

AOP ID and Title:

SNAPSHOT

Created at: 2017-12-15 13:10

AOP 23: Androgen receptor agonism leading to reproductive dysfunction

Short Title: Androgen receptor agonism leading to reproductive dysfunction

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Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment	EAGMST Approved	1.12	Included in OECD Work Plan

Abstract

This adverse outcome pathway details the linkage between binding and activation of androgen receptor as a nuclear transcription factor in females and reproductive dysfunction as evidenced through reductions cumulative fecundity and spawning in repeat-spawning fish species. Androgen receptor mediated activities are one of the major activities of concern to endocrine disruptor screening programs worldwide. Cumulative fecundity is the most apical endpoint considered in the OECD 229 Fish Short Term Reproduction Assay. The OECD 229 assay serves as screening assay for endocrine disruption and associated reproductive impairment (OECD 2012). Cumulative fecundity is one of several variables known to be of demographic significance in forecasting fish population trends. Therefore, this AOP has utility in supporting the application of measures of androgen receptor binding and activation as a nuclear transcription factor as a means to identify chemicals with known potential to adversely affect fish populations. At present this AOP is largely supported by evidence conducted with small laboratory model fish species such as *Pimephales promelas*, *Oryzias latipes*, and *Fundulus heteroclitus*. While many aspects of the biology underlying this AOP are largely conserved across vertebrates, particularly oviparous vertebrates, the relevance of this AOP to vertebrate classes other than fish as well as to fish species employing different reproductive strategies has not been established at this time. Thus, caution should be used in applying this AOP beyond a fairly narrow range of fish species with life cycles similar to that of the three species noted above.

Background

No additional background

Summary of the AOP

Stressors

Name	Evidence
17beta-Trenbolone	Strong

17beta-Trenbolone

17β-trenbolone is a prototypical stressor for this AOP.

Molecular Initiating Event

Title	Short name
Agonism, Androgen receptor (https://aopwiki.org/events/25)	Agonism, Androgen receptor

AOP23

25: Agonism, Androgen receptor (<https://aopwiki.org/events/25>)

Short Name: Agonism, Androgen receptor

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	MolecularInitiatingEvent

Stressors

Name
17beta-Trenbolone
Spironolactone
5alpha-Dihydrotestosterone

Biological Organization

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Characterization of chemical properties: Androgen receptor binding chemicals can be grouped into two broad structural domains, steroidal and non-steroidal (Yin et al. 2003). Steroidal androgens consist primarily of testosterone and its derivatives (Yin et al. 2003). Many of the non-steroidal AR binding chemicals studied are derivatives of well known non-steroidal AR antagonists like bicalutamide, hydroxyflutamide, and nilutamide (Yin et al. 2003). Nonetheless, a number of QSARs and SARs that consider AR binding of both these pharmaceutical agents as well as environmental chemicals have been developed (Waller et al. 1996; Serafimova et al. 2002; Todorov et al. 2011; Hong et al. 2003; Bohl et al. 2004). However, it has been shown that very minor structural differences can dramatically impact function as either an agonist or antagonist (Yin et al. 2003; Bohl et al. 2004; Norris et al. 2009), making it difficult at present to predict agonist versus antagonist activity based on chemical structure alone.

In vivo considerations: A variety of steroidal androgens can be converted to estrogens in vitro through the action of cytochrome P450 19 (aromatase). Structures subject to aromatization may behave in vivo as estrogens despite exhibiting potent androgen receptor agonism in vitro.

5alpha-Dihydrotestosterone

Chemical is a non-aromatizable androgen.

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
medaka	Oryzias latipes	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

Taxonomic applicability: Androgen receptor orthologs are primarily limited to vertebrates (Baker 1997; Thornton 2001; Eick and Thornton 2011; Markov and Laudet 2011). Therefore, this MIE would generally be viewed as relevant to vertebrates, but not invertebrates.

How this Key Event Works

Site of action: The molecular site of action is the ligand binding domain of the AR. This particular key event specifically refers to interaction with nuclear AR. Downstream KE responses to activation of membrane ARs may be different. The cellular site of action for the molecular initiating event is undefined.

Responses at the macromolecular level: Binding of a ligand, including xenobiotics that act as AR agonists, to the cytosolic AR mediates a conformational shift that facilitates dissociation from accompanying heat shock proteins and dimerization with another AR (Prescott and Coetzee 2006; Claessens et al. 2008; Centenera et al. 2008). Homodimerization unveils a nuclear localization sequence, allowing the AR-ligand complex to translocate to the nucleus and bind to androgen-response elements (AREs) (Claessens et al. 2008; Cutress et al. 2008). This elicits recruitment of additional transcription factors and transcriptional activation of androgen-responsive genes (Heemers and Tindall 2007).

AR paralogs:

- Most vertebrates have a single gene coding for nuclear AR. However, most fish have two AR genes (AR-A, AR-B) as a result of a whole genome duplication event after the split of Acipenseriformes from teleosts but before the divergence of Osteoglossiformes (Douard et al. 2008).
- AR-B has been lost in Cypriniformes, Siluriformes, Characiformes, and Salmoniformes (Douard et al. 2008).
- In Percomorphs, AR-B has accumulated significant substitutions in the both ligand binding and DNA binding domains (Douard et al. 2008).
- Differential ligand selectivity and subcellular localization has been reported for AR paralogs in some fish species (e.g., Bain et al. 2015), but the difference is not easily generalized based on available data in the literature.

How it is Measured or Detected**Measurement/detection:**

- **In vitro methods:**
 - OECD Test No. 458: Stably transfected human androgen receptor transcriptional activation assay for detection of androgen agonists and antagonists has been reviewed and validated by OECD and is well suited for detection of this key event (OECD 2016 (<http://www.oecd.org/env/test-no-458-stably-transfected-human-androgen-receptor-transcriptional-activation-assay-for-detection-of-androgenic-agonist-9789264264366-en.htm>)).
 - Binding to the androgen receptor can be directly measured in cell free systems based on displacement of a radio-labeled standard (generally testosterone or DHT) in a competitive binding assay (e.g., Olsson et al. 2005; Sperry and Thomas 1999; Wilson et al. 2007; Tilley et al. 1989; Kim et al. 2010).
 - Cell based transcriptional activation assays are typically required to differentiate agonists from antagonists, in vitro. A number of reporter gene assays have been developed and used to screen chemicals for AR agonist and/or antagonist activity (e.g., (Wilson et al. 2002; van der Burg et al. 2010; Mak et al. 1999; Araki et al. 2005).
 - Expression of androgen responsive proteins like spiggin in primary cell cultures has also been used to detect AR agonist activity (Jolly et al. 2006).
- **In vivo methods:**
 - In fish, phenotypic masculinization of females has frequently been used as an indirect measurement of in vivo androgen receptor agonism.
 - Development of nuptial tubercles, a dorsal fatpad, and a characteristic banding pattern has been observed in female fathead minnows exposed to androgen agonists (Ankley et al. 2003; Jensen et al. 2006; Ankley et al. 2010; LaLone et al. 2013; OECD 2012 (http://www.oecd-ilibrary.org/environment/test-no-229-fish-short-term-reproduction-assay_9789264185265-en)).
 - Anal fin elongation in female western mosquitofish (*Gambusia affinis*) has similarly been viewed as evidence of AR activation (Raut et al. 2011; Sone et al. 2005).
 - In medaka, development of papillary processes, which normally only appear on the second to seventh or eighth fin ray of the anal fin, has also been used as an indirect measure of androgen receptor agonism (OECD 2012 (http://www.oecd-ilibrary.org/environment/test-no-229-fish-short-term-reproduction-assay_9789264185265-en)).
 - Production of the nest building glue, spiggin, in three female 3-spined sticklebacks (*Gasterosteus aculeatus*) has also been well documented as an indicator of androgen receptor agonism (Jakobsson et al. 1999; Hahlbeck et al. 2004). Quantification of the spiggin protein in exposed female 3-spined stickleback or green fluorescence protein expression in a transgenic spg1-gfp medaka line (Sébillot et al. 2014) can be used to detect androgen receptor agonism.

• High Throughput Screening

- Measures of AR agonism have been included in high throughput screening programs, such as US EPA's Toxcast program. Toxcast assays relevant for screening chemicals for their ability to bind and/or activate the AR include:
 - ATG_AR_TRANS A cell based assay that can differentiate agonism from antagonism
 - NVS_NR_hAR A cell free assay using recombinant human AR. Can detect binding, but cannot distinguish agonism from antagonism.
 - NVS_NR_rAR A cell free assay using recombinant rat AR. Can detect binding, but cannot distinguish agonism from antagonism.
 - OT_AR_ARELUC_AG_1440 A cell based assay that measures expression of a reporter gene under control of androgen-responsive elements. Can distinguish agonism from antagonism.
 - Tox21_AR_BLA_Agonist_ratio A cell based assay with an inducible reporter. Can distinguish agonists from antagonists.
 - Tox21_AR_LUC_MDAKB2_agonist A cell based assay with an inducible reporter. Can distinguish agonists from antagonists.
- Assay descriptions (<https://actorws.epa.gov/actorws/edsp21/v02/assays>)

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Key Events

Title	Short name
Reduction, Gonadotropins, circulating concentrations (https://aopwiki.org/events/129)	Reduction, Gonadotropins, circulating concentrations
Reduction, Testosterone synthesis by ovarian theca cells (https://aopwiki.org/events/274)	Reduction, Testosterone synthesis by ovarian theca cells
Reduction, 17beta-estradiol synthesis by ovarian granulosa cells (https://aopwiki.org/events/3)	Reduction, 17beta-estradiol synthesis by ovarian granulosa cells
Reduction, Plasma 17beta-estradiol concentrations (https://aopwiki.org/events/219)	Reduction, Plasma 17beta-estradiol concentrations
Reduction, Vitellogenin synthesis in liver (https://aopwiki.org/events/285)	Reduction, Vitellogenin synthesis in liver
Reduction, Plasma vitellogenin concentrations (https://aopwiki.org/events/221)	Reduction, Plasma vitellogenin concentrations
Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development (https://aopwiki.org/events/309)	Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development
Reduction, Cumulative fecundity and spawning (https://aopwiki.org/events/78)	Reduction, Cumulative fecundity and spawning

129: Reduction, Gonadotropins, circulating concentrations (<https://aopwiki.org/events/129>)

Short Name: Reduction, Gonadotropins, circulating concentrations

Key Event Component

AOP23

Process	Object	Action
	Luteinizing hormone	decreased
	Follicle stimulating hormone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent

Stressors

Name
n/a

Biological Organization

Level of Biological Organization
Organ

Organ term

Organ term
blood plasma

Evidence for Perturbation by Stressor

n/a

n/a

Evidence Supporting Applicability of this Event

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

A functional hypothalamic-pituitary-gonadal axis involving GnRH and gonadotropin-mediated regulation of reproductive functions is a vertebrate trait (Sower et al. 2009). The taxonomic applicability of this key event is limited to chordates.

How this Key Event Works

Gonadotropin (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) secretion from the pituitary is a key regulator of gonadal steroid biosynthesis. LH and FSH are heterodimeric glycoproteins composed of a hormone-specific beta subunit and a common alpha subunit (Norris 2007). The subunits are synthesized in pituitary gonadotropes and stored in secretory vesicles. Gonadotropin secretion by pituitary gonadotropes

is regulated via gonadotropin releasing hormone (GnRH) signaling from the hypothalamus as well as by intrapituitary regulators of gonadotropin expression (e.g., activin, follistatin, inhibin) (Norris 2007; Habibi and Huggard 1998).

How it is Measured or Detected

- Circulating concentrations of gonadotropins in humans and common mammalian models (e.g., rodents, many livestock species) can be directly measured using either commercial or custom immunoassays (e.g., enzyme-linked immunosorbent assays, radioimmunoassays, etc.).
- Similar immunoassay-based methods have been developed for quantifying gonadotropins in fish (e.g., (Govoroun et al. 1998; Amano et al. 2000; Kah et al. 1989; Prat et al. 1996)). However, at present, antibodies specific for distinguishing LH and FSH are only available for a limited number of species, primarily salmonids (Levavi-Sivan et al. 2010).
- Expression of mRNAs coding for luteinizing hormone beta subunit (lhb) and follicle-stimulating hormone beta subunit (fshb) tend to fluctuate in parallel in repeat-spawning fish and plasma concentrations LH and FSH in tilapia were also shown to fluctuate in parallel (reviewed in (Levavi-Sivan et al. 2010)). Consequently, the two gonadotropins are treated non-specifically for the purposes of the current key event.
- For small fish species limited plasma volumes relative to the sensitivity of the available immunoassay methods may impose limits on the ability to measure this key event directly.

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274: Reduction, Testosterone synthesis by ovarian theca cells (<https://aopwiki.org/events/274>)

Short Name: Reduction, Testosterone synthesis by ovarian theca cells

Key Event Component

Process	Object	Action
testosterone biosynthetic process	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Cell term

Cell term
theca cell

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Not Specified

This key event is relevant to vertebrates and amphioxus, but not invertebrates.

- Cytochrome P45011a (Cyp11a), a rate limiting enzyme for the production of testosterone, is specific to vertebrates and amphioxus (Markov et al. 2009; Baker et al. 2011; Payne and Hales, 2004).
- Cyp11a does not occur in invertebrates, as a result, they do not synthesize testosterone, nor other steroid intermediates required for testosterone synthesis (Markov et al. 2009; Payne and Hales, 2004).
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How this Key Event Works

Testosterone is synthesized in ovarian theca cells through a series of enzyme catalyzed reactions that convert cholesterol to androgens (see KEGG reference pathway 00140 for details; www.genome.jp/kegg; (Payne and Hales 2004; Magoffin 2005; Young and McNeilly 2010). Binding of luteinizing hormone to luteinizing hormone receptors located on the surface of theca cell membranes leads to increased expression of steroidogenic cytochrome P450s, steroidogenic acute regulatory protein, and consequent increases in androgen production (Payne and Hales 2004; Miller 1988; Miller and Strauss 1999).

How it is Measured or Detected

Steroid production by isolated primary theca cells can be measured using radioimmunoassay or enzyme linked immunosorbent assay approaches (e.g., (Benninghoff and Thomas 2006; Campbell et al. 1998). However, the isolation and culture methods are not trivial. Similarly, development of immortalized theca cell lines has proven challenging (Havelock et al. 2004). Consequently, this key event is perhaps best evaluated by examining T 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)). Reductions in T production by ovarian tissue explants can indicate either direct inhibition of steroidogenic enzymes involved in T synthesis, or indirect impacts due to feedback along the hypothalamic-pituitary-gonadal axis (in cases where chemical exposures occur in vivo). However, because T synthesis in the theca cells is closely linked to estradiol (E2) synthesis by granulosa cells, reductions in T production by intact ovary tissue can also be due to increased aromatase activity and the resulting increased rate of converting T to E2.

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3: Reduction, 17beta-estradiol synthesis by ovarian granulosa cells (<https://aopwiki.org/events/3>)

Short Name: Reduction, 17beta-estradiol synthesis by ovarian granulosa cells

Key Event Component

Process	Object	Action
estrogen biosynthetic process	17beta-estradiol	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
7: Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female (https://aopwiki.org/aops/7)	KeyEvent
25: Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	KeyEvent
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent
122: Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	KeyEvent
123: Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Cell term

Cell term
granulosa cell

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Term	Scientific Term	Evidence	Links
Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

Key enzymes needed to synthesize 17 β -estradiol first appear in the common ancestor of amphioxus and vertebrates (Markov et al. 2009; Baker 2011). Consequently, it is plausible that this key event is applicable to most vertebrates. This key event is not applicable to invertebrates, which lack the enzymes required to synthesize 17 β -estradiol.

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 (17 α -hydroxylase/17,20-lyase), 3 β hydroxysteroid dehydrogenase, 17 β 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 radioimmunoassay (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) (http://www.oecd-ilibrary.org/environment/test-no-456-h295r-steroidogenesis-assay_9789264122642-en) is the validated test guideline for an in vitro screen for chemical effects on steroidogenesis, specifically the production of 17 β -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) (http://ecvam-dbalm.jrc.ec.europa.eu/beta/index.cfm/methodsAndProtocols/index?id_prot=266), Granulosa and Theca Cell Culture Systems (EURL ECVAM Method Summary No. 92) (http://ecvam-dbalm.jrc.ec.europa.eu/beta/index.cfm/methodsAndProtocols/index?id_met=535).

References

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219: Reduction, Plasma 17beta-estradiol concentrations (<https://aopwiki.org/events/219>)

Short Name: Reduction, Plasma 17beta-estradiol concentrations

Key Event Component

Process	Object	Action
	17beta-estradiol	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
7: Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female (https://aopwiki.org/aops/7)	KeyEvent
25: Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	KeyEvent
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent
122: Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	KeyEvent
123: Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Organ term

Organ term
blood plasma

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
fathead minnow	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Term	Scientific Term	Evidence	Links
Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult	Strong

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

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

How this Key Event Works

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 17 β -estradiol in plasma can be measured by radioimmunoassay (e.g., (Jensen et al. 2001)), enzyme-linked immunosorbent assay (available through many commercial vendors), or by analytical chemistry (e.g., LC/MS; 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, chorionogenins, 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).

References

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285: Reduction, Vitellogenin synthesis in liver (<https://aopwiki.org/events/285>)

Short Name: Reduction, Vitellogenin synthesis in liver

Key Event Component

Process	Object	Action
gene expression	vitellogenins	decreased

AOPs Including This Key Event

AOP23

AOP ID and Name	Event Type
25: Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	KeyEvent
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent
30: Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	KeyEvent
122: Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	KeyEvent
123: Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	KeyEvent

Biological Organization

Level of Biological Organization
Tissue

Cell term

Cell term
hepatocyte

Organ term

Organ term
liver

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Pimephales promelas	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)
Oryzias latipes	Oryzias latipes	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

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)

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 polymerase chain reaction (PCR; transcripts) or enzyme linked immunosorbent assay (ELISA; protein).

References

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

221: Reduction, Plasma vitellogenin concentrations (<https://aopwiki.org/events/221>)

Short Name: Reduction, Plasma vitellogenin concentrations

Key Event Component

Process	Object	Action
	vitellogenins	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
25: Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	KeyEvent
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent
30: Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	KeyEvent
122: Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	KeyEvent
123: Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Organ term

Organ term
blood plasma

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Oryzias latipes	Oryzias latipes	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)
Danio rerio	Danio rerio	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

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.

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.

References

- 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.
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- 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 17 β -estradiol treatment. Environmental Toxicology and Chemistry 19(4): 972-981.
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- Wahli W. 1988. Evolution and expression of vitellogenin genes. Trends in Genetics. 4:227-232.

309: Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development (<https://aopwiki.org/events/309>)

Short Name: Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development

Key Event Component

Process	Object	Action
receptor-mediated endocytosis	vitellogenins	decreased
oocyte growth		decreased
oocyte development		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
25: Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	KeyEvent
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent
30: Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	KeyEvent
122: Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	KeyEvent
123: Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

Cell term

Cell term
oocyte

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Oryzias latipes	Oryzias latipes	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

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.

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

AOP23

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.

References

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

78: Reduction, Cumulative fecundity and spawning (<https://aopwiki.org/events/78>)

Short Name: Reduction, Cumulative fecundity and spawning

Key Event Component

Process	Object	Action
egg quantity		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
29: Estrogen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/29)	KeyEvent
25: Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	KeyEvent
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	KeyEvent
30: Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	KeyEvent
122: Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	AdverseOutcome
123: Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	AdverseOutcome

Biological Organization

Level of Biological Organization
Individual

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	<i>Pimephales promelas</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Fundulus heteroclitus	<i>Fundulus heteroclitus</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Term	Scientific Term	Evidence	Links
Oryzias latipes	Oryzias latipes	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

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

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 Test No. 229; OECD Test No. 240), spawning and cumulative fecundity can be directly measured through daily observation of egg deposition and egg counts.

In some cases, fecundity may be estimated based on gonado-somatic index (OECD 2008 ([http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2008\)22&doclanguage=en](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2008)22&doclanguage=en))).

Regulatory Examples Using This Adverse Outcome

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 (http://www.oecd-ilibrary.org/environment/test-no-229-fish-short-term-reproduction-assay_9789264185265-en)). Fecundity is also an important apical endpoint in the Medaka Extended One Generation Reproduction Test (MEOGRT; OECD Test Guideline 240 (http://www.oecd-ilibrary.org/environment/test-no-240-medaka-extended-one-generation-reproduction-test-meogrt_9789264242258-en); OECD 2015).

A variety of fish life cycle tests also include cumulative fecundity as an endpoint (OECD 2008 ([http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2008\)22&doclanguage=en](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2008)22&doclanguage=en))).

References

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

Adverse Outcomes

Title	Short name
Decrease, Population trajectory (https://aopwiki.org/events/360)	Decrease, Population trajectory

360: Decrease, Population trajectory (<https://aopwiki.org/events/360>)

Short Name: Decrease, Population trajectory

Key Event Component

AOP23

Process	Object	Action
population growth rate		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
23: Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	AdverseOutcome
25: Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	AdverseOutcome
29: Estrogen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/29)	AdverseOutcome
30: Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	AdverseOutcome
100: Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior (https://aopwiki.org/aops/100)	AdverseOutcome
122: Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	AdverseOutcome
123: Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	AdverseOutcome
155: Deiodinase 2 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/155)	AdverseOutcome
156: Deiodinase 2 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/156)	AdverseOutcome
157: Deiodinase 1 inhibition leading to reduced young of year survival via posterior swim bladder inflation (https://aopwiki.org/aops/157)	AdverseOutcome
158: Deiodinase 1 inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/158)	AdverseOutcome
159: Thyroperoxidase inhibition leading to reduced young of year survival via anterior swim bladder inflation (https://aopwiki.org/aops/159)	AdverseOutcome

Biological Organization

Level of Biological Organization
Population

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=0)

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

AOP23

Sex	Evidence
Unspecific	Not Specified

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

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.

Regulatory Examples Using This Adverse Outcome

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

References

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

Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Agonism, Androgen receptor	directly leads to	Reduction, Gonadotropins, circulating concentrations	Weak	Weak
Reduction, Gonadotropins, circulating concentrations	directly leads to	Reduction, Testosterone synthesis by ovarian theca cells	Strong	Weak
Reduction, Testosterone synthesis by ovarian theca cells	directly leads to	Reduction, 17beta-estradiol synthesis by ovarian granulosa cells	Strong	Weak
Reduction, 17beta-estradiol synthesis by ovarian granulosa cells	directly leads to	Reduction, Plasma 17beta-estradiol concentrations	Strong	Weak
Reduction, Plasma 17beta-estradiol concentrations	directly leads to	Reduction, Vitellogenin synthesis in liver	Strong	Moderate
Reduction, Vitellogenin synthesis in liver	directly leads to	Reduction, Plasma vitellogenin concentrations	Strong	Moderate
Reduction, Plasma vitellogenin concentrations	directly leads to	Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development	Moderate	Weak
Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development	directly leads to	Reduction, Cumulative fecundity and spawning	Moderate	Moderate
Reduction, Cumulative fecundity and spawning	directly leads to	Decrease, Population trajectory	Moderate	Moderate
Agonism, Androgen receptor	indirectly leads to	Reduction, Testosterone synthesis by ovarian theca cells	Moderate	Weak

AOP23

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Agonism, Androgen receptor	indirectly leads to	Reduction, 17beta-estradiol synthesis by ovarian granulosa cells	Moderate	Weak
Agonism, Androgen receptor	indirectly leads to	Reduction, Vitellogenin synthesis in liver	Strong	Weak
Reduction, Plasma 17beta-estradiol concentrations	indirectly leads to	Reduction, Plasma vitellogenin concentrations	Strong	Moderate

Agonism, Androgen receptor leads to Reduction, Gonadotropins, circulating concentrations
(<https://aopwiki.org/relationships/31>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Weak	Weak

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Not Specified

Sex Applicability

Sex	Evidence
Female	Not Specified

At present, this relationship is assumed to operate in repeat spawning fish species with asynchronous oocyte development.

The extent to which the negative feedback mechanism proposed here is operable for fish species employing other reproductive strategies and/or for other vertebrates has not been extensively examined, to date.

How Does This Key Event Relationship Work

See biological plausibility, below.

Weight of Evidence

Biological Plausibility

- Circulating concentrations of steroid hormones are tightly regulated via positive and negative feedback loops that operate through endocrine, autocrine, and/or paracrine mechanisms within the hypothalamic-pituitary-gonadal axis (Norris 2007).
- Gonadotropin (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) secretion from the pituitary is a key regulator of gonadal steroid biosynthesis.
- Negative feedback of androgens or estrogens at the level of the hypothalamus and/or pituitary can reduce gonadotropin secretion by pituitary gonadotropes either indirectly due to decreased GnRH signaling from the hypothalamus or directly through intrapituitary regulators of gonadotropin expression (e.g., activin, follistatin, inhibin) (Norris 2007; Habibi and Huggard 1998).
- While similar processes of negative feedback of sex steroids on gonadotropin expression and release have been established in fish (Levavi-Sivan et al. 2010), there are many remaining uncertainties about the exact mechanisms through which feedback takes place in fish as well as other vertebrates. For example, feedback is thought to involve a complex interplay of neurotransmitter signaling, kisspeptins, and the follistatin/inhibin/activin system (Trudeau et al. 2000; Trudeau 1997; Oakley et al. 2009; Cheng et al. 2007).
- In addition, the nature of the feedback produced by androgens is dependent on the concentration, form of the androgen (e.g., aromatizable

versus non-aromatizable), life-stage and likely species (Habibi and Huggard 1998; Trudeau et al. 2000; Gopurappilly et al. 2013).

- The specific mechanisms through which negative feedback of AR agonists on the hypothalamus and pituitary are mediated in fish are not fully understood.
 - It is thought that GABAergic and dopaminergic neurons may be important mediators of sex steroid feedback on gonadotropin releasing hormone (GnRH) release from the hypothalamus (Trudeau et al. 2000; Trudeau 1997).
 - More recent evidence also suggests an important role of kisspeptin neurons, which have been shown to express both AR and ER α are important mediators of feedback response to circulating androgen concentrations (Oakley et al. 2009).
 - Follistatin expression in the pituitary has also been cited as a key regulator of gonadotropin expression that is directly regulated by androgens and estrogens (Cheng et al. 2007).
- Regardless of the exact mechanisms, negative feedback of androgens on GnRH and/or gonadotropin release from the hypothalamus and/or pituitary is a well established endocrine phenomenon.

Empirical Support for Linkage

- There is a relatively strong body of evidence demonstrating that gonadectomy and/or treatment with potent AR antagonists can increase circulating concentrations of gonadotropins in fish and that those effects can be reversed by treatment with testosterone (reviewed in (Habibi and Huggard 1998; Levavi-Sivan et al. 2010)).
- However, we are currently unaware of any studies conducted with xenobiotic or pharmaceutical androgen agonists that measured effects on circulating gonadotropins.
- Empirical support for this linkage is largely lacking for most fish species, as antibodies capable of specifically detecting and distinguishing luteinizing hormone and follicle stimulating hormone have not yet been developed, despite many attempts.
- FSH and LH can be specifically measured for salmonids, but measurement methods for most other species are lacking.

Uncertainties or Inconsistencies

Due to uncertainties regarding the exact mechanisms through which exogenous androgens mediate a negative feedback response this initiation of a negative feedback response is not directly observable. Negative feedback would generally be inferred through a decrease in gonadotropin release and associated declines in circulating gonadotropin concentrations.

Quantitative Understanding of the Linkage

Given the uncertainties in the specific mechanism(s) of negative feedback that are involved and the lack of data on circulating gonadotropin concentrations following exposure to exogenous androgen agonists there is currently no quantitative understanding that would translate relative binding affinity and/or effect concentrations in an AR-mediated transcriptional activation assay into expected impacts on circulating gonadotropin concentrations.

Quantitative understanding of this linkage is largely absent for fish due to lack of established methods for measuring the gonadotropins and uncertainties about the exact mechanisms through which androgens or androgen receptor activation may exert negative feedback control on gonadotropin secretion.

References

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Reduction, Gonadotropins, circulating concentrations leads to Reduction, Testosterone synthesis by ovarian theca cells (<https://aopwiki.org/relationships/143>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Strong	Weak

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Weak	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Not Specified

Sex Applicability

Sex	Evidence
Female	Not Specified

- A functional hypothalamic-pituitary-gonadal axis involving GnRH and gonadotropin-mediated regulation of reproductive functions is a vertebrate trait (Sower et al. 2009).
- The taxonomic applicability of this key event is limited to chordates.
- CYP11a, one of the critical enzymes for testosterone synthesis has only been found in amphioxus or vertebrates (Baker 2011). Consequently, taxonomic relevance of this KER is likely restricted to that domain.

How Does This Key Event Relationship Work

See biological plausibility below.

Weight of Evidence**Biological Plausibility**

- In mammals androgen production by theca cells is largely under control of LH (Norris 2007; Young and McNeilly 2010).
- In fish, the differential role of LH versus FSH has been more difficult to define, in part due to the inability to specifically measure LH and FSH in most fish species and the parallel fluctuations of LH and FSH expression in many species.
- Regardless of the differential effects of the two gonadotropins there is little dispute that gonadotropins stimulate gonadal steroid production and that the production of androgens (e.g., androstenedione, testosterone), which are the precursors for estrogen synthesis occurs in the theca cells (Payne and Hales 2004; Young and McNeilly 2010; Miller 1988; Nagahama et al. 1993).

Empirical Support for Linkage

There is a strong weight of evidence establishing the role of gonadotropins in stimulating gonadal steroidogenesis. This relationship is widely accepted.

Uncertainties or Inconsistencies

No significant inconsistencies identified to date, although comprehensive literature review as not conducted.

Quantitative Understanding of the Linkage

Predictive quantitative relationships between circulating gonadotropin concentrations in fish and testosterone synthesis by ovarian theca cells have not been established.

References

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Reduction, Testosterone synthesis by ovarian theca cells leads to Reduction, 17beta-estradiol synthesis by ovarian granulosa cells (<https://aopwiki.org/relationships/302>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Strong	Weak

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Fundulus heteroclitus	Fundulus heteroclitus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

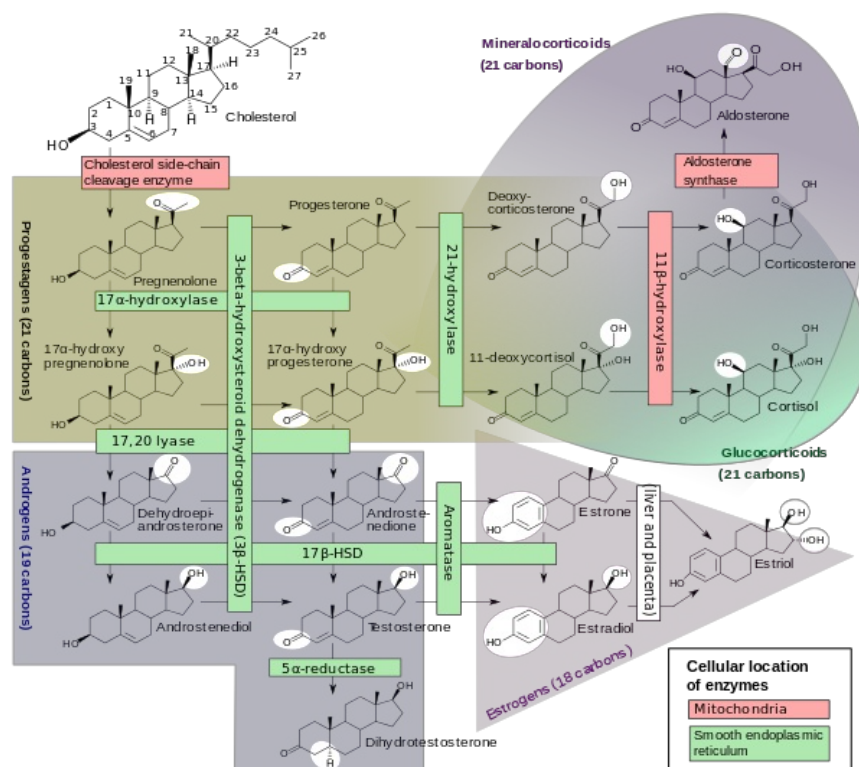
Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Enzymes required for testosterone and 17 β -estradiol synthesis are only found in vertebrates and amphioxus (Markov et al. 2009; Baker 2011). They are not present in invertebrates. Consequently, this KER is not applicable to invertebrates.

How Does This Key Event Relationship Work



(<https://upload.wikimedia.org/wikipedia/commons/thumb/1/13/Steroidogenesis.svg/600px-Steroidogenesis.svg.png>)

Figure 1. Overview of steroid biosynthesis pathway. Note, testosterone is converted to 17 β -estradiol through aromatization catalyzed by cyp19 (aromatase).

Weight of Evidence

Biological Plausibility

Theca cell-derived androgens (e.g., testosterone, androstenedione) are precursors for estrogen (e.g., 17 β -estradiol, estrone) synthesis. Androgens secreted from the theca cells are aromatized to estrogens in the ovarian granulosa cells (Norris 2007; Senthilkumaran et al. 2004). Consequently, reductions in theca cell testosterone synthesis can be expected to reduce the rate of estradiol synthesis by the ovarian granulosa cells (Payne

and Hales 2004; Miller 1988; Nagahama et al. 1993).

Empirical Support for Linkage

- Ex vivo T production by ovary tissue collected from female fathead minnows exposed in vivo to 33 or 472 ng 17 β -trenbolone/L was significantly reduced after 24 or 48 h of exposure (Ekman et al. 2011). Reductions in ex vivo T production preceded significant reductions in ex vivo E2 production.
- Ketoconazole is a fungicide thought to inhibit CYP11A and CYP17 (both involved in theca cell androgen production) with greater potency than it inhibits CYP19 (aromatase) (Villeneuve et al. 2007). Ex vivo E2 and T production were significantly reduced following exposure to 30 or 300 μ g ketoconazole/L (Ankley et al. 2012).

Uncertainties or Inconsistencies

No significant inconsistencies identified to date. However, the literature review on this topic has not been comprehensive.

Quantitative Understanding of the Linkage

At present we are unaware of any well established quantitative relationships between ex vivo T production (as an indirect measure of theca cell T synthesis) and ex vivo E2 production (as an indirect measure of granulosa cell E2 synthesis). 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 that may be adaptable to support a quantitative understanding of this linkage (Breen et al. 2007; Shoemaker et al. 2010; Quignot and Bois 2013).

References

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Reduction, 17beta-estradiol synthesis by ovarian granulosa cells leads to Reduction, Plasma 17beta-estradiol concentrations (<https://aopwiki.org/relationships/5>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female (https://aopwiki.org/aops/7)	directly leads to	Strong	
Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	directly leads to	Strong	Moderate
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Strong	Weak
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	directly leads to	Strong	Moderate

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	directly leads to		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
fathead minnow	Pimephales promelas	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

Key enzymes needed to synthesize 17 β -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 17 β -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.

How Does This Key Event Relationship Work

See plausibility, below.

Weight of Evidence

Updated 03/20/2017.

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.

Empirical Support for Linkage

Include consideration of temporal concordance here

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).
- Ekman et al. (2011) reported significant reductions in ex vivo E2 production and plasma E2 concentrations in female fathead minnows exposed to 0.05 µg/L 17β-trenbolone. The effect on plasma E2 was observed at an earlier time point (24 h, versus 48 h for E2 production).
- Rutherford et al. (2015) reported significant reductions in both E2 production and circulating E2 concentrations in female *Fundulus heteroclitus* exposed to 5α-dihydrotestosterone or 17α-methyltestosterone for 14 d. The effects were equipotent in the case of 17α-methyltestosterone, but in the case of 5α-dihydrotestosterone, the effect on plasma E2 could be detected at a lower dose (10 µg/L) than that at which a significant effect on E2 production was detected (100 µg/L).
- In female *Fundulus heteroclitus* exposed to 17α-methyltestosterone for 7 or 14 d, both E2 production and plasma E2 were impacted at the same exposure concentrations (Sharpe et al. 2004).

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 17α-ethynylestradiol or 17β-trenbolone.

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Reduction, Plasma 17beta-estradiol concentrations leads to Reduction, Vitellogenin synthesis in liver
(<https://aopwiki.org/relationships/252>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	directly leads to	Strong	Moderate
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Strong	Moderate
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	directly leads to	Strong	Moderate
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	directly leads to		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Not Specified

Sex Applicability

Sex	Evidence
Female	Strong

Key enzymes needed to synthesize 17 β -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.

How Does This Key Event Relationship Work

See Plausibility below.

Weight of Evidence

Updated 2017-03-17.

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 responsive 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).

Empirical Support for Linkage

- Empirical support for estrogen-dependent regulation of vitellogenin synthesis:
 - 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 17 β -trenbolone, significant reductions in plasma E2 concentrations preceded significant reductions in plasma VTG (Ekman et al. 2011).
 - Intra-arterial injection of the estrogen 17 α 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).
- Specific empirical support for reductions in plasma E2 leading to reductions in hepatic vitellogenin synthesis:
 - 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 era, 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.
 -

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.

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Reduction, Vitellogenin synthesis in liver leads to Reduction, Plasma vitellogenin concentrations

(<https://aopwiki.org/relationships/315>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	directly leads to	Strong	Moderate
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Strong	Moderate
Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	directly leads to	Strong	Moderate

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	directly leads to	Strong	Strong
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	directly leads to		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

This KER primarily applies to taxa that synthesize vitellogenin in the liver which is transported elsewhere in the body via plasma (i.e., oviparous vertebrates).

How Does This Key Event Relationship Work

See biological plausibility, below.

Weight of Evidence

Updated 03/20/2017.

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).

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Reduction, Plasma vitellogenin concentrations leads to Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development (<https://aopwiki.org/relationships/255>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Moderate	Weak
Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	directly leads to	Moderate	Weak
Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	directly leads to	Moderate	Weak
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	directly leads to	Moderate	
Unknown MIE leading to reproductive dysfunction via increased HIF-1 α transcription (https://aopwiki.org/aops/123)	directly leads to		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Oryzias latipes	Oryzias latipes	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

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.

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 (Wallace and Selman 1981; 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.

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AOP23

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Reduction, Vitellogenin accumulation into oocytes and oocyte growth/development leads to Reduction, Cumulative fecundity and spawning (<https://aopwiki.org/relationships/337>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	directly leads to	Moderate	Moderate
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Moderate	Moderate
Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	directly leads to	Moderate	Moderate
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	directly leads to		
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	directly leads to		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	<i>Pimephales promelas</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
<i>Oryzias latipes</i>	<i>Oryzias latipes</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

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.

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.
- Ankley et al. (2003) reported significant reductions in VTG accumulation in oocytes along with significant reductions in cumulative fecundity, although fecundity was significantly impacted at a lower dose (0.05 ug/L 17beta-trenbolone versus 0.5 ug/L for VTG accumulation).
- Kang et al. (2008) reported significant reductions in both VTG accumulation in oocytes and cumulative fecundity in Japanese medaka, with cumulative fecundity being impacted at slightly lower concentrations (0.047 ug 17alpha-methyltestosterone/L versus 0.088 ug/L).
-

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.

References

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Reduction, Cumulative fecundity and spawning leads to Decrease, Population trajectory
(<https://aopwiki.org/relationships/94>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	directly leads to	Moderate	Moderate
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	directly leads to	Moderate	Moderate
Estrogen receptor antagonism leading to reproductive dysfunction (https://aopwiki.org/aops/30)	directly leads to	Moderate	Moderate
Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation (https://aopwiki.org/aops/122)	directly leads to		
Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription (https://aopwiki.org/aops/123)	directly leads to		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

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.

How Does This Key Event Relationship Work

SEE BIOLOGICAL PLAUSIBILITY BELOW

Weight of Evidence

Updated 03/20/2017

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).

References

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Agonism, Androgen receptor leads to Reduction, Testosterone synthesis by ovarian theca cells
(<https://aopwiki.org/relationships/32>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	indirectly leads to	Moderate	Weak

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	<i>Pimephales promelas</i>	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Fundulus heteroclitus	<i>Fundulus heteroclitus</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

This KER is potentially relevant to sexually mature female vertebrates and amphioxus. It is not relevant to invertebrates.

- Androgen receptor orthologs are primarily limited to vertebrates (Baker 1997; Thornton 2001; Eick and Thornton 2011; Markov and Laudet 2011).
- Cytochrome P45011a (Cyp11a), a rate limiting enzyme for the production of testosterone, is specific to vertebrates and amphioxus (Markov et al. 2009; Baker et al. 2011; Payne and Hales, 2004).
- Cyp11a does not occur in invertebrates, as a result, they do not synthesize testosterone, nor other steroid intermediates required for testosterone synthesis (Markov et al. 2009; Payne and Hales, 2004).

How Does This Key Event Relationship Work

At present, a direct structural/functional linkage between androgen receptor agonism and reduced testosterone production by ovarian theca cells is not known. This linkage is thought to operate indirectly through endocrine feedback along the hypothalamic-pituitary-gonadal axis. Consequently, the relationship is supported primarily via association/correlation.

Weight of Evidence

Biological Plausibility

Synthesis of the steroidogenic enzymes that catalyze the formation of testosterone from cholesterol as a precursor is stimulated by gonadotropins, particularly luteinizing hormone, whose synthesis and secretion are in turn regulated by gonadotropin releasing hormone (GnRH) released from the hypothalamus (Payne and Hales 2004; Norris 2007; Miller 1988). Negative feedback of circulating androgens (e.g., testosterone) on GnRH release from the hypothalamus and/or gonadotropin release from the pituitary is a well established physiological phenomenon in vertebrate endocrinology (Norris 2007). While similar processes of negative feedback of sex steroids on gonadotropin expression and release have been established in fish (Levavi-Sivan et al. 2010), there are many remaining uncertainties about the exact mechanisms through which feedback takes place in fish as well as other vertebrates. For example, feedback is thought to involve a complex interplay of neurotransmitter signaling, kisspeptins, and the follistatin/inhibin/activin system (Trudeau et al. 2000; Trudeau 1997; Oakley et al. 2009; Cheng et al. 2007). In addition, the nature of the feedback produced by androgens is dependent on the concentration, form of the androgen (e.g., aromatizable versus non-

aromatizable), life-stage and likely species (Habibi and Huggard 1998; Trudeau et al. 2000; Gopurappilly et al. 2013). At present, such negative feedback responses in vivo provide a biologically plausible connection between androgen receptor agonism and reduced testosterone production in ovarian theca cells, but uncertainty regarding the details of the underlying biology and the relevant applicability domain remain.

Empirical Support for Linkage

Direct evidence based on measurements of T production following exposure to established AR agonists:

- Ekman et al. (2011) reported significant reductions in ex vivo T production by ovary tissue following 24 or 48 h of in vivo exposure to the synthetic, non-aromatizable, AR agonist 17 β -trenbolone.
- Glinka et al. (2015) reported significant reductions in T production by ovary tissue collected from *Fundulus heteroclitus* that had been exposed to the model (non-aromatizable) androgen 5 α -dihydrotestosterone.
- Sharpe et al. (2004) reported that testosterone biosynthetic capacity was inhibited in female *Fundulus heteroclitus* following 7 d of in vivo exposure to 0.25 μ g methyl testosterone/L or 14 d of exposure to 0.1 μ g methyl testosterone/L. Note - methyl testosterone is an aromatizable androgen.

Indirect evidence based on measurements of circulating T following exposure to established AR agonists:

- Ankley et al. (2003) showed that 17 β -trenbolone binds the *Pimephales promelas* androgen receptor, induced tubercle formation (an androgen regulated male secondary sex characteristic in females), and reduced circulating concentrations of T and 11-ketotestosterone following 21 d of continuous exposure.
- Jensen et al. (2006) reported tubercle formation in females and reduced circulating concentrations of T in female *Pimephales promelas* exposed to 17 α -trenbolone for 21 d.
- LaLone et al. (2013) reported tubercle formation in female *Pimephales promelas* and papillary complexes on the anal fin (a male secondary sex characteristic) of female *Oryzias latipes* following 21 d of exposure to spironolactone, along with significant reductions in plasma T measured in *Pimephales promelas* (that endpoint was not measured in medaka due to limited plasma volumes).

Uncertainties or Inconsistencies

See biological plausibility section above regarding current uncertainties in the mechanisms through which AR agonists may reduce gonadotropin secretion.

- Rutherford et al. (2015) reported an increase in plasma T concentrations in female and no change in gonadal T production in *Fundulus heteroclitus* following 14 d of exposure to 100 μ g/L 5 α -dihydrotestosterone.

Quantitative Understanding of the Linkage

- Li et al. (2011) describe a computational model of the female fathead minnow (*Pimephales promelas*) hypothalamic-pituitary-gonadal axis that can be used to simulate impacts on plasma T, plasma E2, and plasma vitellogenin concentrations following exposure to 17 β -trenbolone. However, to date, that model has not been robustly tested to determine applicability to other species, or other types of AR agonists.
- At present, the scope of data for associating AR-activation potency with decreased T production is not sufficient to describe a quantitative response-response relationship.

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Agonism, Androgen receptor leads to Reduction, 17beta-estradiol synthesis by ovarian granulosa cells
(<https://aopwiki.org/relationships/1384>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	indirectly leads to	Moderate	Weak

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	<i>Pimephales promelas</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
<i>Fundulus heteroclitus</i>	<i>Fundulus heteroclitus</i>	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Female	Strong

This KER is potentially applicable to sexually mature, female, vertebrates.

- Androgen receptor orthologs are primarily limited to vertebrates (Baker 1997; Thornton 2001; Eick and Thornton 2011; Markov and Laudet 2011).
- Key enzymes needed to synthesize 17 β -estradiol first appear in the common ancestor of amphioxus and vertebrates (Markov et al. 2009; Baker 2011).

How Does This Key Event Relationship Work

At present, a direct structural/functional link between androgen receptor agonism and reduced estradiol synthesis by ovarian granulosa cells is not known. The linkage is thought to operate indirectly via endocrine feedback along the hypothalamic-pituitary-gonadal axis and subsequent effects on the regulation of enzymes involved in ovarian steroidogenesis. This relationship is primarily supported by association/correlation.

Weight of Evidence

Updated 2017-03-17.

Biological Plausibility

Synthesis of the steroidogenic enzymes that catalyze the formation of testosterone from cholesterol as a precursor as well as 17 β -estradiol (E2) from testosterone is stimulated by gonadotropins whose synthesis and secretion are in turn regulated by gonadotropin releasing hormone (GnRH) released from the hypothalamus (Payne and Hales 2004; Norris 2007; Miller 1988). Strong AR agonists are thought to exert negative feedback along the hypothalamic-pituitary-gonadal axis, leading to decreased stimulation of the steroidogenic pathway and subsequent declines in E2 production.

Empirical Support for Linkage

Direct support for the effect of AR agonists on estrogen production by ovary tissue:

- Ekman et al. (2011) reported reductions in ex vivo E2 production by ovary tissue collected from fathead minnows exposed to 17 β -trenbolone in vivo. However, among four exposure durations assessed (1, 2, 4, 8 d), E2 production was only reduced following 2 d of exposure.
- Glinka et al. (2015) reported reductions in E2 production in female *Fundulus heteroclitus* following 21 d of exposure to 0.5 or 5.0 ug 5 α -dihydrotestosterone.
- Rutherford et al. (2015) demonstrate reduced E2 production in *Fundulus heteroclitus* following exposure to 100 ug 5 α -dihydrotestosterone (a non-aromatizable androgen) or 0.1 and 1.0 ug methyltestosterone (an aromatizable androgen) for 14 d.
- Sharpe et al. (2004) reported reductions in E2 production in female *Fundulus heteroclitus* following exposure to 0.25 or 1.0 ug methyltestosterone/L for 7d, and in females exposed to 0.001, 0.01, or 0.1 ug methyltestosterone/L for 14 d.

Uncertainties or Inconsistencies

The work of Ekman et al. (2011) demonstrates the effects can be transient due to complex compensatory behaviors.

Quantitative Understanding of the Linkage

- At present, the scope of data for associating AR-activation potency with decreased E2 production is not sufficient to describe a quantitative response-response relationship.

References

- Baker ME. 1997. Steroid receptor phylogeny and vertebrate origins. *Molecular and cellular endocrinology* 135(2): 101-107.
- Baker ME. 2011. Origin and diversification of steroids: Co-evolution of enzymes and nuclear receptors. *Mol Cell Endocrinol* 334: 14-20.
- Eick GN, Thornton JW. 2011. Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. *Molecular and cellular endocrinology* 334(1-2): 31-38.
- Ekman DR, Villeneuve DL, Teng Q, Ralston-Hooper KJ, Martinović-Weigelt D, Kahl MD, Jensen KM, Durhan EJ, Makynen EA, Ankley GT, Collette TW. Use of gene expression, biochemical and metabolite profiles to enhance exposure and effects assessment of the model androgen 17 β -trenbolone in fish. *Environ Toxicol Chem*. 2011 Feb;30(2):319-29. doi: 10.1002/etc.406.
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Agonism, Androgen receptor leads to Reduction, Vitellogenin synthesis in liver (<https://aopwiki.org/relationships/1385>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	indirectly leads to	Strong	Weak

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Danio rerio	Danio rerio	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
medaka	Oryzias latipes	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8090)
Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

This KER is potentially applicable to reproductively mature, adult, oviparous vertebrates.

- Androgen receptor orthologs are primarily limited to vertebrates (Baker 1997; Thornton 2001; Eick and Thornton 2011; Markov and Laudet 2011).
- 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.

How Does This Key Event Relationship Work

At present, a direct structural/functional linkage between androgen receptor agonism and reduced plasma vitellogenin concentrations is not known. Consequently, the relationship is supported primarily via association/correlation.

Weight of Evidence

Updated 2017-03-17.

Biological Plausibility

Synthesis of the steroidogenic enzymes that catalyze the formation of testosterone from cholesterol as a precursor as well as 17 β -estradiol (E2) from testosterone is stimulated by gonadotropins whose synthesis and secretion are in turn regulated by gonadotropin releasing hormone (GnRH) released from the hypothalamus (Payne and Hales 2004; Norris 2007; Miller 1988). Strong AR agonists are thought to exert negative feedback along the hypothalamic-pituitary-gonadal axis, leading to decreased stimulation of the steroidogenic pathway and subsequent declines in E2 production. E2 is known to be a major regulator of hepatic vitellogenin production (Tyler et al. 1996; Tyler and Sumpter 1996; Arukwe and Goksøyr 2003).

Empirical Support for Linkage

Direct support for the effect of AR agonists on plasma vitellogenin (VTG) concentrations:

- Ekman et al. (2011) reported significant reductions in plasma VTG in female fathead minnows exposed to 0.5 ng 17 β -trenbolone/L for 4 or 8 d, or to 0.05 ng/L for 8 d.
- Ankley et al. (2010) showed significant reductions in plasma VTG in four independent experiments in which female fathead minnows were exposed to 17 β -trenbolone for 14 d and a fifth experiment where they were exposed for 21 d.
- Seki et al. (2006) showed significant reductions in plasma VTG in three different species of female fish (Oryzias latipes, Danio rerio, Pimephales promelas) following 21 d of exposure to 17 β -trenbolone.
- Villeneuve et al. (2016) reported significant reductions in plasma VTG following 22 d of exposure to 0.5 ng 17 β -trenbolone/L.
- Jensen et al. (2006) observed significant reductions in plasma VTG in female fathead minnows following exposure to 17 α -trenbolone for 21 d.
- LaLone et al. (2013) reported significant reductions in plasma VTG in female fathead minnows exposed to 5 ug/L or higher concentrations of spironolactone.
- Rutherford et al. (2015) reported significant reductions in plasma VTG in female Fundulus heteroclitus after 14 d of exposure to 100 ug 5 α -dihydrotestosterone/L as well as after exposure to 1 ug methyltestosterone/L.

- Sharpe et al. (2004), detected significant reductions in plasma VTG in female *Fundulus heteroclitus* exposed to 0.25 ug/L methyltestosterone for 7 d or 0.01 ug/L for 14 d.

Uncertainties or Inconsistencies

None noted.

Quantitative Understanding of the Linkage

- Li et al. (2011) describe a computational model of the female fathead minnow (*Pimephales promelas*) hypothalamic-pituitary-gonadal axis that can be used to simulate impacts on plasma T, plasma E2, and plasma vitellogenin concentrations following exposure to 17 β -trenbolone. However, to date, that model has not been robustly tested to determine applicability to other species, or other types of AR agonists.
- At present, the scope of data for associating AR-activation potency with decreased plasma VTG is not sufficient to describe a quantitative response-response relationship.

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Reduction, Plasma 17beta-estradiol concentrations leads to Reduction, Plasma vitellogenin concentrations (<https://aopwiki.org/relationships/1386>)

AOPs Referencing Relationship

AOP Name	Directness	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to reproductive dysfunction (https://aopwiki.org/aops/23)	indirectly leads to	Strong	Moderate
Aromatase inhibition leading to reproductive dysfunction (https://aopwiki.org/aops/25)	indirectly leads to	Strong	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)
Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8078)

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Strong

Sex Applicability

Sex	Evidence
Female	Strong

This key event relationship likely applies to oviparous vertebrates only.

- Key enzymes needed to synthesize 17 β -estradiol first appear in the common ancestor of amphioxus and vertebrates (Baker 2011).
- Vitellogenesis is common to a range of egg-laying vertebrates and invertebrates. However, in the case of invertebrates, vitellogenins are transported via hemolymph rather than plasma and vitellogenesis is regulated by invertebrate hormones, not estradiol.

How Does This Key Event Relationship Work

There is not a direct structural/functional relationship between reduced concentrations of 17 β -estradiol in plasma and reduced plasma VTG concentrations. The relationship is thought to be mediated through additional events of hepatic estrogen receptor activation, vitellogenin protein synthesis in the liver, and subsequent secretion of vitellogenin into the plasma.

Weight of Evidence

Updated 2017-03-17

Biological Plausibility

The mechanisms through which 17 β -estradiol stimulates the transcription and translation of hepatic vitellogenin are well understood.

- In fish, see: Tyler et al. 1996; Tyler and Sumpter 1996; Arukwe and Goksøyr 2003; Teo et al. 1998
- In frogs: Chang et al. 1992; Wang and Knowland 1975
- In reptiles: Ho et al. 1980
- Ho (1987)
- In birds: Deeley et al. 1975;

17 β -estradiol is not synthesized in significant amounts in the liver. Its synthesis originates in other tissues, principally the gonads. It is then transported to the liver and other tissues via circulation (Norris 2007; Payne and Hales 2004; Miller 1988; Nagahama et al. 1993).

Empirical Support for Linkage

- Under conditions of continuous flow through exposure to 17 β -trenbolone (a non-aromatizable androgen receptor agonist), plasma E2 concentrations were reduced in female fathead minnows after 2, 4, or 8 d of exposure to concentrations of 0.05 ug/L or greater. Plasma VTG concentrations were significantly reduced only after 4 or 8 d of exposure, and at 4 d, only at a concentration of 0.5 ug/L, not 0.05 ug/L (Ekman et al. 2011).
- In the same study by Ekman et al. (2011), once exposure ceased, plasma E2 concentrations returned to control levels within 48 h, while plasma VTG concentrations remained significantly depressed until d. 4, post-exposure.
- Ankley et al. (2003) detected reductions in both plasma E2 and plasma VTG in female fathead minnows following 21 d of continuous exposure to 17 β -trenbolone. At 21 d, plasma E2 concentrations were impacted at concentrations of 0.5 ug/L or greater, while plasma VTG was significantly reduced at 0.05 ug/L or greater.

- Villeneuve et al. (2016) observed significant reductions in both plasma E2 and plasma VTG in female fathead minnows exposed to 0.5 ug/L 17 β -trenbolone for 14 d.
- Jensen et al. (2006) observed significant reductions in both plasma E2 and plasma VTG following exposure to 0.03 ug/L 17alpha-trenbolone for 21 d.
- Following 21 d of continuous exposure to spironolactone, plasma E2 and plasma VTG were both significantly reduced in female fathead minnows. The lowest effect concentration for plasma E2 was 0.5 ug/L, while that for plasma VTG was 5 ug/L (LaLone et al. 2013).
- In female *Fundulus heteroclitus* exposed to 5alpha-dihydrotestosterone for 14 d, plasma E2 was significantly reduced following exposure to 10 ug/L, while plasma VTG was reduced at 100 ug/L (Rutherford et al. 2015).
- In two experiments in which female *Fundulus heteroclitus* were exposed to 17alpha-methyltestosterone, both plasma E2 and plasma VTG were significantly reduced. In both cases, plasma E2 was impacted at lower concentrations (0.25 ug/L in a 7 d study; 0.01 ug/L in a 14 d study) than plasma VTG (1 ug/L in the 7 d study; 0.1 ug/L in the 14 d study; Sharpe et al. 2004).
- In two experiments where plasma E2 and plasma VTG were measured in female fathead minnows (*Pimephales promelas*) in a time-course following continuous exposure the aromatase inhibitor fadrozole, both plasma VTG and plasma E2 were depressed (Villeneuve et al. 2009; 2013). In both cases, following cessation of exposure, plasma E2 concentrations recovered to control levels before plasma VTG concentrations recovered (Villeneuve et al. 2009; 2013).
- Schroeder et al. (in preparation) reported effects on plasma E2 concentrations within 4 h of initiating exposure to 5 or 50 ug/L fadrozole. Plasma VTG concentrations did not decline until 24 h or later (Schroeder et al. 2009; Villeneuve et al. 2009; 2013).
- In female fathead minnows exposed to 300 ug/L prochloraz, plasma E2 concentrations were significantly reduced after 12 h of exposure, while plasma VTG concentrations were not significantly reduced until 24 h of exposure (Skolness et al. 2011).
- Ankley et al. (2009) reported significant reductions in plasma E2 in female fathead minnows following 24 h of exposure to 30 ug/L prochloraz. In the same study, plasma VTG concentrations did not significantly decline until 48 h of exposure, and then only at 300 ug/L prochloraz.
- In a 21 d exposure to prochloraz, plasma E2 was significantly reduced in females exposed to 300 ug prochloraz/L, while plasma VTG was significantly reduced in females exposed to 100 ug/L (Ankley et al. 2005).

Uncertainties or Inconsistencies

- In several studies, significant decreases in plasma vitellogenin are detected at lower concentrations than those that result in significant decreases in plasma E2. However, detection of differences in plasma VTG is often enhanced by the greater dynamic range in the concentrations of the protein that occur in plasma, compared to the dynamic range of steroid hormone concentrations.

Quantitative Understanding of the Linkage

- A computational model developed by Cheng et al. (2016) is capable of simulating altered plasma VTG concentrations associated with changes in plasma E2 concentrations in female fathead minnows. This model has been used to generate a quantitative response-response relationship that can predict steady state plasma VTG concentrations for a given steady state plasma E2 concentration (Conolly et al. 2017).
 - The model and response-response relationship were developed based on data from exposures to the model aromatase inhibitor fadrozole. The validity of the model-based predictions/relationships for other stressors and species has not yet been established.
- Li et al. (2011) also developed a physiologically-based computational model of the adult female fathead minnow (*Pimephales promelas*) hypothalamic-pituitary-gonadal axis. Conceptually, this model could also be applied to derive a quantitative response-response relationship between plasma E2 and plasma VTG concentrations. The Li et al. model was calibrated based on data from exposures to 17alpha-ethynylestradiol and 17 β -trenbolone. Neither its validity for other stressors or species, nor its agreement with the Cheng et al. (2016) model have been examined in detail.

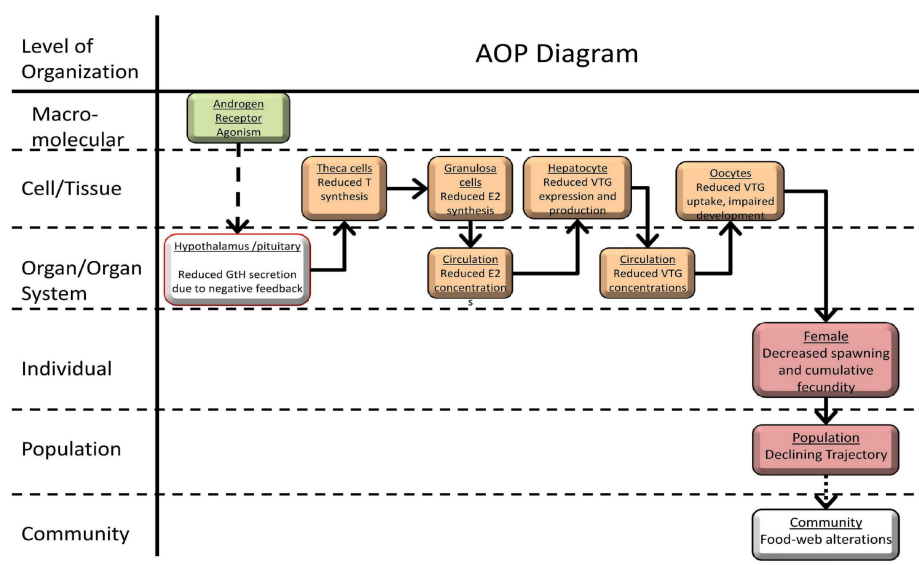
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Graphical Representation



Overall Assessment of the AOP

Annex 1 Table, Assessment of the relative level of confidence in the overall AOP based on rank ordered weight of evidence elements is attached in PDF format.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Pimephales promelas	Pimephales promelas		NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=90988)

Sex Applicability

Sex	Evidence
Female	

Domain(s) of Applicability

Chemical: This AOP applies to non-aromatizable androgens. Compounds which can bind the AR in vitro, but are converted to high potency estrogens in vivo through aromatization do not produce the profile of effects described in the present AOP (e.g., methyltestosterone [Ankley et al. 2001; Pawlowski et al. 2004]; androstenedione [OECD 2007]).

Sex: The AOP applies to females only.

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, photoperiod, etc. in a laboratory setting).

Taxonomic: At present, the assumed taxonomic applicability domain of this AOP is iteroparous teleost fish species.

- However, to date the majority of toxicological data on which this AOP is based has been limited to several small fish species, fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and mummichog (*Fundulus heteroclitus*) with asynchronous oocyte development and a repeat spawning reproductive strategy.
- Species dependent differences in endocrine feedback responses, likely associated with different reproductive strategies, have been reported. Thus, the applicability domain may prove more restricted than currently assumed. In particular, the applicability to fish species with synchronous or group synchronous oocyte development patterns (see Wallace and Selman 1981) is unclear.
- European eel may be an exception to the generalizability of the negative feedback response to a non-aromatizable xenoandrogen (Huang et al. 1997).
- Reductions in plasma VTG concentrations and/or hepatic VTG mRNA abundance in females following exposure to 17 β -trenbolone has been observed in *Pimephales promelas*, *Oryzias latipes*, *Danio rerio*, (Seki et al. 2006), *Cyprinodon variegatus* (Hemmer et al. 2008), *Gambusia holbrooki* and *Gambusia affinis* (Brockmeier et al. 2013)

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

Essentiality of the Key Events

- In general, few studies have directly addressed the essentiality of the proposed sequence of key events.
- Ekman et al. 2011 provide evidence that in fathead minnow, cessation of trenbolone exposure resulted in recovery of plasma E2 and VTG concentrations which were depressed by continuous exposure to 17 β trenbolone. This provides some support for the essentiality of these two key events.
- Essentiality of the proposed negative feedback key event is supported by experimental work that evaluated the ability of AR agonists to reduce T or E2 production in vitro. In vitro exposure of fathead minnow ovary tissue to 17 β -trenbolone or spironolactone does not cause reductions in T or E2 synthesis at concentrations comparable to those that produce significant responses in vivo (i.e., at non-cytotoxic concentrations; D.L. Villeneuve, unpublished data; C.A. LaLone unpublished data), nor are there any known reports of 17 β -trenbolone directly inhibiting steroid biosynthesis. When tested in an in vitro steroidogenesis assay using H295R adrenal carcinoma cells, trenbolone caused a concentration-dependent increase in estradiol production, as opposed to any reductions in steroid hormone concentrations, an effect that was concurrent with increased transcription of CYP19 (aromatase) in the cell line (Gracia et al. 2007).

Weight of Evidence Summary**Biological Plausibility**

- The biochemistry of steroidogenesis and the predominant role of the gonad in synthesis of the sex steroids are 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.
- 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). 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).
- At present, negative feedback is the most biologically plausible explanation for the reductions in ex vivo T and E2 production following exposure to 17 β -trenbolone. In vitro exposure of fathead minnow ovary tissue to 17 β -trenbolone or spironolactone does not cause reductions in T or E2 synthesis at concentrations comparable to those that produce significant responses in vivo (i.e., at non-cytotoxic concentrations; D.L. Villeneuve, unpublished data; C.A. LaLone unpublished data), nor are there any known reports of 17 β -trenbolone directly inhibiting steroid biosynthesis. When tested in an in vitro steroidogenesis assay using H295R adrenal carcinoma cells, trenbolone caused a concentration-dependent increase in estradiol production, as opposed to any reductions in steroid hormone concentrations, an effect that was concurrent with increased transcription of CYP19 (aromatase) in the cell line (Gracia et al. 2007). Given the lack of any established direct effect on steroidogenic enzyme activity, negative feedback is currently the most likely explanation for the consistent effects observed in vivo. That said, many uncertainties regarding the exact mechanisms through which an exogenous, non-aromatizable, AR agonist elicits negative feedback remain.

Concordance of dose-response relationships: See Concordance Table (<https://aopwiki.org/aops/23/pictures>) (available in Excel and PDF format)

There are a limited number of studies in which multiple key events were considered in the same study following exposure to known, non-aromatizable, AR agonists. 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. For exposures to 17 β -trenbolone, key events related to steroid production and circulating estradiol and vitellogenin concentrations were impacted at the same dose at which effects on cumulative fecundity were observed. Effects on vitellogenin transcription were only observed at greater concentrations, but data for comparable species and dose ranges were unavailable at present. For two other AR agonists tested in fish, available studies examined a single time-point only. Consequently, it was unclear whether lower effect concentrations for certain downstream KEs, relative to upstream were due to a lack of dose-response concordance, or due to decreased sensitivity of the upstream later in the exposure time-course.

While not directly addressing dose-response concordance, the dependence of the key events on the concentration of the androgen agonist has been established for all key events starting at and down-stream of reduced T synthesis. However, to date we are not aware of any studies that have established a concentration-response relationship between exposure to non-endogenous AR agonists (e.g., xenobiotics, pharmaceuticals) and circulating gonadotropin concentrations in fish or other vertebrates.

- Exposure of female fathead minnows to the AR agonist 17 β -trenbolone for 21 d caused concentration-dependent reductions in circulating T, E2, and VTG concentrations over a range from 0.005 to 0.5 μ g/L. The concentration response for all three variables had a "U"-shaped concentration response curve which may indicate concentration-dependent differences in the feedback response and/or compensatory processes. Histological evidence of reduced VTG uptake and reduced gonad stage were evident, although the concentration-response of histological effects was not determined. Despite the "U"-shaped concentration-response at the biochemical level, concentration-dependent reductions in cumulative fecundity were observed (Ankley et al. 2003). Effective concentrations were consistent with those causing phenotypic masculinization in female fish.
- Jensen et al. (2006) also demonstrated concentration-dependent reductions in circulating T, E2, and VTG following 21 d of in vivo exposure to 17 α -trenbolone (Jensen et al. 2006).
- In a time-course experiment in which female fathead minnows were exposed to 33 or 472 ng 17 β -trenbolone/L ex vivo T, ex vivo E2, plasma E2, and plasma VTG all showed concentration-dependent reductions that were consistent with the AOP (Ekman et al. 2011).
- Exposure of female fathead minnows to spironolactone, a pharmaceutical that binds the fathead minnow AR, for 21 d caused concentration-dependent reductions in cumulative fecundity, plasma VTG and VTG mRNA expression, and plasma E2 concentrations. The frequency and severity of females with decreased yolk accumulation, and increased oocyte atresia was concentration-dependent. The chemical also induced phenotypic masculinization in female fish. (Lalone et al. 2013).
- Exposure of female medaka to spironolactone caused concentration-dependent reductions in cumulative fecundity and VTG mRNA expression (impacts on steroid hormone concentrations were not measured). Spironolactone also caused phenotypic masculinization of female medaka (Lalone et al. 2013).

Temporal concordance among the key events and adverse effect: Temporal concordance between activation of the AR as a nuclear transcription factor and onset of a negative feedback response resulting in decreased gonadotropin secretion has not been established. Temporal concordance of the key events starting with reduced T biosynthesis and proceeding through reductions in plasma vitellogenin has been established (Concordance Table (<https://aopwiki.org/aops/23/pictures>)). Temporal concordance beyond the key event of reductions in plasma vitellogenin has not been established, 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 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 based on currently available data, 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. Due to variability in the cumulative fecundity endpoint and potential compensatory responses ((Villeneuve et al. 2009; Villeneuve et al. 2013; Ankley et al. 2009b; Zhang et al. 2008; Ekman et al. 2012), the cumulative fecundity endpoint can be less sensitive than

key events measured at lower levels of biological organization. Nonetheless, the occurrence of the final adverse outcome when the other key events are observed is very consistent. The final adverse effect 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.

- In general, there is a consistent body of evidence linking exposure to an AR agonist to decreased T synthesis, E2 synthesis, circulating E2 and VTG concentrations, and cumulative fecundity in female fish. For example, the association between 17 β -trenbolone exposure and reduced vitellogenin concentrations in females has been replicated in over a dozen independent experiments (Ekman et al. 2011; Ankley et al. 2003; Jensen et al. 2006; Ankley et al. 2010; Hemmer et al. 2008; Seki et al. 2006; Brockmeier et al. 2013). However, relatively few exogenous, non-aromatizable, AR agonists have been tested. Other than recent work with spironolactone (Lalone et al. 2013), we are not aware of the profile of responses being demonstrated for other AR agonists.

Uncertainties, inconsistencies, and data gaps: There are three major areas of uncertainty and data gaps in the current AOP:

- First, there remains considerable uncertainty as to the specific mechanism(s) through which AR agonism elicits a negative feedback response at the level of the hypothalamus and/or pituitary. There is also a substantial data gap relative to establishing that exposure to an AR agonist like 17 β -trenbolone causes concentration-dependent reductions in circulating gonadotropins. That uncertainty is amplified further by the variation in feedback control along the endocrine axis for fish species employing different reproductive strategies. For example, gonadotropin regulation may be very different in species with synchronous oocyte maturation and annual or once per life-time reproductive strategies. Thus, there are considerable uncertainties related to the taxonomic relevance of this AOP to a broader range of fish species or other vertebrates.
- The second major uncertainty in this AOP relates to 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.
- A third uncertainty pertains to the chemical domain of applicability. In vivo, a number of chemicals that are detected as androgens in in vitro screening assays such as receptor binding assays or ligand-activated transcriptional assay can be aromatized to functional estrogens. Thus, in vivo such compounds may produce a profile of effects more consistent with estrogen receptor activation than AR activation or may produced mixed effects characteristic of either estrogen or androgen exposures (e.g., Pawlowski et al. 2004; Hornung et al. 2004). Examples of such aromatizable androgens include, testosterone, methyltestosterone, and androstenedione. Consequently, caution is warranted in applying this AOP based on in vitro screening data alone, without consideration for possible conversion to estrogens.

Quantitative Consideration

Assessment of quantitative understanding of the AOP: At present, the quantitative understanding of the AOP is insufficient to directly link a measure of chemical potency as an AR agonist (e.g., as measured in a transcriptional activation assay) to a predicted effect concentration at the level of cumulative fecundity. However, a number of mechanistic and statistical models are sufficiently developed to facilitate predictions of cumulative outcomes based on intermediate key event measurements such as circulating vitellogenin concentrations. Because the current models were developed based on a fairly limited range of model compounds and species, the general applicability and degree of accuracy and precision in the model-derived predictions remains uncertain.

Considerations for Potential Applications of the AOP (optional)

optional

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