulosa cells, Reduction	Strong		<input type="checkbox"/> <input type="checkbox"/>
17beta-estradiol synthesis by ovarian granulosa cells, Reduction	Directly Leads to	Plasma 17beta-estradiol concentrations, Reduction	Strong		<input type="checkbox"/> <input type="checkbox"/>
Plasma 17beta-estradiol concentrations, Reduction	Directly Leads to	Transcription and translation of vitellogenin in liver, Reduction	Strong		<input type="checkbox"/> <input type="checkbox"/>
Transcription and translation of vitellogenin in liver, Reduction	Directly Leads to	Plasma vitellogenin concentrations, Reduction	Strong		<input type="checkbox"/> <input type="checkbox"/>
Plasma vitellogenin concentrations, Reduction	Directly Leads to	Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	Moderate		<input type="checkbox"/> <input type="checkbox"/>
Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	Directly Leads to	Cumulative fecundity and spawning, Reduction	Moderate		<input type="checkbox"/> <input type="checkbox"/>
Cumulative fecundity and spawning, Reduction	Directly Leads to	Population trajectory, Decrease	Moderate		<input type="checkbox"/> <input type="checkbox"/>

Add a Record ✕

Event^⓪

Description^⓪

Triggers^⓪

Weight of Evidence^⓪

Quantitative Understanding^⓪

Submit

AOP Wiki – Screen Shot 11b
 Section 6 – Key Event Relationship Table

Linkage table:
 Pairwise-
 relationships
 between key
 events

Relationships Among Key Events and the Adverse Outcome

Event	Description	Triggers	Weight of Evidence	Quantitative Understanding
Aromatase, Inhibition	Directly Leads to	17beta-estradiol synthesis by ovarian granulosa cells, Reduction	Strong	
17beta-estradiol synthesis by ovarian granulosa cells, Reduction	Directly Leads to	Plasma 17beta-estradiol concentrations, Reduction	Strong	
Plasma 17beta-estradiol concentrations, Reduction	Directly Leads to	Transcription and translation of vitellogenin in liver, Reduction	Strong	
Transcription and translation of vitellogenin in liver, Reduction	Directly Leads to	Plasma vitellogenin concentrations, Reduction	Strong	
Plasma vitellogenin concentrations, Reduction	Directly Leads to	Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	Strong	
Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	Directly Leads to	Cumulative fecundity and spawning, Reduction	Moderate	
Plasma vitellogenin concentrations, Reduction	Directly Leads to	Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	Moderate	
Cumulative fecundity and spawning, Reduction	Directly Leads to	Population trajectory, Decrease	Moderate	
Plasma 17beta-estradiol concentrations, Reduction	Indirectly Leads to	Plasma vitellogenin concentrations, Reduction		

Key Event Relationship Overview

Please follow link to [widget page](#) to edit this section.

Description of Relationship

Upstream Event	Downstream Event/Outcome
Aromatase, Inhibition	17beta-estradiol synthesis by ovarian granulosa cells, Reduction

AOPs Referencing Relationship

AOP Name	Type of Relationship	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leading to reproductive dysfunction (in fish)	Directly Leads to	Strong	

Note – KER description pages may link to more than one AOP.

Taxonomic Applicability

Name	Scientific Name	Evidence	Links	Taxonomic applicability

AOP Wiki – Screen Shot 12b

Section 6 – Key Event Relationship Page (textual content)

How Does This Key Event Relationship Work

Weight of Evidence

Biological Plausibility **Biological Plausibility**

Within the ovary, aromatase expression and activity is primarily localized in the granulosa cells (reviewed in Nicolis 2007; Yaron 1996; Havelock et al. 2004) and others). C-19 androgens diffuse from the theca cells into granulosa cells where aromatase can catalyze their conversion to C-18 estrogens. Therefore, inhibition of ovarian aromatase activity can generally be assumed to directly impact E2 synthesis by the granulosa cells.

Empirical Support for Linkage **Empirical Support**

Include consideration of temporal concordance here

- Known aromatase inhibitors including fadrozole and prochloraz were shown to cause concentration-dependent inhibition of aromatase activity in fathead minnow ovary homogenates (Vileneuve et al. 2006; Ankley et al. 2005).
- Fadrozole and prochloraz also cause concentration-dependent decreases in E2 production by fathead minnow ovary explants exposed in vitro (Vileneuve et al. 2007).
- Following in vivo exposure to fadrozole or prochloraz, ex vivo E2 production is significantly decreased in a concentration-dependent manner early in the time-course following exposure, although depending on the concentration, compensatory responses may offset the direct impact later in the exposure time-course (Vileneuve et al. 2006; Vileneuve et al. 2009; Ankley et al. 2009a; Skolness et al. 2011).

Uncertainties or Inconsistencies **Uncertainties or Inconsistencies**

Quantitative Understanding of the Linkage **Quantitative Understanding**

Several mechanistically-based models of ovarian steroidogenesis have been developed (Breen et al. 2013; Breen et al. 2007; Shoemaker et al. 2010; Quignot and Bois 2013).

- The Breen et al. 2007 model was developed based on in vitro experiments with fathead minnow ovary tissue, and considers effects on steroidogenesis within the ovary only.
- The Breen et al. 2013 model was developed based on in vivo time-course data for fathead minnow and incorporates prediction of compensatory responses resulting from feedback mechanisms operating as part of the hypothalamic-pituitary-gonadal axis.
- The Shoemaker et al. 2010 model is chimeric and includes signaling pathways and aspects of transcriptional regulation based on a mixture of fish-specific and mammalian sources.
- The Quignot and Bois 2013 model was designed to predict rat ovarian steroid secretion based on in vitro experiments with endocrine disrupting chemicals.
- These may be adaptable to predict in vitro E2 production and/or plasma E2 concentrations from in vitro or in vivo measurements of aromatase inhibition.

Evidence Supporting Taxonomic Applicability **Taxonomic applicability**

References

AOP Wiki – Screen Shot 13a
Section 7 – Overall Assessment of the AOP

Life Stage Applicability

Add Life Stage

Life Stage	Evidence	Links
Adult, reproductively mature		

Add a Record

Life Stage Affected^⓪

Evidence^⓪

Submit

Taxonomic Applicability

Add Species

Name	Scientific Name	Evidence	Links
medaka	Oryzias latipes	Moderate	NCBI
zebrafish	Danio rerio	Moderate	NCBI
fathead minnow	Pimephales promelas	Strong	NCBI

Add a Record

Species^⓪

Search

Scientific Name^⓪

Evidence^⓪

Submit

Sex Applicability

Add a Sex

Sex	Evidence	Links
Female	Strong	

Add a Record

Affected Sex^⓪

Evidence^⓪

Submit

Overall Assessment of the AOP [\[edit\]](#)

Domain of Applicability [\[edit\]](#)

[Life Stage Applicability](#), [Taxonomic Applicability](#), [Sex Applicability](#)

Elaborate on the domains of applicability listed in the summary section above. Specifically, provide the literature supporting, or excluding, certain domains.

Essentiality of the Key Events [\[edit\]](#)

[Molecular Initiating Event Summary](#), [Key Event Summary](#)

Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

Weight of Evidence Summary [\[edit\]](#)

[Summary Table](#)

Provide an overall summary of the weight of evidence based on the evaluations of the individual line

Quantitative Considerations [\[edit\]](#)

[Summary Table](#)

Provide an overall discussion of the quantitative information available for this AOP. Support calls for Relationship table above.

Considerations for Potential Applications of the AOP (optional) [\[edit\]](#)

References [\[edit\]](#)

Life Stage Applicability [\[edit\]](#)

Life Stage	Evidence	Links
adult, reproductively mature		

Taxonomic Applicability [\[edit\]](#)

Name	Scientific Name	Evidence	Links
medaka	Oryzias latipes	Moderate	NCBI ↗
zebrafish	Danio rerio	Moderate	NCBI ↗
fathead minnow	Pimephales promelas	Strong	NCBI ↗

Sex Applicability [\[edit\]](#)

Sex	Evidence	Links
Female	Strong	



Overall Assessment of the AOP [\[edit\]](#)

Domain of Applicability [\[edit\]](#)

[Life Stage Applicability](#), [Taxonomic Applicability](#), [Sex Applicability](#)

Elaborate on the domains of applicability listed in the summary section above. Specifically, provide the literature supporting, or excluding, certain domains.

Essentiality of the Key Events [\[edit\]](#)

[Molecular Initiating Event Summary](#), [Key Event Summary](#)

Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

Weight of Evidence Summary [\[edit\]](#)

[Summary Table](#)

Provide an overall summary of the weight of evidence based on the evaluations of the individual linkages from the Key Event Relationship pages.

Quantitative Considerations [\[edit\]](#)

[Summary Table](#)

Provide an overall discussion of the quantitative information available for this AOP. Summarize the quantitative information available for this AOP. Summarize the Relationship table above.

Considerations for Potential Applications of the AOP (optional)

References [\[edit\]](#)

Summary of the AOP

Molecular Initiating Event

[Add Molecular Initiating Event to Table](#)

Molecular Initiating Event	Support for Essentiality
Aromatase, Inhibition	

Key Events

[Add Event to Table](#)

Event	Support for Essentiality
Plasma 17beta-estradiol concentrations, Reduction	
Transcription and translation of vitellogenin in liver, Reduction	
Plasma vitellogenin concentrations, Reduction	
Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	
Cumulative fecundity and spawning, Reduction	
17beta-estradiol synthesis by ovarian granulosa cells, Reduction	

AOP Wiki – Screen Shot 14b

Section 7 – Overall Assessment of the AOP

Relationships Among Key Events and the Adverse Outcome

[Add record to table](#)

Step	Event	Description	Triggers	Weight of Evidence	Quantitative Understanding	
1	Aromatase, Inhibition	Leads to	17beta-estradiol synthesis by ovarian granulosa cells, Reduction	Strong		 
2	17beta-estradiol synthesis by ovarian granulosa cells, Reduction	Leads to	Plasma 17beta-estradiol concentrations, Reduction	Strong		 
3	Plasma 17beta-estradiol concentrations, Reduction	Leads to	Transcription and translation of vitellogenin in liver, Reduction	Strong		 
3.1	Plasma 17beta-estradiol concentrations, Reduction	Indirectly Leads to	Plasma vitellogenin concentrations, Reduction	Strong		 
4	Transcription and translation of vitellogenin in liver, Reduction	Leads to	Plasma vitellogenin concentrations, Reduction	Strong		 
5	Plasma vitellogenin concentrations, Reduction	Leads to	Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	Moderate		 
6	Vitellogenin uptake into oocytes and oocyte growth/development, Reduction	Leads to	Cumulative fecundity and spawning, Reduction	Moderate		 
7	Cumulative fecundity and spawning, Reduction	Leads to	Population trajectory, Decrease	Moderate		 

Overall Assessment of the AOP [\[edit\]](#)

Domain of Applicability [\[edit\]](#)

[Life Stage Applicability](#), [Taxonomic Applicability](#), [Sex Applicability](#)

Elaborate on the domains of applicability listed in the summary section above.

Essentiality of the Key Events [\[edit\]](#)

[Molecular Initiating Event Summary](#), [Key Event Summary](#)

Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

Weight of Evidence Summary [\[edit\]](#)

[Summary Table](#)

Provide an overall summary of the weight of evidence based on the evaluations of the individual linkages from the Key Event Relationship pages.

Quantitative Considerations [\[edit\]](#)

[Summary Table](#)

Provide an overall discussion of the quantitative information available for this AOP. Support calls for the individual relationships can be included in the Key Event Relationship table above.

Considerations for Potential Applications of the AOP (optional) [\[edit\]](#)

References [\[edit\]](#)