OECD Series on Adverse Outcome Pathways No. 4

Adverse Outcome Pathway on Aromatase Inhibition Leading to Reproductive Dysfunction (in Fish)

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OECD

Foreword

This Adverse Outcome Pathway (AOP) on Aromatase inhibition leading to reproductive dysfunction (in fish) has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Group of the National Coordinators for the Test Guidelines Programme (WNT). The AOP has been reviewed internally by the EAGMST, externally by experts nominated by the WNT, and has been endorsed by the WNT and the Task Force on hazard Assessment (TFHA) in April 2016.

Through endorsement of this AOP, the WNT and the TFHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to declassification of this AOP on 17 June 2016.

This document is being published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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ADVERSE OUTCOME PATHWAY ON AROMATASE INHIBITION LEADING TO REPRODUCTIVE DYSFUNCTION (IN FISH)

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

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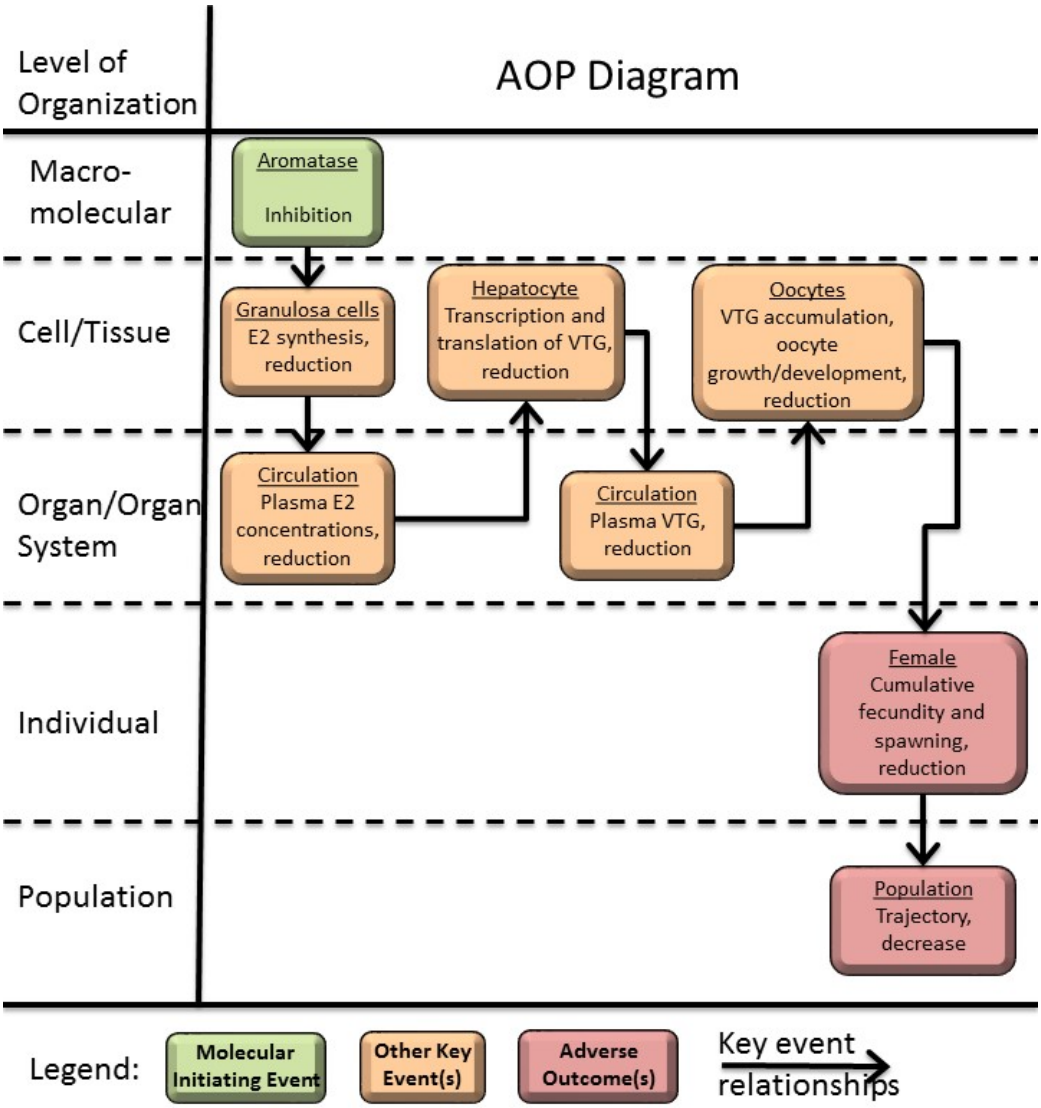
Abstract

This adverse outcome pathway details the linkage between inhibition of gonadal aromatase activity in females and reproductive dysfunction, as measured through the adverse effect of reduced cumulative fecundity and spawning. 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 and potentially other oviparous vertebrates.

Summary of the AOP: Graphical Representation



Key events

Molecular Initiating Event

Molecular Initiating Event
Aromatase, Inhibition

Aromatase, inhibition

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Aromatase inhibition leading to reproductive dysfunction (in fish)	MIE	Strong

Chemical Initiators

The following are chemical initiators that operate directly through this Event:

1. Fadrozole
2. Letrozole
3. Prochloraz

How this Key Event works

Inhibition of cytochrome P450 aromatase (CYP19; specifically cyp19a1a in fish).
Site of action: The site of action for the molecular initiating event is the ovarian granulosa cells.
While many vertebrates have a single isoform of aromatase, fish are known to have two isoforms. CYP19a1a is predominantly expressed in ovary while cyp19a1b is predominantly expressed in brain (Callard et al., 2001; Cheshenko et al., 2008). For the purposes of this MIE, when applied to fish, the assumed effect is on cyp19a1a. However, given that both isoforms show similar sensitivity to aromatase inhibitors (Hinfray et al., 2006) and catalyze the same reaction, discrimination of specific isoforms is not viewed as critical in relative to determining downstream key events resulting from aromatase inhibition in ovarian granulosa cells.
Responses at the macromolecular level: Aromatase catalyzes three sequential oxidation steps (i.e. KEGG reactions R02501, R04761, R03087 or R01840, R04759, R02351; http://www.genome.jp/kegg/pathway.html) involved in the conversion of C-19 androgens (e.g. testosterone, androstenedione) to C-18 estrogens (e.g. 17β-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 17β-estradiol (and presumably estrone) production by the ovary.

How it is Measured or Detected

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

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 (Callard et al., 2001). Given that cyp19a1a is dominant isoform expressed in ovary and both isoforms appear to show similar sensitivity to aromatase inhibitors (Hinfray et al., 2006), for the purpose of this key event which focuses on gonadal aromatase activity, distinction of effects on one isoform versus the other are considered negligible. Total activity, without regard to isoform can be considered.

Evidence for chemical initiation of this Molecular Initiating Event

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 Δ4 positioned double bonds generally acting as stronger inhibitors than those with Δ5 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

Petkov PI, Temelkov S, Villeneuve DL, Ankley GT, Mekenyan OG. 2009. Mechanism-based categorization of aromatase inhibitors: a potential discovery and screening tool. SAR QSAR Environ Res 20(7-8): 657-678.

Lephart ED, Simpson ER. 1991. Assay of aromatase activity. Methods Enzymol 206: 477-483.

Letcher RJ, van Holsteijn I, Drenth H-J, Norstrom RJ, Bergman A, Safe S, et al. 1999. Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. Toxico App Pharm 160: 10-20.

Sanderson J, Seinen W, Giesy J, van den Berg M. 2000. 2-chloro-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity. Toxicol Sci 54: 121-127.

Villeneuve DL, Knoebl I, Kahl MD, Jensen KM, Hammermeister DE, Greene KJ, et al. 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (Pimephales promelas). Aquat Toxicol 76(3-4): 353-368.

Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, et al. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (Pimephales promelas). Toxicol Sci 67: 121-130.

Castro LF, Santos MM, Reis-Henriques MA. 2005. The genomic environment around the Aromatase gene: evolutionary insights. BMC Evol Biol 5: 43.

Callard GV, Tchoudakova AV, Kishida M, Wood E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J Ster Biochem Mol Biol 79: 305-314.

Cheshenko K, Pakdel F, Segner H, Kah O, Eggen RI. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen Comp Endocrinol. 2008 Jan 1;155(1):31-62.

Hinfray N, Porcher JM, Brion F. Inhibition of rainbow trout (Oncorhynchus mykiss) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comp Biochem Physiol C Toxicol Pharmacol. 2006 Nov;144(3):252-62

Key events

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

1. Plasma 17beta-estradiol concentrations, Reduction

AOPs including this Key Event

AOP Name	Event Type	Essentiality
PPARγ activation leading to impaired fertility in adult female	KE	Strong
Aromatase inhibition leading to reproductive dysfunction (in fish)	KE	Strong
Androgen receptor agonism leading to reproductive dysfunction	KE	Strong
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	KE	Moderate
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	KE	

How this Key Event works

Level of biological organisation
Individual

Estradiol synthesized by the gonads is transported to other tissues via blood circulation. The gonads are generally considered to be the primary source of estrogens in systemic circulation.

How it is Measured or Detected

Total concentrations of 17β-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; Owen et al., 2014). Total steroid hormones are typically extracted from plasma or serum via liquid-liquid or solid phase extraction prior to analysis.

Given that there are numerous genes, like those coding for vertebrate vitellogenins, choriongenins, cyp19a1b, etc. which are known to be regulated by estrogen response elements, targeted qPCR or proteomic analysis of appropriate targets could also be used as an indirect measure of reduced circulating estrogen concentrations. However, further support for the specificity of the individual gene targets for estrogen-dependent regulation should be established in order to support their use.
A line of transgenic zebrafish employing green fluorescence protein under control of estrogen response elements could also be used to provide direct evidence of altered estrogen, with decreased GFP signal in estrogen responsive tissues like liver, ovary, pituitary, and brain indicating a reduction in circulating estrogens (Gorelick and Halpern, 2011).

Evidence supporting taxonomic applicability

Name	Scientific Name	Evidence	Links
rat	Rattus sp.	Strong	NCBI
human	Homo sapiens	Strong	NCBI

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

References

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.

Owen LJ, Wu FC, Keevil BG. 2014. A rapid direct assay for the routine measurement of oestradiol and oestrone by liquid chromatography tandem mass spectrometry. Ann. Clin. Biochem. 51(pt 3):360-367.

Gorelick DA, Halpern ME. Visualization of estrogen receptor transcriptional activation in zebrafish. Endocrinology. 2011 Jul;152(7):2690-703. doi: 10.1210/en.2010-1257. Epub 2011 May 3. PubMed PMID: 21540282

2. Vitellogenin synthesis in liver, Reduction

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Aromatase inhibition leading to reproductive dysfunction (in fish)	KE	Moderate
Androgen receptor agonism leading to reproductive dysfunction	KE	Moderate
Estrogen receptor antagonism leading to reproductive dysfunction	KE	Moderate
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	KE	Moderate
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	KE	

How this Key Event works

Vitellogenin is an egg yolk precursor protein synthesized by hepatocytes of oviparous vertebrates. In vertebrates, transcription of vitellogenin genes is predominantly regulated by estrogens via their action on nuclear estrogen receptors. During vitellogenic periods of the reproductive cycle, when circulating estrogen concentrations are high, vitellogenin transcription and synthesis are typically orders of magnitude greater than during non-reproductive conditions.

How it is Measured or Detected

Relative abundance of vitellogenin transcripts or protein can be readily measured in liver tissue from organisms exposed in vivo (e.g. Biales et al., 2007)), or in liver slices (e.g. Schmieder et al., 2000) or hepatocytes (e.g. (Navas and Segner, 2006) exposed in vitro, using real-time quantitative PCR (transcripts) or ELISA (protein).

Evidence supporting taxonomic applicability

Oviparous vertebrates. Although vitellogenin is conserved among oviparous vertebrates and many invertebrates, liver is not a relevant tissue for the production of vitellogenin in invertebrates (Wahli, 1988)

References

Biales AD, Bencic DC, Lazorchak JL, Lattier DL. 2007. A quantitative real-time polymerase chain reaction method for the analysis of vitellogenin transcripts in model and nonmodel fish species. Environ Toxicol Chem 26(12): 2679-2686.

Schmieder P, Tapper M, Linnum A, Denny J, Kolanczyk R, Johnson R. 2000. Optimization of a precision-cut trout liver tissue slice assay as a screen for vitellogenin induction: comparison of slice incubation techniques. Aquat Toxicol 49(4): 251-268.

Navas JM, Segner H. 2006. Vitellogenin synthesis in primary cultures of fish liver cells as endpoint for in vitro screening of the (anti)estrogenic activity of chemical substances. Aquat Toxicol 80(1): 1-22.

Wahli W. 1988. Evolution and expression of vitellogenin genes. Trends in Genetics. 4:227-232.

3. Vitellogenin accumulation into oocytes and oocyte growth/development, Reduction

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Aromatase inhibition leading to reproductive dysfunction (in fish)	KE	Weak
Androgen receptor agonism leading to reproductive dysfunction	KE	Weak
Estrogen receptor antagonism leading to reproductive dysfunction	KE	Weak
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	KE	Moderate
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	KE	

How this Key Event works

Vitellogenin from the blood is selectively taken up by competent oocytes via receptor-mediated endocytosis. Although vitellogenin receptors mediate the uptake, opening of intercellular channels through the follicular layers to the oocyte surface as the oocyte reaches a “critical” size is thought to be a key trigger in allowing vitellogenin uptake (Tyler and Sumpter, 1996). Once critical size is achieved, concentrations in the plasma and temperature are thought to impose the primary limits on uptake (Tyler and Sumpter, 1996). Uptake of vitellogenin into oocytes causes considerable oocyte growth during vitellogenesis, accounting for up to 95% of the final egg size in many fish (Tyler and Sumpter, 1996). Given the central role of vitellogenesis in oocyte maturation, vitellogenin accumulation is a prominent feature used in histological staging of oocytes (e.g. (Leino et al., 2005; Wolf et al., 2004).

How it is Measured or Detected

Relative vitellogenin accumulation can be evaluated qualitatively using routine histological approaches (Leino et al., 2005; Wolf et al., 2004). Oocyte size can be evaluated qualitatively or quantitatively using routine histological and light microscopy and/or imaging approaches.

Evidence supporting taxonomic applicability

Oviparous vertebrates and invertebrates. Although hormonal regulation of vitellogenin synthesis and mechanisms of vitellogenin transport from the site of synthesis to the ovary vary between vertebrates and invertebrates (Wahli, 1988), in both vertebrates and invertebrates, vitellogenin is incorporated into oocytes and cleaved to form yolk proteins.

References

Tyler C, Sumpter J. 1996. Oocyte growth and development in teleosts. Reviews in Fish Biology and Fisheries 6: 287-318.

Leino R, Jensen K, Ankley G. 2005. Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow. Environmental Toxicology and Pharmacology 19: 85-98.

Wolf JC, Dietrich DR, Friederich U, Caunter J, Brown AR. 2004. Qualitative and quantitative histomorphologic assessment of fathead minnow Pimephales promelas gonads as an endpoint for evaluating endocrine-active compounds: a pilot methodology study. Toxicol Pathol 32(5): 600-612.

4. 17beta-estradiol synthesis by ovarian granulosa cells, Reduction

AOPs including this Key Event

AOP Name	Event Type	Essentiality
PPARγ activation leading to impaired fertility in adult female	KE	Strong
Aromatase inhibition leading to reproductive dysfunction (in fish)	KE	Strong
Androgen receptor agonism leading to reproductive dysfunction	KE	Moderate
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	KE	Moderate
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	KE	

How this Key Event works

Like all steroids, estradiol is a cholesterol derivative. Estradiol synthesis in ovary is mediated by a number of enzyme catalyzed reactions involving cyp11 (cholesterol side chain cleavage enzyme), cyp 17 (17alpha-hydroxylase/17,20-lyase), 3beta hydroxysteroid dehyrogenase, 17beta hydroxysteroid dehydrogenase, and cyp19 (aromatase). Among those enzyme catalyzed reactions, conversion of testosterone to estradiol, catalyzed by aromatase, is considered to be rate limiting for estradiol synthesis. Within the ovary, aromatase expression and activity is primarily localized in the granulosa cells (reviewed in Norris, 2007; Yaron, 1995; Havelock et al., 2004; and others). Reactions involved in synthesis of C-19 androgens are primarily localized in the theca cells and C-19 androgens diffuse from the theca into granulosa cells where aromatase can catalyze their conversion to C-18 estrogens.

How it is Measured or Detected

Due to the importance of both theca and granulosa cells in ovarian steroidogenesis, it is generally impractical to measure E2 production by isolated granulosa cells (Havelock et al., 2004). However, this key event can be evaluated by examining E2 production by intact ovarian tissue explants either exposed to chemicals in vitro (e.g. Villeneuve et al., 2007; McMaster ME, 1995) or in vivo (i.e. via ex vivo steroidogenesis assay; e.g. Ankley et al., 2007). Estradiol released by ovarian tissue explants into media can be quantified by RIA (e.g. Jensen et al., 2001), ELISA, or analytical methods such as LC-MS (e.g. Owen et al., 2014).
OECD TG 456 (OECD, 2011) is the validated test guideline for an in vitro screen for chemical effects on steroidogenesis, specifically the production of 17ß-estradiol (E2) and testosterone (T).

The synthesis of E2 can be measured in vitro cultured ovarian cells. The methods for culturing mammalian ovarian cells can be found in the Database Service on Alternative Methods to animal experimentation (DB-ALM): Culture of Human Cumulus Granulosa Cells (EURL ECVAM Protocol No. 92), Granulosa and Theca Cell Culture Systems (EURL ECVAM Method Summary No. 92).

Evidence supporting taxonomic applicability

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

References

Norris DO. 2007. Vertebrate Endocrinology. Fourth ed. New York: Academic Press.

Havelock JC, Rainey WE, Carr BR. 2004. Ovarian granulosa cell lines. Molecular and cellular endocrinology 228(1-2): 67-78.

Yaron Z. 1995. Endocrine control of gametogenesis and spawning induction in the carp. Aquaculture 129: 49-73.

Villeneuve DL, Ankley GT, Makynen EA, Blake LS, Greene KJ, Higley EB, et al. 2007. Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine-active chemicals. Ecotoxicol Environ Saf 68(1): 20-32.

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Ankley GT, Jensen KM, Kahl MD, Makynen EA, Blake LS, Greene KJ, et al. 2007. Ketoconazole in the fathead minnow (Pimephales promelas): reproductive toxicity and biological compensation. Environ Toxicol Chem 26(6): 1214-1223.

Villeneuve DL, Mueller ND, Martinovic D, Makynen EA, Kahl MD, Jensen KM, et al. 2009. Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor. Environ Health Perspect 117(4): 624-631.

Baker ME. 2011. Origin and diversification of steroids: co-evolution of enzymes and nuclear receptors. Molecular and cellular endocrinology 334(1-2): 14-20.

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.

Owen LJ, Wu FC, Keevil BG. 2014. A rapid direct assay for the routine measurement of oestradiol and oestrone by liquid chromatography tandem mass spectrometry. Ann. Clin. Biochem. 51(pt 3):360-367.

OECD (2011), Test No. 456: H295R Steroidogenesis Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264122642-en

EURL ECVAM Protocol no 92 Culture of Human Cumulus Granulosa Cells. Primary cell culture method. Contact Person: Dr. Mahadevan Maha M.

EURL ECVAM Method Summary no 92. Granulosa and Theca Cell Culture Systems - Summary

5. Cumulative fecundity and spawning, Reduction

AOPs including this Key Event

AOP Name	Event Type	Essentiality
Estrogen receptor agonism leading to reproductive dysfunction	KE	Strong
Aromatase inhibition leading to reproductive dysfunction (in fish)	KE	Moderate
Androgen receptor agonism leading to reproductive dysfunction	KE	Moderate
Estrogen receptor antagonism leading to reproductive dysfunction	KE	Moderate
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	AO	
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	AO	

How this Key Event works

Spawning refers to the release of eggs. Cumulative fecundity refers to the total number of eggs deposited by a female, or group of females over a specified period of time.

How it is Measured or Detected

In laboratory-based reproduction assays (e.g. OECD 229; OECD 240), spawning and cumulative fecundity can be directly measured through daily observation of egg deposition and egg counts.

Evidence supporting taxonomic applicability

Cumulative fecundity and spawning can, in theory, be evaluated for any egg laying animal.

References

OECD. 2012a. Test no. 229: Fish short term reproduction assay. Paris, France: Organisation for Economic Cooperation and Development.

6. Plasma vitellogenin concentrations, Reduction

AOPs including this Key Event

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

Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	KE	Moderate
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	KE	

How this Key Event works

Vitellogenin synthesized in the liver is secreted into the blood and circulates to the ovaries for uptake.

How it is Measured or Detected

Vitellogenin concentrations in plasma are typically detected using enzyme linked Immunosorbent assay (ELISA; e.g. Korte et al., 2000; Tyler et al., 1996; Holbech et al., 2001; Fenske et al., 2001). Although less specific and/or sensitive, determination of alkaline-labile phosphate or Western blotting has also been employed.

Evidence supporting taxonomic applicability

Oviparous vertebrates synthesize yolk precursor proteins that are transported in the circulation for uptake by developing oocytes. Many invertebrates also synthesize vitellogenins that are taken up into developing oocytes via active transport mechanisms. However, invertebrate vitellogenins are transported in hemolymph or via other transport mechanisms rather than plasma.

References

Korte JJ, Kahl MD, Jensen KM, Mumtaz SP, Parks LG, LeBlanc GA, et al. 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17B-estradiol treatment. Environmental Toxicology and Chemistry 19(4): 972-981.

Tyler C, van der Eerden B, Jobling S, Panter G, Sumpter J. 1996. Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. Journal of Comparative Physiology and Biology 166: 418-426.

Wahli W. 1988. Evolution and expression of vitellogenin genes. Trends in Genetics. 4:227-232.

Holbech H, Andersen L, Petersen GI, Korsgaard B, Pedersen KL, Bjerregaard P. Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (Danio rerio). Comp Biochem Physiol C Toxicol Pharmacol. 2001 Sep;130(1):119-31.

Fenske M, van Aerle R, Brack S, Tyler CR, Segner H. Development and validation of a homologous zebrafish (Danio rerio Hamilton-Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals. Comp Biochem Physiol C Toxicol Pharmacol. 2001. Jul;129(3):217-32.

Adverse Outcome

Adverse Outcome
Population trajectory, Decrease

Population trajectory, Decrease

AOPs including this Key Event

AOP Name	Event Type	Essentiality
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	
Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior	AO	
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	AO	
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	AO	

How this Key Event works

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

How it is Measured or Detected

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

Evidence supporting taxonomic applicability

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

References

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

Key event relationships: Scientific evidence supporting the linkages in the AOP

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

1 Aromatase, Inhibition leads to 17beta-estradiol synthesis by ovarian granulosa cells, Reduction

How Does This Key Event Relationship Work

Biological Plausibility

Within the ovary, aromatase expression and activity is primarily localized in the granulosa cells (reviewed in Norris, 2007; Yaron, 1995; 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.

Weight of Evidence

Empirical Support for Linkage

- Known aromatase inhibitors including fadrozole and prochloraz were shown to cause concentration-dependent inhibition of aromatase activity in fathead minnow ovary homogenates (Villeneuve 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 (Villeneuve 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 (Villeneuve et al., 2006; Villeneuve et al., 2009; Ankley et al., 2009a; Skolness et al., 2011).

Uncertainties or Inconsistencies

Based on the limited set of studies available to date, there are no known inconsistencies.

Quantitative Understanding of the Linkage

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

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).

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2. 17beta-estradiol synthesis by ovarian granulosa cells, Reduction leads to Plasma 17beta-estradiol concentrations, Reduction

How Does This Key Event Relationship Work

Biological Plausibility

While brain, interrenal, adipose, and breast tissue (in mammals) are capable of synthesizing estradiol, the gonads are generally considered the major source of circulating estrogens in vertebrates, including fish (Norris, 2007). Consequently, if estradiol synthesis by ovarian granulosa cells is reduced, plasma E2 concentrations would be expected to decrease unless there are concurrent reductions in the rate of E2 catabolism. Synthesis in other tissues generally plays a paracrine role only, thus the contribution of other tissues to plasma E2 concentrations can generally be considered negligible.

Weight of Evidence

Empirical Support for Linkage

Fish

- In multiple studies with aromatase inhibitors (e.g. fadrozole, prochloraz), significant reductions in ex vivo E2 production have been linked to, and shown to precede, reductions in circulating E2 concentrations (Villeneuve et al., 2009; Skolness et al., 2011). It is also notable that compensatory responses at the level of ex vivo steroid production (i.e. rate of E2 synthesis per unit mass of tissue) tend to precede recovery of plasma E2 concentrations following an initial insult (Villeneuve et al., 2009; Ankley et al., 2009a; Villeneuve et al., 2013).
- Ex vivo E2 production by ovary tissue collected from female fish exposed to 30 or 300 µg ketoconazole/L showed significant decreases prior to significant effects on plasma estradiol being observed (Ankley et al., 2012).

Mammals

- MEHP /DEHP, mice, ex vivo DEHP (10 -100 µg/ml); MEHP (0.1 and 10 µg/ml) dose dependent reduction E2 production (Gupta et al., 2010)
- DEHP, rat, in vivo 300-600 mg/kg/day, dose dependent reduction of E2 plasma levels (Xu et al., 2010)

Evidence for rodent and human models is summarized in Table 1.

Compound class	Species	Study type	Dose	E2 production/levels	Reference
Phthalates (DEHP)	rat	ex vivo	1500 mg/kg/day	Reduced/increased E2 production in ovary culture	(Laskey & Berman, 1993)
Phthalates (MEHP)	rat	in vitro	From 50 µM	Reduced E2 production (concentration and time dependent in Granulosa cell)	(Davis, Weaver, Gaines, & Heindel, 1994)
Phthalates (MEHP)	rat	in vitro	100-200µM	reduction E2 production (dose dependent)	(Lovekamp & Davis, 2001)
Phthalates (DEHP)	rat	in vivo	300-600 mg/kg/day	reduction E2 levels dose dependent	(Xu et al., 2010),
Phthalates (MEHP)	human	in vitro	IC(50)= 49- 138 µM (dependent on the stimulant)	reduction E2 production (dose dependent)	(Reinsberg, Wegener-Toper, van der Ven, van der Ven, & Klingmueller, 2009)
Phthalates (MEHP/DEHP)	mice	ex vivo	DEHP (10 -100 µg/ml); MEHP (0.1 and 10 µg/ml)	reduction E2 production (dose dependent)	(Gupta et al., 2010)

Table 1. Summary of the experimental data for decrease E2 production and decreased E2 levels. IC50- half maximal inhibitory concentration values reported if available, otherwise the concentration at which the effect was observed.

Uncertainties or Inconsistencies

Based on the limited set of studies available to date, there are no known inconsistencies.

Quantitative Understanding of the Linkage

At present we are unaware of any well-established quantitative relationships between ex vivo E2 production (as an indirect measure of granulosa cell E2 synthesis) and plasma E2 concentrations. There are considerable data available which might support the development of such a relationship. Additionally, there are a number of existing mathematical/computational models of ovarian steroidogenesis (Breen et al., 2013; Shoemaker et al., 2010) and/or physiologically-based pharmacokinetic models of the hypothalamic-pituitary-gonadal axis (e.g. Li et al., 2011a) that may be adaptable to support a quantitative understanding of this linkage.

• 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 Li et al. (2011) model is a PBPK-based model that was calibrated from data from fathead minnows, including controls and fish exposed to either 17alpha ethynylestradiol or 17beta trenbolone.

Evidence supporting taxonomic applicability

Name	Scientific Name	Evidence	Links
human	Homo sapiens		NCBI
mouse	Mus musculus	Moderate	NCBI
rat	Rattus sp.	Strong	NCBI

Key enzymes needed to synthesize 17β-estradiol first appear in the common ancestor of amphioxus and vertebrates (Baker, 2011). While some E2 synthesis can occur in other tissues, the ovary is recognized as the major source of 17β-estradiol synthesis in female vertebrates. Endocrine actions of ovarian E2 are facilitated through transport via the plasma. Consequently, this key event relationship is applicable to most female vertebrates.

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Fish

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3. Plasma 17beta-estradiol concentrations, Reduction leads to Transcription and translation of vitellogenin in liver, Reduction

How Does This Key Event Relationship Work

Biological Plausibility

Vitellogenin synthesis in fish is localized in the liver and is well documented to be regulated by estrogens via interaction with estrogen receptors (Tyler et al., 1996; Tyler and Sumpter, 1996; Arukwe and Goksøyr, 2003). The vitellogenin gene contains estrogen repsonsive elements in its promoter region and site directed mutagenesis has shown these to be essential for estrogen-dependent expression of vitellogenin (Chang et al., 1992; Teo et al., 1998). Liver is not regarded as a major site of E2 synthesis (Norris, 2007), therefore the majority of E2 in liver comes from the circulation.

- Estrogen regulates expression of the vitellogenin gene in the amphibian Xenopus laevis (Skipper and Hamilton, 1977).

Weight of Evidence

Empirical Support for Linkage

- In a number of time-course experiments with aromatase inhibitors (e.g. fadrozole, prochloraz), decreases in plasma estradiol concentrations precede decreases in plasma

vitellogenin concentrations (Villeneuve et al., 2009; Skolness et al., 2011; Ankley et al., 2009b). Recovery of plasma E2 concentrations also precedes recovery of plasma VTG concentrations after cessation of exposure (Villeneuve et al., 2009; Ankley et al., 2009a; Villeneuve et al., 2013).

- It was demonstrated in Danio rerio that in vivo exposure to the aromatase inhibitor letrozole significantly reduced the expression of mRNA transcripts coding for vtg1, vtg2, and erα, all of which are known to be regulated by estrogens (Sun et al., 2010). However, similar effects were not observed in primary cultured hepatocytes from Danio rerio, indicating that letrozole's effects on vtg transcription were not direct.
- Many studies have demonstrated that exposure of hepatocytes to estrogens in vitro or in vivo induce vitellogenin mRNA synthesis (e.g. see reviews by Navas and Segner, 2006; Iguchi et al., 2006).
- In female fathead minnows exposed to 17β-trenbolone, significant reductions in plasma E2 concentrations preceded significant reductions in plasma VTG (Ekman et al., 2011).
- Intra-arterial injection of the estrogen 17α ethynyl estradiol into male rainbow trout causes vitellogenin induction with about a 12 h lag time before increasing from basal levels (Schultz et al., 2001).

Uncertainties or Inconsistencies

Based on the limited set of studies available to date, there are no known inconsistencies.

Quantitative Understanding of the Linkage

- At least two computational models that include functions which link circulating concentrations of E2 to VTG production by the liver have been published (Li et al., 2011a; Murphy et al., 2005; Murphy et al., 2009), although both models focus on predicting plasma VTG concentrations rather than transcription or translation within the liver. A significant positive correlation (r=0.87) between plasma E2 concentrations corresponding plasma VTG concentrations in female fathead minnows held under laboratory conditions has also been reported (Ankley et al., 2008).

- There are multiple isoforms of vitellogenin. The sensitivity and inducibility of each of those isoforms may vary somewhat. Consequently, response-response relationships may vary somewhat depending on the specific isoform for which QPCR primers or antibodies were developed.

Evidence supporting taxonomic applicability

Key enzymes needed to synthesize 17β-estradiol first appear in the common ancestor of amphioxus and vertebrates (Baker, 2011). However, non-oviparous vertebrates do not require vitellogenin. Consequently, this KER is applicable to oviparous vertebrates.

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4. Cumulative fecundity and spawning, Reduction leads to Population trajectory, Decrease

How Does This Key Event Relationship Work

SEE BIOLOGICAL PLAUSIBILITY BELOW

Weight of Evidence

Biological Plausibility

Using a relatively simple density-dependent population model and assuming constant young of year survival with no immigration/emigration, reductions in cumulative fecundity have been predicted to yield declines in population size over time (Miller and Ankley, 2004). Under real-world environmental conditions, outcomes may vary depending on how well conditions conform with model assumptions. Nonetheless, cumulative fecundity can be considered one vital rate that contributes to overall population trajectories (Kramer et al., 2011).

Empirical Support for Linkage

- Using a relatively simple density-dependent population model and assuming constant young of year survival with no immigration/emigration, reductions in cumulative fecundity have been predicted to yield declines in population size over time (Miller and Ankley, 2004). However, it should be noted that the model was constructed in such a way that predicted population size is dependent on cumulative fecundity, therefore this is a fairly weak form of empirical support.
- In a study in which an entire lake was treated with 17alpha-ethynyl estradiol, Kidd et al. (2007) declines in fathead minnow population size were associated with signs of reduced fecundity.

Uncertainties or Inconsistencies

- Wester et al. (2003) and references cited therein suggest that although egg production is an endpoint of demographic significance, incomplete reductions of egg production may not translate in a simple manner to population reductions. Compensatory effects of reduced predation and reduced competition for limited food and/or habitat resources may offset the effects of incomplete reductions in egg production.
- Fish and other egg laying animals employ a diverse range of reproductive strategies and life histories. The nature of the relationship between reduced spawning frequency and cumulative fecundity and overall population trajectories will depend heavily on the life history and reproductive strategy of the species in question. Relationships developed for one species will not necessarily hold for other species, particularly those with differing life histories.

Quantitative Understanding of the Linkage

- Cumulative fecundity is one example of a vital rate that can influence population size over time. A variety of population model constructs can be adapted to utilize measurements or estimates of cumulative fecundity as a predictor of population trends over time (e.g. (Miller and Ankley, 2004; Miller et al., 2013).
- The model of Miller et al. 20014 uses a relatively simple density-dependent population model and assuming constant young of year survival with no immigration/emigration, use measures of cumulative fecundity to predict relative change in in population size over time (Miller and Ankley, 2004).

Evidence supporting taxonomic applicability

Spawning generally refers to the release of eggs and/or sperm into water, generally by aquatic or semi-aquatic organisms. Consequently, by definition, this KER is likely applicable only to organisms that spend a portion of their life-cycle in or near aquatic environments.

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5. Vitellogenin accumulation into oocytes and oocyte growth/development, Reduction leads to Cumulative fecundity and spawning, Reduction

How Does This Key Event Relationship Work

SEE BIOLOGICAL PLAUSIBILITY BELOW

Weight of Evidence

Biological Plausibility

Vitellogenesis is a critical stage of oocyte development and accumulated lipids and yolk proteins make up the majority of oocyte biomass (Tyler and Sumpter, 1996). At least in mammals, maintenance of meiotic arrest is supported by signals transmitted through gap junctions between the granulosa cells and oocytes (Jamnongjit and Hammes, 2005). Disruption of oocyte-granulosa contacts as a result of cell growth has been shown to coincide with oocyte maturation (Eppig, 1994). However, it remains unclear whether the relationship between vitellogenin accumulation and oocyte growth and eventual maturation is causal or simply correlative.

Empirical Support for Linkage

At present, to our best knowledge there are no studies that definitively demonstrate a direct cause-effect relationship between impaired VTG accumulation into oocytes and impaired spawning. There is, however, strong correlative evidence. Across a range of laboratory studies with small fish, there is a robust and statistically significant correlation between reductions in circulating VTG concentrations and reductions in cumulative fecundity (Miller et al., 2007). To date, we are unaware of any fish reproduction studies which show a large reduction in circulating VTG concentrations, but not reductions in cumulative fecundity.

Uncertainties or Inconsistencies

Based on the limited number of studies available that have examined both of these KEs, there are no known, unexplained, results that are inconsistent with this relationship.

Quantitative Understanding of the Linkage

Across a range of laboratory studies with fathead minnow, there is a robust and statistically significant correlation between reductions in circulating VTG concentrations and reductions in cumulative fecundity (Miller et al., 2007). At present it is unclear how well that relationship may hold for other fish species or feral fish under the influence of environmental variables. A model based on a statistical relationship between plasma E2 concentrations, spawning interval, and cumulative fecundity has been developed to predict changes in cumulative fecundity from plasma VTG (Li et al., 2011b). However, to date, such models do not specifically consider vitellogenin uptake into oocytes as a quantitative predictor of fecundity. Furthermore, with the exception of a

few specialized studies, quantitative measures of VTG content in oocytes are rarely measured in toxicity studies. In contrast, plasma VTG is routinely measured.

Evidence supporting taxonomic applicability

On the basis of the taxonomic relevance of the two KEs linked via this KER, this KER is likely applicable to aquatic, oviparous, vertebrates which both produce vitellogenin and deposit eggs/sperm into an aquatic environment.

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6. Plasma vitellogenin concentrations, Reduction leads to Vitellogenin accumulation into oocytes and oocyte growth/development, Reduction

How Does This Key Event Relationship Work

SEE BIOLOGICAL PLAUSIBILITY BELOW

Weight of Evidence

Biological Plausibility

Vitellogenin synthesized in the liver and transported to the ovary via the circulation is the primary source of egg yolk proteins in fish (Tyler and Sumpter, 1996; Arukwe and Goksøyr, 2003). In many teleosts vitellogenesis can account for up to 95% of total egg size (Tyler and Sumpter, 1996).

Empirical Support for Linkage

In some (Ankley et al., 2002; Ankley et al., 2003; Lalone et al., 2013), but not all (Ankley et al., 2005; Sun et al., 2007; Skolness et al., 2013) fish reproduction studies, reductions in plasma vitellogenin have been associated with visible decreases in yolk protein content in oocytes and overall reductions in ovarian stage.

Uncertainties or Inconsistencies

Not all fish reproduction studies showing reductions in plasma vitellogenin have caused visible decreases in yolk protein content in oocytes and overall reductions in ovarian stage (Ankley et al., 2005; Sun et al., 2007; Skolness et al., 2013).
While plasma vitellogenin is well established as the only major source of vitellogenins to the oocyte, the extent to which a decrease will impact an ovary that has already developed vitellogenic staged oocytes is less certain. It would be assumed that the more rapid the turn-over of oocytes in the ovary, the tighter the linkage between these KEs. Thus, repeat spawning species with asynchronous oocyte development that spawn frequently would likely be more vulnerable than annual spawning species with synchronous oocyte development that had already reached late vitellogenic stages.

Quantitative Understanding of the Linkage

- Rates of vitellogenin uptake as a function of ovarian follicle surface area have been estimated for rainbow trout, an annual spawning fish species, and may exceed 700 ng/mm2 follicle surface per hour (Tyler and Sumpter, 1996).
- Comparable data are lacking for repeat-spawning species and kinetic relationships between plasma concentrations and uptake rates within the ovary have not been defined.
- A model based on a statistical relationship between plasma E2 concentrations, spawning interval, and cumulative fecundity has been developed to predict changes in cumulative fecundity from plasma VTG (Li et al., 2011b), but it does not incorporate a model of the kinetics of VTG uptake nor the influence of VTG uptake on oocyte growth.

Evidence supporting taxonomic applicability

This KER is expected to be primarily applicable to oviparous vertebrates that synthesize vitellogenin in hepatic tissue which is ultimately incorporated into oocytes present in the ovary.

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7. Transcription and translation of vitellogenin in liver, Reduction leads to Plasma vitellogenin concentrations, Reduction

How Does This Key Event Relationship Work

Weight of Evidence

Biological Plausibility

Liver is the major source of VTG protein production in fish (Tyler and Sumpter, 1996; Arukwe and Goksøyr, 2003). Protein production involves transcription and subsequent translation. The time-lag between decreases in transcription/translation and decreases in plasma VTG concentrations can be expected to be dependent on vitellogenin elimination half-lives.

Empirical Support for Linkage

- In a number of time-course experiments with aromatase inhibitors, decreases in plasma estradiol concentrations precede decreases in plasma vitellogenin concentrations (Villeneuve et al., 2009; Skolness et al., 2011; Ankley et al., 2009b). Recovery of plasma E2 concentrations also precedes recovery of plasma VTG concentrations after cessation of exposure (Villeneuve et al., 2009; Ankley et al., 2009a; Villeneuve et al., 2013).
- In experiments with strong estrogens, increases in vtg mRNA synthesis precede increases in plasma VTG concentration (Korte et al., 2000; Schmid et al., 2002).
- Elimination half-lives for VTG protein have been determined for induced male fish, but to our knowledge, similar kinetic studies have not been done for reproductively mature females (Korte et al., 2000; Schultz et al., 2001).
- In male sheepshead minnows injected with E2, induction of VTG mRNA precedes induction of plasma VTG (Bowman et al., 2000).
- In male Cichlasoma dimerus exposed to octylphenol for 28 days and then held in clean water, decline in induced VTG mRNA concentrations precedes declines in induced plasma VTG concentrations (Genovese et al., 2012).

Uncertainties or Inconsistencies

There are no known inconsistencies between these KERs which are not readily explained on the basis of the expected dose, temporal, and incidence relationships between these two KERs. This applies across a significant body of literature in which these two KEs have been measured.

Quantitative Understanding of the Linkage

Due to temporal disconnects (lag) between induction of mRNA transcription and translation and significant changes in plasma concentrations as well as variable rates of uptake of VTG from plasma into oocytes, a precise quantitative relationship between VTG transcription/translation and circulating VTG concentrations has not been described. However, models and statistical relationships that define quantitative relationships between circulating E2 concentrations and circulating VTG concentrations have been developed (Li et al., 2011a; Murphy et al., 2005; Murphy et al., 2009; Ankley et al., 2008).

Evidence supporting taxonomic applicability

This KER primarily applies to taxa that synthesize vitellogenin in the liver which is transported elsewhere in the body via plasma.

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Overall Assessment of the AOP

Weight of Evidence Summary

Event	Description	Triggers	Weight of Evidence
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
Cumulative fecundity and spawning, Reduction	Directly Leads to	Population trajectory, Decrease	Moderate
Vitellogenin accumulation into oocytes and oocyte growth/development, Reduction	Directly Leads to	Cumulative fecundity and spawning, Reduction	Moderate
Plasma vitellogenin concentrations, Reduction	Directly Leads to	Vitellogenin accumulation into oocytes and oocyte growth/development, Reduction	Moderate
Transcription and translation of vitellogenin in liver, Reduction	Directly Leads to	Plasma vitellogenin concentrations, Reduction	Strong

Annex 1 Table provides the relative level of confidence in the AOP based on rank ordered elements of weight of evidence

Biological plausibility: Biological plausibility refers to the structural or functional relationship between the key events based on our fundamental understanding of "normal biology". In general, the biological plausibility and coherence linking aromatase inhibition through decreases in circulating concentrations of E2 is very solid. The biochemistry of steroidogenesis and the predominant role of the gonad in synthesis of the sex steroids is well established. Similarly, the role of E2 as the major regulator of hepatic vitellogenin production is widely documented in the literature. The direct link between reduced VTG concentrations in the plasma and reduced uptake into oocytes is highly plausible, as the plasma is the primary source of the VTG. However, the direct connection between reduced VTG uptake and impaired spawning/reduced cumulative fecundity is more tentative. It is not clear, for instance whether impaired VTG uptake limits oocyte growth and failure to reach a critical size in turn impairs physical or inter-cellular signaling processes that promote release of the oocyte from the surrounding follicles. In at least one experiment, oocytes with similar size to vitellogenic oocytes, but lacking histological staining characteristic of vitellogenic oocytes was observed (R. Johnson, personal communication). Regulation of oocyte maturation and spawning involves many factors other than vitellogenin accumulation (Clelland and Peng, 2009). At present, the link between reductions in circulating

VTG concentrations and reduced cumulative fecundity are best supported by the correlation between those endpoints across multiple experiments, including those that impact VTG via other molecular initiating events (Miller et al., 2007).

Concordance of dose-response relationships: Dose response concordance considers the degree to which upstream events are shown to occur at test concentrations equal to or lower than those that cause significant effects on downstream key events, the underlying assumption being that all KEs can be measured with equal precision. There are a limited number of studies in which multiple key events were considered in the same study. These were considered the most useful for evaluating the concordance of dose-response relationships. In general, effects on downstream key events occurred at concentrations equal to or greater than those at which upstream events occurred (Concordance table: [1]). However, there are exceptions. There are cases where no significant effects on estradiol synthesis by ovarian granulosa cells (ovary explants) were observed, but significant effects on plasma E2 or VTG concentrations were observed. Likewise, there are cases where impacts on plasma VTG were observed at concentrations lower than those reported to reduce plasma E2 concentrations. Based on knowledge of the studies in question, the apparent lack of concordance in some cases is driven by two primary factors. First, differences in the sensitivity and dynamic range of the measurements being made. Second, the effects of compensatory responses along the HPG axis. For instance, although ex vivo E2 production is rapidly affected by exposure to fadrozole, it is also a response that is more rapidly corrected through upregulation of aromatase transcripts (see Villeneuve et al., 2009), meaning that it recovers more quickly than plasma concentrations of E2 or plasma VTG concentrations. Thus, at certain time points, one can get an apparent effect on plasma E2 or T without a measurable impact on E2 production by the gonad tissue, because the upstream insult occurred earlier in time and was subsequently offset by a compensatory response, but the compensation has yet to propagate through the pathway. Sensitivity and dynamic range of the measurement methods is also an issue. Vitellogenin concentrations have a highly dynamic range and can change by orders of magnitude. Other endpoints like plasma steroids are regulated in a narrower range, making differences more difficult to distinguish statistically. Therefore, in our assessment, the deviations from concordance do not call the KERs into question.

The concentration-dependence of the key event responses with regard to the concentration of aromatase inhibitor has been established in vitro and/or in vivo for nearly all key events in the AOP.

References

1. Concentration-dependent aromatase inhibition: (Villeneuve et al., 2006; Ankley et al., 2005; M et al., 2004; AM et al., 2000; Shilling et al., 1999)
2. Concentration-dependent decreases in E2 production in vitro, ex vivo: (Ankley et al., 2002; Villeneuve et al., 2007; Villeneuve et al., 2009; Ankley et al., 2005; a Marca Pereira et al., 2011; Lee et al., 2006).
3. Concentration-dependent decreases in circulating E2 concentrations: (Ankley et al. 2002; Villeneuve et al., 2009; Ankley et al., 2005; Ankley et al., 2009a; GT et al., 2001)
4. Concentration-dependent decreases in vitellogenin mRNA expression: (Sun et al., 2010; Sun et al., 2011; Zhang et al., 2008)

5. Concentration-dependent decreases in circulating vitellogenin concentrations: (Ankley et al., 2002; Villeneuve et al., 2009; Ankley et al., 2005; Ankley et al., 2009a; Sun et al., 2007; GT et al., 2001; Ralston-Hooper et al., 2013)
6. Concentration-dependent reductions in VTG uptake into oocytes or impaired oocyte development: Concentration-dependence of these effects has not been well demonstrated. The effects, when seen, have typically been documented at the greatest exposure concentration tested, but concentration-dependence of the severity or frequency of the impact was not documented (e.g. (Ankley et al., 2002; Ankley et al., 2005; Sun et al., 2007)
7. Concentration-dependent reductions in cumulative fecundity: (Ankley et al., 2002; Ankley et al., 2005; Sun et al., 2007; Zhang et al., 2008)
8. Declining population trajectory: Modeled population trajectories show a concentration-dependent reduction in projected population size, however, those results are driven by the concentration-dependence of cumulative fecundity. Population-level effects have not been measured directly.

Temporal concordance: Temporal concordance refers to the degree to which the data support the hypothesized sequence of the key events; i.e., the effect on KE1 is observed before the effect on KE2, which is observed before the effect on KE3 and so on. Temporal concordance of the AOP from aromatase inhibition to decreased E2 production, decreased circulating E2, and decreased plasma VTG concentrations has been established (e.g. (Villeneuve et al., 2009; Ankley et al., 2009a; Skolness et al., 2011). Temporal concordance has not been established beyond that key event, in large part due to disconnect in the time-scales over which the events can be measured. For example, most small fish used in reproductive toxicity testing will can spawn anywhere from once daily to several days per week. Given the variability in daily spawning rates, it is neither practical nor effective to evaluate cumulative fecundity at a time scale shorter than roughly a week. Since the impacts at lower levels of biological organization can be detected within hours of exposure, lack of impact on cumulative fecundity before the other key events are impacted cannot be effectively measured. Overall, among those key events whose temporal concordance can reasonably be evaluated, the temporal profile observed is consistent with the AOP.

Consistency: We are aware of no cases where the pattern of key events described was observed without also observing a significant impact on cumulative fecundity. The final adverse outcome is not specific to this AOP. Many of the key events included in this AOP overlap with AOPs linking other molecular initiating events to reproductive dysfunction in small fish.

Uncertainties, inconsistencies, and data gaps: The current major uncertainty in this AOP is whether there is a direct biological linkage between impaired VTG uptake into oocytes and impaired spawning/reduced cumulative fecundity. Plausible biological connections have been hypothesized, but have not yet been tested experimentally.

Essentiality of the Key Events

Molecular Initiating Event	Support for Essentiality
Aromatase, Inhibition	Strong

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

Molecular Initiating Event Summary, Key Event Summary

Support for the essentiality of a number of key events in the AOP was provided by several time-course, stop-reversibility, experiments with fathead minnows exposed to aromatase inhibitors.

1. Villeneuve et al. (2009 and 2013) examined a time-course of key event responses to fadrozole as well as the time-course of recovery following cessation of fadrozole delivery. Once fadrozole was removed from the system, ex vivo E2 production increased, followed by increases in plasma E2 concentrations, and then increases in plasma vitellogenin concentrations. Additionally, while exposure to the chemical was on-going, compensatory up-regulation of CYP19a1a gene expression resulted in increases in ex vivo E2 production, followed by increased plasma E2 and plasma VTG. The essentiality of aromatase inhibition relative to impaired E2 production was further supported by the observation of an "overshoot" in E2 production, relative to controls, shortly after cessation of fadrozole delivery.

2. Similar support was provided in a study by Ankley et al. (2009a). Cessation of prochloraz delivery resulted in rapid recovery of ex vivo E2 production and plasma E2 concentrations, with recovery of vitellogenin concentrations lagging slightly behind. Increased expression of cyp19a1a mRNA during the exposure period aligned with increased ex vivo E2 production, and increased plasma E2, compared to the first day of exposure.

Rationale for essentiality calls:

- Aromatase, inhibition: [Strong] There is good evidence from stop/reversibility studies that ceasing delivery of the aromatase inhibitor leads to recovery of the subsequent key events.
- 17beta-estradiol synthesis by ovarian granulosa cells, reduction: [Strong] In both exposure studies and stop/reversibility studies, when ex vivo E2 production (as measure of this KE) recovers either through compensation or due to removal of the stressor, subsequent KEs have been shown to recover after a lag period.
- plasma 17beta-estradiol concentrations, reduction: [Strong] In both exposure studies and stop/reversibility studies, when plasma E2 concentrations recover either through compensation or due to removal of the stressor, subsequent KEs have been shown to recover after a lag period.
- vitellogenin production in liver (transcription, translation), reduction: [Moderate] This endpoint was not specifically examined in stop/reversibility studies with aromatase inhibitors, but biological plausibility provides strong support for the essentiality of this event.
- plasma vitellogenin concentrations, reduction: [Strong] Shown to recover in a predictable fashion consistent with the order of events in the AOP in stop/recovery studies.
- vitellogenin accumulation into oocytes and oocyte growth/development, reduction: [Weak] Some contradictory evidence regarding the essentiality of this event. No stop/reversibility studies have explicitly considered this key event.
- cumulative fecundity and spawning, reductions: [Moderate] By definition, some degree of spawning is required to maintain population.

Quantitative Considerations

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

Summary Table

Assessment of quantitative understanding of the AOP:

At present, quantitative understanding of the AOP is approaching the point where an in vitro measurement of aromatase inhibition could be used as an input parameter into a series of coupled computational models that could generate quantitative predictions across multiple key events (e.g. circulating E2 concentrations, circulating VTG concentrations, predicted impacts on cumulative fecundity, and effects on population trajectories). A sequence of supporting models has been coupled together and predictions have been made for novel aromatase inhibitors (identified through high throughput in vitro screening), but those predictions have not yet been validated experimentally. The present models are also unable to account for pharmacokinetic considerations (e.g. adsorption, distribution, metabolism/biotransformation, and elimination) and have demonstrated only partial success in simulating compensatory/feedback responses to aromatase inhibition (e.g. Breen et al., 2013).

Applicability of the AOP

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Female	Strong

Life Stage Applicability, Taxonomic Applicability, Sex Applicability

- **Sex**: The AOP applies to females only. Males have relatively low gonadal aromatase expression and activity and the androgen 11-KT, rather than the estrogen E2 is a stronger driver of reproductive functions in males. That said, at least in fish, there is a potential autocrine and paracrine for estrogens synthesized in the brain in regulating reproductive behaviors. However, those potential effects are addressed through an alternative AOP that shares the MIE of aromatase inhibition.
- **Life stages**: The relevant life stages for this AOP are reproductively mature adults. This AOP does not apply to adult stages that lack a sexually mature ovary, for example as a result of seasonal or environmentally-induced gonadal senescence (i.e. through control of temperature, photo-period, etc. in a laboratory setting).
- **Taxonomic**: At present, the assumed taxonomic applicability domain of this AOP is class Osteichthyes. In all likelihood, the AOP will also prove applicable to all classes of fish (e.g. Agnatha and Chondrithyes as well). Additionally, all the key events described should be conserved among all oviparous vertebrates, suggesting that the AOP may also have relevance for amphibians, reptiles, and birds. However, species-specific differences in reproductive strategies/life histories, ADME (adsorption, distribution, metabolism, and elimination), compensatory reproductive endocrine responses may influence the outcomes, particularly from a quantitative standpoint.

Considerations for Potential Applications of the AOP

- The present AOP can provide potential support for the use of alternatives to the fish short term reproduction assay as a screen for aromatase inhibitors.

- The present AOP can serve as a foundation for tiered testing strategies and IATA related to risk assessments on chemicals identified as aromatase inhibitors.
- The present AOP can be used to guide endpoint selection for effects-based monitoring studies at sites where aromatase inhibition has been identified as a relevant biological activity of interest (e.g. through bioeffects prediction or bioeffects surveillance approaches; see Schroeder et al., 2016).

  Schroeder, A. L., Ankley, G. T., Houck, K. A. and Villeneuve, D. L. (2016), Environmental surveillance and monitoring—The next frontiers for high-throughput toxicology. Environ Toxicol Chem, 35: 513–525. doi:10.1002/etc.3309
- A series of computational models aligned with this AOP (i.e., a quantitative AOP construct) can be applied to estimate in vivo bench-mark doses based on in vitro screening results. Case studies evaluating this application are under way.

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AOP: 25 – Annex 1, assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements.

	Defining Question	High (Strong)	Moderate	Low (Weak)
1. Support for Biological Plausibility of KERS				
	a) Is there a mechanistic 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.	KER is plausible based on analogy to accepted biological relationships, but scientific understanding is incomplete	Empirical support for association between KEs , but the structural or functional relationship between them is not understood.
KE1 (MIE) => KE2: Aromatase inhibition directly leads to 17β-estradiol synthesis by ovarian granulosa cells, reduction	**STRONG.** It is well established that aromatase is rate limiting for 17β-estradiol synthesis and that the granulosa cells of the ovary are the primary site of expression and production.			
KE2 => KE3: 17β-estradiol synthesis by ovarian granulosa cells, reduction directly leads to plasma 17β-estradiol concentrations, reduction	**STRONG.** The biochemistry of steroidogenesis and the predominant role of the gonad in synthesis of the sex steroids is well established			
KE3 => KE4: plasma 17β-estradiol concentrations, reduction directly leads to vitellogenin production in liver (transcription, translation), reduced	**STRONG.** The role of E2 as the major regulator of hepatic vitellogenin production is widely documented in the literature			
KE4 => KE5: vitellogenin production in liver (transcription, translation), reduced directly leads to plasma vitellogenin concentrations, reduced	**STRONG.** It is well established that hepatic synthesis is the major source of plasma vitellogenin in oviparous vertebrates. The central dogma of molecular biology dictates that transcription and translation are needed for protein production.			
KE5 => KE6: plasma vitellogenin concentrations, reduced directly leads to vitellogenin uptake into oocytes and oocyte growth/development, reduction.	**STRONG.** It is well established that the circulation is the primary source of egg yolk proteins in fish.			
KE6 => KE7 (AO): vitellogenin uptake into oocytes and oocyte growth/development, reduction directly leads to cumulative fecundity and spawning, reduction	**MODERATE.** The direct connection between reduced VTG accumulation and impaired spawning/reduced cumulative fecundity is somewhat tentative. It is not clear, for instance whether impaired VTG accumulation limits oocyte growth and failure to reach a critical size in turn impairs physical or inter-cellular signaling processes that promote release of the oocyte from the surrounding follicles. In at least one experiment, oocytes with similar size to vitellogenic oocytes, but lacking histological staining characteristic of vitellogenic oocytes was observed (R. Johnson, personal communication). At present, the link between reductions in circulating VTG concentrations and reduced cumulative fecundity are best supported by the correlation between those endpoints across multiple experiments, including those that impact VTG via other molecular initiating events (Miller et al. 2007). Reference: Miller DH, Jensen KM, Villeneuve DL, Kahl MD, Makynen EA, Durhan EJ, Ankley GT. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (Pimephales promelas). Environ Toxicol Chem. 2007 Mar;26(3):521-7.			

AOP: 25 – Annex 1, assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements.

KE7 (AO) => KE8 (AO): cumulative fecundity and spawning, reduction directly leads to population trajectory, decrease	**MODERATE.** Using a relatively simple density-dependent population model and assuming constant young of year survival with no immigration/emigration, reductions in cumulative fecundity have been predicted to yield declines in population size over time (Miller and Ankley 2004). Under real-world environmental conditions, outcomes may vary depending on how well conditions conform with model assumptions. Nonetheless, cumulative fecundity can be considered one vital rate that contributes to overall population trajectories. Reference: Miller DH, Ankley GT. Modeling impacts on populations: fathead minnow (Pimephales promelas) exposure to the endocrine disruptor 17beta-trenbolone as a case study. Ecotoxicol Environ Saf. 2004 Sep;59(1):1-9.

2. Support for Essentiality of KEs	Defining Question	High (Strong)	Moderate	Low (Weak)
	Are downstream KEs and/or the AO prevented if an upstream KE is blocked?	Direct evidence from specifically designed experimental studies illustrating essentiality for at least one of the important KEs	Indirect evidence that sufficient modification of an expected modulating factor attenuates or augments a KE	No or contradictory experimental evidence of the essentiality of any of the KEs.

Essentiality of the KEs was assessed for the AOP as a whole – rationale for the individual KE calls is provided.	Support for the essentiality of a number of key events in the AOP was provided by several time-course, stop-reversibility, experiments with fathead minnows exposed to aromatase inhibitors. 1. Villeneuve et al. 2009 and 2013 examined a time-course of key event responses to fadrozole as well as the time-course of recovery following cessation of fadrozole delivery. Once fadrozole was removed from the system, ex vivo E2 production increased, followed by increases in plasma E2 concentrations, and then increases in plasma vitellogenin concentrations. Additionally, while exposure to the chemical was on-going, compensatory up-regulation of CYP19a1a gene expression resulted in increases in ex vivo E2 production, followed by increased plasma E2 and plasma VTG. The essentiality of aromatase inhibition relative to impaired E2 production was further supported by the observation of an "overshoot" in E2 production, relative to controls, shortly after cessation of fadrozole delivery. 2. Similar support was provided in a study by Ankley et al. (2009a). Cessation of prochloraz delivery resulted in rapid recovery of ex vivo E2 production and plasma E2 concentrations, with recovery of vitellogenin concentrations lagging slightly behind. Increased expression of cyp19a1a mRNA during the exposure period aligned with increased ex vivo E2 production, and increased plasma E2, compared to the first day of exposure. Rationale for essentiality calls: • *Aromatase, inhibition*: [Strong] There is good evidence from stop/reversibility studies that ceasing delivery of the aromatase inhibitor leads to recovery of the subsequent key events. • *17beta-estradiol synthesis by ovarian granulosa cells, reduction*: [Strong] In both exposure studies and stop/reversibility studies, when ex vivo E2 production (as measure of this KE) recovers either through compensation or due to removal of the stressor, subsequent KEs have been shown to recover after a lag period. • *plasma 17beta-estradiol concentrations, reduction*: [Strong] In both exposure studies and stop/reversibility studies, when plasma E2 concentrations recover either through compensation or due to removal of the stressor, subsequent KEs have been shown to recover after a lag period. • *vitellogenin production in liver (transcription, translation), reduction*: [Moderate] This endpoint was not specifically examined in stop/reversibility studies with aromatase inhibitors, but biological plausibility provides strong support for the essentiality of this event. • *plasma vitellogenin concentrations, reduction*: [Strong] Shown to recover in a predictable fashion consistent with the order of events in the AOP in stop/recovery studies. • *vitellogenin accumulation into oocytes and oocyte growth/development, reduction*: [Weak] Some contradictory evidence regarding the essentiality of this event. No stop/reversibility studies have explicitly considered this key event. • *cumulative fecundity and spawning, reductions*: [Moderate] By definition, some degree of spawning is required to maintain population. **REFERENCES** Villeneuve DL, Breen M, Bencic DC, Cavallin JE, Jensen KM, Makynen EA, Thomas LM, Wehmas LC, Conolly RB, Ankley GT. Developing predictive approaches to characterize adaptive responses of the reproductive endocrine axis to aromatase inhibition: I. Data generation in a small fish model. Toxicol Sci. 2013 Jun;133(2):225-33. doi: 10.1093/toxsci/kft068. Villeneuve DL, Mueller ND, Martinović D, Makynen EA, Kahl MD, Jensen KM, Durhan EJ, Cavallin JE, Bencic D, Ankley GT. Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor. Environ Health Perspect. 2009 Apr;117(4):624-31. doi: 10.1289/ ehp.11891.

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3. Empirical Support for KERs	Defining Questions	High (Strong)	Moderate	Low (Weak)
	Does 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}? Inconsistencies?	Multiple studies showing dependent change in both events following exposure to a wide range of specific stressors. No or few critical data gaps or conflicting data	Demonstrated dependent change in both events following exposure to a small number of stressors. Some inconsistencies with expected pattern that can be explained by various factors.	Limited or no studies reporting dependent change in both events following exposure to a specific stressor; and/or significant inconsistencies in empirical support across taxa and species that don't align with hypothesized AOP

KE1 (MIE) => KE2: Aromatase inhibition directly leads to 17β-estradiol synthesis by ovarian granulosa cells, reduction	**MODERATE** Direct measurement of aromatase inhibition following in vivo exposures are difficult to achieve, therefore identification of aromatase inhibition as a relevant MIE is most often based on in vitro experiments. Reductions in the rate of E2 production by ovary tissue or steroid producing cells following exposure to chemicals identified in vitro as aromatase inhibitors provides support. **Dose-response:** There is little direct support for dose-response concordance of these key events in vivo. However, using in vitro systems concentrations that reduce aromatase activity tend to elicit reductions in E2 production. **Temporality:** E2 production by ovary explants obtained from fish exposed to known aromatase inhibitors declines rapidly, following exposure, and has also been shown to recover rapidly upon cessation of the delivery of known aromatase inhibitors. **Uncertainties**: Because E2 synthesis is at the fairly terminal end of the steroid biosynthesis pathway, impacts of chemicals on other enzymes in the steroid biosynthesis pathway can lead to reduced E2 synthesis. There is also compelling evidence for fairly rapid in vivo compensation for aromatase inhibition via up-regulated transcription of aromatase mRNA expression. Consequently, complementary data from multiple types of in vitro assays are likely superior to in vivo evidence for establishing this KER.
KE2 => KE3: 17β-estradiol synthesis by ovarian granulosa cells, reduction directly leads to plasma 17β-estradiol concentrations, reduction	**STRONG** The rate of E2 production by ovarian explants and circulating concentrations of estradiol can generally both be measured for individual animals exposed in an experiment. Therefore, there is a fair amount of concurrent data for these endpoints. **Dose Response:** Effects on KE2 are generally observed at or near the same concentrations that impact KE3. There are exceptions, but these are typically explained by the higher variability (and thus lower statistical power) associated with the ex vivo steroid production assays often used to measure KE2. **Temporality:** Data from several time course studies, with at least two different aromatase inhibitors, support the idea that impacts on KE2 are detected (statistically) at earlier time-points than impacts on KE3. Data from these studies also show that KE2 recovers before KE3 both as the result of compensatory responses during an exposure period and following cessation of delivery of an aromatase inhibitor. **Incidence:** Particularly for experiments of longer duration (> 4 d), there are cases where impacts on KE3 are detected without concurrent effects on KE2. These are plausibly explained by the fact that compensatory responses in vivo lead to more rapid "recovery" of KE2 than KE3. It also reflects the fact that measures of KE2 represent a rate of steroid production per unit mass of tissue, while KE3 reflects total output of the whole organ into circulation. Small reductions in the rate of production per unit mass of tissue, which are not statistically detectable, can still lead to statistically detectable reductions in circulating concentrations.
KE3 => KE4: plasma 17β-estradiol concentrations, reduction directly leads to vitellogenin production in liver (transcription, translation), reduced	**WEAK** Circulating E2 concentrations and the relative abundance of hepatic vitellogenin transcripts can generally be concurrently measured for individual animals from the same experiment. Although methodologically more challenging, hepatic vitellogenin protein abundance can also be measured from the same fish. However, based on the empirical evidence currently assembled, relatively few studies have included a measurement of either VTG mRNA abundance or VTG protein abundance as an endpoint (see Tables 1 and 2). **Dose Response:** In one study that examined both KE3 and KE4, impacts on KE4 were observed at much lower concentrations. However, the measurement technology (mass spectroscopy-based proteomics) employed for measuring KE4 may be significantly more quantitative and precise than that employed for measuring KE3. **Temporality:** There are currently no time-course studies in which KE3 and KE4 were both measured. **Incidence:** In the only study that examined both KE3 and KE4, effects on both KEs were observed.
KE4 => KE5: vitellogenin production in liver (transcription, translation),	**WEAK** Few studies with aromatase inhibitors have reported impacts on hepatic vitellogenin transcription or translation, thus empirical data for evaluating this KER are limited.

reduced directly leads to plasma vitellogenin concentrations, reduced	**Dose Response:** In the one study that examined both KE4 and KE5, the impact on the upstream event (KE4) occurred at a lower concentration than that at which the effect on the downstream KE (KE5) was observed. However, it should be noted that the measurement methods may not be comparable in terms of precision; mass-spectroscopy-based proteomics for KE4 versus an ELISA for KE5. **Temporality:** There are not sufficient empirical data to evaluate the temporal concordance of these key events. **Incidence:** In the only study that examined both KE3 and KE4, effects on both KEs were observed.
KE3 => KE5: plasma 17β-estradiol concentrations, reduction indirectly leads to plasma vitellogenin concentrations, reduced	**STRONG** Circulating E2 (KE3) and VTG concentrations (KE5) are readily measured in plasma samples collected from the same individual animals exposed in an experiment. Measurements of both KEs have frequently been made. **Dose Response:** Generally speaking effects on the downstream KE (KE5) were observed at concentrations equal to or greater than those at which effects on the upstream event (KE3) were reported. There were several exceptions. However exceptions are plausibly explained by a number of factors. First, vitellogenin concentrations in plasma have a much greater dynamic range (i.e., often change by orders of magnitude) than circulating steroid concentrations (changes are typically within 1-2 orders of magnitude). Second, compensatory responses elicited in response to aromatase inhibition have been shown to impact KE3 more rapidly than KE5, which can lead to a disconnect in the apparent dose needed to elicit a response at a given time-point. **Temporality:** In several independent time-course studies with multiple aromatase inhibitors, impacts on KE3 reliably precede those on KE5. Likewise, "recovery" of KE3 as a result of compensatory responses during exposure or cessation of chemical delivery consistently precede that of KE5. **Incidence:** Taking the temporal relationship between the two KEs into account, there is strong concordance in the incidence of KE3 and KE5 across several studies.
KE5 => KE6: plasma vitellogenin concentrations, reduced directly leads to vitellogenin accumulation into oocytes and oocyte growth/development, reduction.	**WEAK** Conceptually, both plasma vitellogenin concentrations and ovarian histology measurements can be made in the same individuals exposed in a given experiment. However, among the studies available to date, examination of both endpoints has generally been limited to the longer duration studies. Given that ovulation and spawning are the major routes through which oocytes containing vitellogenin are lost from the ovary, one or more spawning events may need to occur in order for existing vitellogenic oocytes to be "cleared" from the ovary or to undergo atresia, before the impacts on KE6 can be detected. **Dose Response:** For the one study in which both plasma vitellogenin and ovarian histology were examined, effects on uptake of VTG into oocytes were detected at concentrations greater than those that impacted plasma steroid concentrations. **Temporality:** Impacts on circulating vitellogenin have been observed at time points earlier than those at which significant histological evidence of reduced VTG uptake into oocytes has been detected. **Incidence:** Given the limited data set, incidence concordance cannot be thoroughly evaluated.
KE6 => KE7 (AO): vitellogenin accumulation into oocytes and oocyte growth/development, reduction directly leads to cumulative fecundity and spawning, reduction	**WEAK** There are only a few studies in which KE6 and KE7 were examined concurrently. **Dose Response**: In the one study in which concurrent measures for KE6 and KE7 were reported, effects were detected at the same concentration. **Temporality**: At present, there are no time-course data that directly address the temporal concordance between KE6 and KE7. **Incidence**: Given the limited data set, incidence concordance cannot be robustly evaluated.
KE7 (AO) => KE8 (AO): cumulative fecundity and spawning, reduction directly leads to population trajectory, decrease	**WEAK** There is limited direct evidence in the literature that population size will decrease if fecundity is decreased. There are no empirical data suitable for evaluating the dose-response, temporal, or incidence concordance between KE7 and KE8.

AOP: 25 – Annex 1, assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements.

KER	Integrative Assessment leading to the final weight of evidence call for each KER
KE1 (MIE) => KE2: Aromatase inhibition directly leads to 17β-estradiol synthesis by ovarian granulosa cells, reduction	STRONG Strong biological plausibility supported by moderate empirical support and well established essentiality for both KEs.
KE2 => KE3: 17β-estradiol synthesis by ovarian granulosa cells, reduction directly leads to plasma 17β-estradiol concentrations, reduction	STRONG Strong biological plausibility supported by strong empirical support.
KE3 => KE4: plasma 17β-estradiol concentrations, reduction directly leads to vitellogenin production in liver (transcription, translation), reduced	STRONG Even though the empirical support available is quite limited, plausibility provides a very strong basis upon which to build confidence in this key event relationship. Estrogen-dependent regulation of vitellogenin production is very well established. Additionally, there is strong support for the indirect relationship linking KE3 and KE5, which together with plausibility lends strong support for this KER.
KE4 => KE5: vitellogenin production in liver (transcription, translation), reduced directly leads to plasma vitellogenin concentrations, reduced	STRONG Even though the empirical support available is quite limited, plausibility provides a very strong basis upon which to build confidence in this key event relationship. Estrogen-dependent regulation of vitellogenin production is very well established. Additionally, there is strong support for the indirect relationship linking KE3 and KE5, which together with plausibility lends strong support for this KER.
KE5 => KE6: plasma vitellogenin concentrations, reduced directly leads to vitellogenin accumulation into oocytes and oocyte growth/development, reduction.	MODERATE While plausibility is fairly strong, the empirical support for the relationship is relatively weak. There are few studies in which both plasma VTG and ovarian histology have been examined. Because VTG is the only major source of VTG to the developing oocytes the connection is highly plausible. However, it remains unclear how much decreases in plasma VTG impacts accumulation if the decrease happens after oocytes have already reached vitellogenic stage. Presumably, the more rapid the oocyte turn over, the tighter the linkage, but uncertainties remain.
KE6 => KE7 (AO): vitellogenin accumulation into oocytes and oocyte growth/development, reduction directly leads to cumulative fecundity and spawning, reduction	MODERATE The plausibility is only moderate and only a few studies have examined KE6 and KE7 concurrently in the same experiment.
KE7 (AO) => KE8 (AO): cumulative fecundity and spawning, reduction directly leads to population trajectory, decrease	MODERATE The relationship is plausible, but not necessarily generalizable to real-world situation or a diversity of life histories and reproductive strategies. Direct evidence is quite limited.