Adverse Outcome Pathway on Aromatase inhibition leading to male-biased sex ratio via impacts on gonad differentiation

Series on Adverse Outcome Pathways No. 34

AOP No. 346 in the AOP-Wiki platform

This AOP shares several Key Events and Key Events Relationships with AOP 35 in the Series on Adverse Outcome Pathways (AOP 376 in the AOP-Wiki), linking activation of the androgen receptor to male biased sex ratios.

Foreword

This Adverse Outcome Pathway (AOP) on Aromatase inhibition leading to male-biased sex ratio via impacts on gonad differentiation, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Advisory Group on Emerging Science in Chemicals Assessment (ESCA), which is an advisory group under the Working Party of the National Coordinators for the Test Guidelines Programme (WNT) and the Working Party on Hazard Assessment (WPHA).

The scientific review was conducted by the scientific journal Environmental Toxicology and Chemistry (ET&C), following the OECD AOP review principles outlined in the Guidance Document on the scientific review of AOPs. This AOP was endorsed by the WNT and the WPHA on 13 October 2023.

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

The OECD's Chemicals and Biotechnology Committee agreed to declassification of this AOP on 30 November 2023.

This document is being published under the responsibility of the OECD's Chemicals and Biotechnology Committee.

The outcome of the scientific review is publicly available in the AOP-Wiki at the following links: [discussion page]. The related AOP Report has been accepted by ET&C and is available on the website of the Society of Environmental Toxicology and Chemistry (SETAC) - [Link].

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Abstract

This adverse outcome pathway links inhibition of aromatase activity in teleost fish during gonadogenesis to increased differentiation to testis resulting in a male-biased sex ratio in the population, and ultimately, reduced population sustainability. Most gonochoristic fish species develop either as males or females and do not change sex throughout their life span. However, in species where sexual differentiation is controlled at least to some degree by environmental factors, there can be a window of development during gonadal differentiation that is sensitive to a variety of exogenous conditions, including exposure to some chemicals. For example, treatment with sex steroids in conjunction with the period of sexual differentiation has been showed to favor ovary or testis development in fish exposed to estrogens or androgens, respectively. Altered synthesis and regulation of endogenous steroids can also affect sexual differentiation in fish. In most vertebrate taxa, aromatase (cytochrome P450 [CYP]19a1) is the rate-limiting enzyme for the conversion of 17β-estradiol (E2) from testosterone (T). Endocrine-active chemicals such as fadrozole, letrozole and exemestane (pharmaceuticals) or prochloraz and propiconazole (fungicides) inhibit aromatase activity. Exposure of some fish species to aromatase inhibitors during sex differentiation can reduce endogenous E2 synthesis, thereby resulting in phenotypic males, the default sex in the absence of estrogen signaling during gonadal differentiation. Given the critical role of female fecundity in determining total numbers of offspring, the resultant male-biased sex ratio can reduce population size, especially if sustained over multiple generations.

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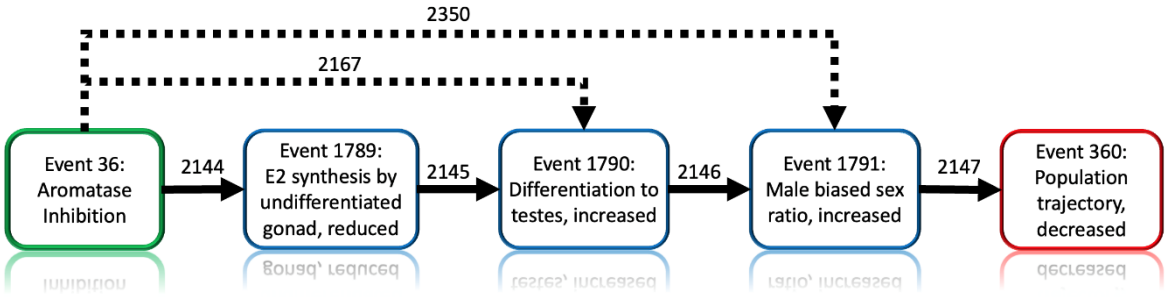
Background

In fish sexual differentiation occurs post hatch and can be influenced by exogenous factors such as chemicals, temperature, pH, population density, social cues and more. As a result, the gonadal sex phenotype in oviparous fish can be altered by environmental conditions experienced during development, particularly in conjunction with sexual differentiation (Scholz and Klüver, 2009). At this stage, the bipotential gonad can differentiate to either testes or ovaries depending both on genetic and environmental factors (Strüssmann and Nakamura, 2002). Sex steroids are among the factors that influence sex differentiation in non-mammalian vertebrates; in many fish species exogenous androgens and estrogens act, respectively, to enhance the development of testes and ovaries in exposed animals (Nakamura 2010). In teleost fish, the relative balance between endogenous estrogens and androgens during sexual differentiation is critical to ensuring normal sex ratios and, ultimately, viable populations. Various homeostatic mechanisms ensure that steroid biosynthesis is appropriately controlled during development. A key biosynthetic enzyme is CYP19a1 (aromatase), which is responsible for the conversion of C19 androgens (e.g., T) to C18 estrogens (e.g., E2) in brain and gonadal tissues of vertebrates (Payne and Hales, 2004; Simpson et al. 1994). In fish, there are two CYP19a1 isoforms, with CYP19a1a mostly expressed in the gonads and CYP19a1b largely expressed in the brain (Callard et al. 2001).

Since the mid-90s, there has been concern about the potential impacts of endocrine disrupting chemicals (EDCs) in fish and wildlife. Many EDCs can exert effects in early life stages that can lead to potential impacts at the population level. For example, some chemicals have been shown to alter the sexual phenotype of fish by affecting steroidogenic enzymes such as aromatase. Inhibition of CYP19a1 expression or activity can alter the production of estrogens in developing gonads, affecting processes such as gonadal differentiation. In many fish species the "default" gonad type is testes, so when estrogen signaling is reduced there is a resultant bias toward male-biased sex ratios (Guiguen et al. 2010). When male biased sex ratios occur, the number of breeding females can decrease over time and have negative impacts on population growth and sustainability. The present AOP provides the evidence framework of the negative impacts of aromatase inhibition at early developmental stages of teleost fish during the critical period of sexual differentiation and how this could lead to population-level effects.

Graphical representation



Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Type	Event ID	Title	Short name
MIE	36	Inhibition, Aromatase	Inhibition, Aromatase
KE	1789	Reduction, 17beta-estradiol synthesis by the undifferentiated gonad	Reduction, E2 Synthesis by the undifferentiated gonad
KE	1790	Increased, Differentiation to Testis	Increased, Differentiation to Testis
KE	1791	Increased, Male Biased Sex Ratio	Increased, Male Biased Sex Ratio
AO	360	Decrease, Population growth rate	Decrease, Population growth rate

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Inhibition, Aromatase	adjacent	Reduction, 17beta-estradiol synthesis by the undifferentiated gonad	High	
Reduction, 17beta-estradiol synthesis by the undifferentiated gonad	adjacent	Increased, Differentiation to Testis	Moderate	
Increased, Differentiation to Testis	adjacent	Increased, Male Biased Sex Ratio	High	
Increased, Male Biased Sex Ratio	adjacent	Decrease, Population growth rate	Low	
Inhibition, Aromatase	non-adjacent	Increased, Differentiation to Testis	High	
Inhibition, Aromatase	non-adjacent	Increased, Male Biased Sex Ratio	Moderate	

Prototypical Stressors

Name	Evidence
Fadrozole	High
Letrozole	High
Exemestane	Moderate
Stressor:292 Clotrimazole	Low
Prochloraz	High

Stressor:292 Clotrimazole ; Brown et al., 2015

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Development	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
Oreochromis niloticus	Oreochromis niloticus	High	NCBI
Chinook salmon	Oncorhynchus tshawytscha	Low	NCBI
fathead minnow	Pimephales promelas	Low	NCBI
European sea bass	Dicentrarchus labrax	Low	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage

The life stage to which this AOP applies is developing embryos/juveniles during gonadal differentiation. Since the sexually dimorphic expression of aromatase has been shown to play a crucial role in the differentiation to testis vs ovary of the undifferentiated bipotential gonad (Guiguen et al. 2010), the AOP is applicable to the stage of development during which aromatase might influence this process. The precise timing of the sensitive period relevant to this AOP will vary by species, but the AOP is not applicable to differentiated juveniles or to adults.

Studies with zebrafish (*Danio rerio*) have shown that both brain and gonadal aromatase expression can be observed at 20 days post-fertilization (dpf) with an increase in expression at 25 dpf in fish destined to become females, coinciding with the onset of gonadal differentiation period (Lau et al. 2016). In Nile tilapia (*Oreochromis niloticus*), aromatase expression can be observed as early as 3-4 dpf with an increase in expression starting at 11 dpf in genetic females (Kwon et al. 2001). Additionally, it has been shown that the period of 7-14 dpf is the most sensitive to chemical inhibition of CYP19a1 activity, and a continuous exposure of 2-3 weeks is sufficient for the masculinization of the majority of genetic female tilapia (Kwon et al. 2000). This clearly indicates alteration of differentiation from ovary to testis results during sex differentiation (OECD 2011).

Sex

The molecular initiating event for this AOP occurs during gonad differentiation. Therefore, the AOP is only applicable to sexually undifferentiated individuals.

Taxonomic

Most evidence for the taxonomic applicability of this AOP comes from species in the class Osteichthyes. Aromatase itself is well conserved among vertebrates (e.g., Wilson et al. 2005; LaLone et al. 2018). However, the degree to which aromatase and subsequent

production of endogenous estrogens such as E2 are involved in sex determination or sexual differentiation varies with species. Many fish, amphibian, and reptile species have environmental sex determination, and regulation of aromatase expression and sex steroids profiles are closely tied to sex-determining environmental factors (Angelopoulou et al. 2012). Alternatively, vertebrates that largely rely on genetic sex determination (birds, mammals) would be anticipated to be less vulnerable to effects of aromatase inhibitors during gonad differentiation, although there remains compelling evidence for an important role of steroid signaling during the process (Angelopoulou et al. 2012). Overall, regardless of differing roles for aromatase in sexual differentiation, expression appears universal among vertebrates during this life stage (Angelopoulou et al. 2012; Sarre et al. 2004; Uller and Helantera, 2011; Ramsey and Crews, 2009). Thus, in principle, components of the present AOP may have some degree of applicability to all vertebrates. Given the substantial diversity of sex determination and differentiation strategies in fish, amphibians and reptiles (including those from closely related phylogenetic groups; Sarre et al. 2004; Angelopoulou et al. 2012), quantitative sensitivity, and taxonomic domain of applicability of the present AOP are hard to generalize, although there is reason to believe it should have broad applicability in bony fishes.

Essentiality of the Key Events

Direct support for the essentiality of several of the key events in the AOP has been provided by gene modification/knockout studies of the *cyp19a1* gene in zebrafish and Nile tilapia. Specifically:

1. Lau et al. (2016) generated insertion/deletion mutations in the zebrafish *cyp19a1a* gene using TALEN (transcription activator-like effector nuclease) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 approaches. All mutant *cyp19a1a*$^{-/-}$ fish developed as males. Histological examination (at 120 dpf) of the *cyp1a1a*$^{-/-}$ mutants showed that they exhibited normal spermatogenesis in the testis with no observable difference between the wild type (+/+) and heterozygous (+/-) males. To confirm the necessity of E2 synthesis for ovarian differentiation, they performed an experiment to "rescue" the phenotype of *cyp19a1a* mutants by E2 treatment (0.05, 0.50 and 5.00 nM) encompassing the period of gonadal differentiation (15–30 days pdf). Treatment with the estrogen resulted in normal functioning ovaries with fully developed perinucleolar oocytes and small amount of stromal tissue, even in some individuals at the lowest E2 concentration (0.05 nM). This supports the essentiality of aromatase inhibition relative to E2 synthesis reduction as a critical step for testis differentiation.
2. In a similar study also with zebrafish, Muth-Köhne et al. (2016) generated *cyp19a1a* and *cyp19a1b* gene mutant lines and a *cyp19a1a*;*cyp19a1b* double-knockout line using TALENs. All *cyp19a1a* mutants and *cyp19a1a;cyp19a1b* double mutants developed as males, whereas *cyp1a1b* double mutant (-/-) had a 1:1 sex ratio similar to the wild type controls. This again supports the essentiality of gonadal aromatase inhibition for testis differentiation that would lead to a male biased sex ratio. Additionally, a small rescue experiment performed using E2 on all male mutant *cyp1a1a*$^{-/-}$ indicated that E2 treatment could restore a near normal sex ratio (9 females among 14 fish).
3. Studies in Nile tilapia similar to those conducted in zebrafish were described by Zhang et al. (2017), who worked with genetic female mutants for *cypa19a* and *cyp19a1b*. Results showed that all *cyp19a1a*$^{+/-}$ XX and *cyp19a1a*$^{+/+}$ XX fish developed as females, whereas all *cyp19a1a*$^{-/-}$ XX and *cyp19a1a*-/- XY fish developed as males,

based on gonad differentiation. The *cyp19a1a*$^{-/-}$ XX tilapia shifted to the male pathway as early as 5 dph and ultimately were fertile. This again provides strong support for the critical role of gonadal aromatase relative to ovarian development.

Key Event	Evidence	Essentiality/Assessment
Inhibition, Aromatase	strong	There is good evidence from gene knockout experiments of the two different isoforms of aromatase that support the specificity of gonadal aromatase inhibition for the subsequent key events to occur.
E2 Synthesis by the undifferentiated gonad	weak	There is evidence from a stop (by *cyp19a1* knockout) and recovery (through compensation) experiment where E2 can rescue the sex ratio altered due to the gonadal aromatase gene knockout suggesting that E2 depletion is necessary for the subsequent key events to occur.
Differentiation to Testis	strong	By definition, differentiation to testis is required for a male reproductive phenotype.
Male Biased Sex Ratio	moderate	Breeding females (and both sexes) are necessary for population sustainability. A male biased sex population suggests a reduced offspring production and consequentially reduced population sustainability.
Population Sustainability	n/a	This is the terminal key event in the AOP. Its essentiality for progression to downstream events in the sequence cannot be evaluated.

Weight of Evidence Summary

Biological Plausibility

Aromatase catalyzes the conversion of T to E2, so the biological plausibility of aromatase inhibition leading to reductions in available E2 is clear. Additionally, the role of E2 as a major regulator of normal female gonad development is well documented (Gorelick et al. 2011; Guiguen et al. 2010). The link between E2 reductions leading to increased differentiation of the bipotential gonad to testis is highly plausible. As E2 signaling is reduced, ER responsive genes required for ovarian differentiation will be downregulated in the bipotential gonad resulting in a default development of testes (Yin et al. 2017; Zhang et al. 2017). Therefore, it is plausible that E2 reduction in the undifferentiated gonad at the onset of sexual differentiation would promote testis formation. The direct link between increased differentiation to testis leading to a male biased sex ratio is also well supported by biological plausibility. If the conditions that favor a male producing phenotype (in this case, the aromatase inhibitor) overlap with the critical period of sex differentiation in a given population, it is reasonable that relatively more male offspring will be produced (D'Cotta et al., 2001, Kwon et al., 2000; Luzio et al. 2016). Therefore, exposure of sensitive

species to aromatase inhibition for an extended period of time during reproductive development plausibly would result in a male-biased population. Empirical evidence supporting the direct link between male biased cohorts and a reduced population sustainability in fish species is limited. However, biased sex ratios can definitely impact fish populations (Marty et al. 2017). For example, a male-biased sex ratio would logically lead to a reduction in the number of breeding females such that over time decreases in offspring would result in population declines (Brown et al. 2015; Grayson et al. 2014). Miller et al. (2022) recently developed a model specifically designed to capture the effects of male-biased sex ratios on population trajectories in fathead minnows (*Pimephales promelas*).

Concordance of Dose Response Relationships

There have been a number of in vitro and in vivo studies, primarily in fish, that have examined the effects of known aromatase inhibitors on different key events in the AOP. Most of these studies only measured one key event in the AOP so cannot be directly used to explore dose-response concordance between key events.

The differential sensitivity to inhibition of aromatase is most easily measured in vitro. Doering et al. (2019b) determined the effects of different concentrations of several known aromatase inhibitors (e.g., fadrozole, prochloraz) on brain aromatase activity in a taxonomically-diverse set of fish species, and found that while absolute potency of the chemicals varied across species, rank order potency of the test chemicals was generally similar. Importantly, relative potencies measured in vitro reflected those observed in in vivo studies such as those described below, thus providing indirect evidence of dose concordance between the MIE and downstream Key Events.

There have been several in vivo studies evaluating the effects of varying degrees of aromatase inhibition on different key events in the AOP. However, there are limitations to these studies in the context of determining dose-dependency across all key events in the AOP. For example, E2 levels typically have not been or measured or determined at a time relevant to gonadal differentiation. However, a few have measured multiple key events, although typically only at one time point. One study assessed dose-reponse relationships between different concentrations of the model aromatase inhibitor exemestane and expression of the enzyme. Immunohistochemical analyses revealed that gonad tissue of Nile tilapia (*Oreochromis niloticus*) exposed from 9-35 days post-hatch (dph) to 100, 500, 1000 and 2000 μg/g feed had no cross-reaction with P450arom at the three highest doses, but gonad tissue samples exhibited a strong immunopositive responses against P450arom at a lower dose of exemestane (100 μg/g feed), similar to the differentiating ovaries of the control fish (Ruksana et al. 2010). No ovarian development was noted in fish in the 500, 1000 and 2000 mg/kg treatments, and the 1000 and 2000 treatments resulted in 100% phenotypic males.

Uchida et al. (2004) evaluated two key events in the AOP in an experiment with fadrozole using zebrafish genetic females exposed from 15-40 dph via the diet. They observed ovarian transition to testis in all exposed animals, culminating in 62.5, 100 and 100% males in 10, 100 and 1000 mg/kg treatments, respectively.

Another study showed a dose-dependent rate of increased differentiation to testes in zebrafish exposed from 0-63 dph to different concentrations of fadrozole (10, 32, 100 ug/L) via the water (Muth-Köhne et al. 2016).

The most commonly reported dose response relationship for this AOP was for the non-adjacent relationship between aromatase inhibition and an increased male biased sex ratio. For example, Nile tilapia, zebrafish, fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), yellow catfish (*Pelteobagrus fulvidraco*) and Japanese flounder

(*Paralichthys olivaceus*) exposed to different concentrations of known aromatase inhibitors (exemestane, fadrozole, letrozole, prochloraz) via the diet or water reported dose-dependent increases in the relative number of males (Kwon et al. 2000; Kitano et al. 2000; Thorpe et al. 2011; Holbech et al. 2012; Gao et al. 2010; Shen et al. 2013).

Finally, there are models that demonstrate a dose-dependent decrease in population size corresponding with an increasing proportion of males in zebrafish and fathead minnows (Brown et al. 2015; Miller et al. 2022).

Temporal Concordance

Because this AOP involves actions during a specific development transition from an undifferentiated to differentiated gonad, the temporal concordance of the events is implicit. A male biased sex ratio cannot be observed until the population has undergone sexual differentiation. Likewise, reproduction and associated population growth rate cannot be assessed until the animals achieve sexual maturity.

Consistency

There have been a number of in vitro and in vivo studies, primarily in fish, that have examined the effects of known aromatase inhibitors on different key events in the AOP. Some of these studies measured only one key event in the AOP and/or employed just a single dose of a given stressor, so cannot be directly used to explore dose-response concordance. However, even with these limitations, they demonstrate that the overall AOP is consistent with expectations in a variety of species exposed to known chemical inhibitors of aromatase (see Dose Concordance table). For example, studies with chinook salmon (*Oncorhynchus tshawytscha*), Japanese fugu (*Takifugu rubripes*), Japanese medaka (*Oryzias latipes*), Nile tilapia, zebrafish, fathead minnow, bluegill, yellow catfish and Japanese flounder exposed to known aromatase inhibitors (exemestane, fadrozole, letrozole, prochloraz) via the diet or water during sexual differentiation have reported increases in differentiation to testis and/or the relative number of males (Piferrer et al. 1994; Kwon et al. 2000; Rashid et al. 2007; Kitano et al. 2000; Thorpe et al. 2011; Thresher et al. 2011; Holbech et al. 2012; Gao et al. 2010; Shen et al. 2013).

Male-biased sex ratios are not specific to this AOP. Many of the key events included overlap with another AOP (#376 in the AOP-Wiki) linking activation of the androgen receptor to male biased sex ratios.

Uncertainties, inconsistencies, and data gaps

Currently the major uncertainty in this AOP is the biological linkage between E2 synthesis reduction by the undifferentiated gonad leading to an increased, differentiation to testis. Biological plausibility connections have been established, but experimental measurements of E2 during the particular period of differentiation are lacking. Also, as noted in the Domain of Applicability section, the taxonomic range of applicability of the AOP is uncertain.

Quantitative Consideration

There is not yet a sufficient quantitative understanding of this overall AOP to predict the degree to which aromatase inhibition would result in population-level impacts. That said, there are models available suitable for the quantitative prediction of changes in E2 levels caused by degree of aromatase inhibition in some small fish species (Conolly et al. 2018; Doering et al. 2019a), as well as the effects of different (male-biased) sex ratios on fathead minnow population size (Miller et al. 2022). However, there currently are no quantitative data/models relating reductions in E2 to the degree of (increased) differentiation to male gonads and/or male-biased cohorts of fish.

Considerations for Potential Applications of the AOP

Altered sex ratios in fish can be a useful diagnostic endpoint for identifying EDCs both in field and lab settings. For example, the Fish Sexual Development Test (FSDT) has formally been adopted by the Organisation of Economic Cooperation and Development (OECD) as a test guideline (No. 234) for the detecting EDCs (OECD, 2011b). The FDST is conducted in zebrafish during early development, including sexual differentiation, and uses gonadal differentiation and skewed sex ratios to detect estrogen, androgen and steroidogenesis activity of test chemicals (Dang & Kienzler 2019). This AOP directly supports the mechanistic basis for assays such as the FDST. The AOP also supports the use of in vitro assays that measure aromatase inhibition by test chemicals as a basis for predicting apical impacts on fish (e.g., Conolly et al. 2018; Doering et al. 2019a; 2019b).

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Appendix 1 - MIE, KEs and AO

List of MIEs in this AOP

Event: 36: Inhibition, Aromatase

Short Name: Inhibition, Aromatase

Key Event Component

Process	Object	Action
aromatase activity	aromatase	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:25 - Aromatase inhibition leading to reproductive dysfunction	Molecular Initiating Event
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	Molecular Initiating Event

Stressors

Name
Fadrozole
Letrozole
Prochloraz

Biological context

Level of Biological Organization
Molecular

Cell term

Cell term
granulosa cell

Organ term

Organ term
ovary growing follicle

Evidence for Perturbation by Prototypic Stressor

Overview for Molecular Initiating Event

Characterization of chemical properties: Chemicals are known to inhibit aromatase activity through two primary molecular mechanisms. Steroid-like structures can inhibit the enzyme at its active site, with structures having Δ4 positioned double bonds generally acting as stronger inhibitors than those with Δ5 positioned double bonds (Petkov et al. 2009). Non-steroidal aromatase inhibitors generally act by interfering with electron transfer via the cytochrome P450 heme group of the aromatase enzyme, with greater nucleophilicity of the heteroatom contributing to greater potency as an inhibitor (Petkov et al. 2009). Petkov et

al. (Petkov et al. 2009) have provided a detailed analysis of structural categorization of chemicals as potential steroidal or non-steroidal aromatase inhibitors.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

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

Fishes generally have two aromatase isoforms, cyp19a1a which is predominantly expressed in ovary and cyp19b, predominantly expressed in brain (Callard et al. 2001). Given that cyp19a1a is dominant isoform expressed in ovary and both isoforms appear to show similar sensitivity to aromatase inhibitors (Hinfray et al., 2006), for the purpose of this key event which focuses on gonadal aromatase activity, distinction of effects on one isoform versus the other are considered negligible. Total activity, without regard to isoform can be considered.

Life stage applicability: Aromatase activity can be measured at any life stage after the onset of endogenous steroid biosynthesis, generally shortly after birth or hatch.

Sex applicability: Although expression and activity tends to be greater in females, aromatase activity can be measured in both male and female vertebrates.

Key Event Description

Inhibition of cytochrome P450 aromatase (CYP19; specifically cyp19a1a in fish).

Site of action: The site of action for the molecular initiating event is the ovarian granulosa cells.

While many vertebrates have a single isoform of aromatase, fish are known to have two isoforms. CYP19a1a is predominantly expressed in ovary while cyp19a1b is predominantly expressed in brain (Callard et al. 2001; Cheshenko et al. 2008). For the purposes of this MIE, when applied to fish, the assumed effect is on cyp19a1a. However, given that both isoforms show similar sensitivity to aromatase inhibitors (Hinfray et al. 2006) and catalyze the same reaction, discrimination of specific isoforms is not viewed as critical in relative to determining downstream key events resulting from aromatase inhibition in ovarian granulosa cells.

Responses at the macromolecular level: Aromatase catalyzes three sequential oxidation steps (i.e., KEGG reactions R02501, R04761, R03087 or R01840, R04759, R02351; http://www.genome.jp/kegg/pathway.html) involved in the conversion of C-19 androgens (e.g., testosterone, androstenedione) to C-18 estrogens (e.g., 17β-estradiol, estrone). Aromatase inhibitors interfere with one or more of these reactions, leading to reduced efficiency in converting C-19 androgens into C-18 estrogens. Therefore, inhibition of aromatase activity results in decreased rate of 17β-estradiol (and presumably estrone) production by the ovary.

How it is Measured or Detected

Measurement/detection: Aromatase activity is typically measured by evaluating the production of tritiated water released upon the aromatase catalyzed conversion of radio-labeled androstenedione to estrone (Lephart and Simpson 1991). Aromatase activity can be measured in cell lines exposed in vitro (e.g., human placental JEG-3 cells and JAR choriocarcinoma cells, (Letcher et al. 1999); H295R human adrenocortical carcinoma cells (Sanderson et al. 2000)). Aromatase activity can also be quantified in tissue (i.e., ovary or brain) from vertebrates exposed in vivo (e.g., (Villeneuve et al. 2006; Ankley et al. 2002). In vitro aromatase assays are amenable to high throughput and have been included in nascent high throughput screening programs like the US EPA Toxcast™ program. Specific ToxCast assays indicative of potential aromatase inhibition include:

NVS_ADME_hCYP19A1

ERF_ENZ_hCYP19A1_dn

TOX21_Aromatase_Inhibition

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List of Key Events in the AOP

Event: 1789: Reduction, 17beta-estradiol synthesis by the undifferentiated gonad

Short Name: Reduction, E2 Synthesis by the undifferentiated gonad

Key Event Component

Process	Object	Action
estrogen biosynthetic process	17beta-estradiol	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	Key Event

Biological context

Level of Biological Organization
Cellular

Cell term

Cell term
primordial germ cell

Organ term

Organ term
gonad

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Development	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Low

Taxonomic applicability: Most of the key enzymes involved in the process of E2 biosynthesis are well conserved among vertebrates (Callard et al. 2001; Thornton et al. 2001; Eick et al. 2011; Coumailleau et al. 2015). Estrogens play a key role in embryonic development particularly during gonadogenesis for most vertebrates (Coumailleau et al., 2015; Callard et al., 2015). Therefore, it is possible that this key event is applicable to most vertebrate taxa. In contrast, this key event is not applicable to organisms that lack the necessary enzymes for estrogen synthesis such as invertebrates and plants (Jones et al. 2017).

Life stage applicability: Endogenous steroid biosynthesis generally begins shortly after birth or hatch.

Sex applicability: This key event applies to the undifferentiated gonad. Therefore, sex is non-specific.

Key Event Description

Estrogens are essential for normal ovarian differentiation, growth and maintenance. When estrogens bind to estrogen receptors (ER), these then regulate the transcription of downstream estrogen-responsive genes necessary for proper gonad development (Guiguen et al. 2010; Gorelick et al. 2011). Among the different forms of estrogens, 17β-estradiol (E2) is considered the most fundamental in gonad differentiation in most vertebrates, as it is responsible for inducing and maintaining ovarian development (Bondesson et al. 2015; Li et al. 2019). Consequently, disruption of the E2 synthesis by the undifferentiated gonad has been linked to altered gonad differentiation and development in many vertebrates.

How it is Measured or Detected

Estrogen concentrations can be measured via radioimmunoassay (e.g., US EPA 2002) or by analytical methods such as LC/MS/MS (e.g., Gravitte et al. 2021; Jalabert et al. 2021; Nouri et al. 2020). Measurement in the undifferentiated gonad would generally require extraction of tissue homogenates. This tissue mass can be very limited during primordial stages.

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Event: 1790: Increased, Differentiation to Testis

Short Name: Increased, Differentiation to Testis

Key Event Component

Process	Object	Action
male gonad development	immature gonad	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	Key Event
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	Key Event

Biological context

Level of Biological Organization
Tissue

Organ terms

Organ term
testis

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Development	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate

The primordial bipotential gonad and basic molecular machinery/pathways responsible for differentiation of testis and ovary are well conserved across all vertebrates (Cutting et al. 2013; DeFalco and Capel 2009). Although timing/expression of key genes controlling pathways involved in male versus female gonadal differentiation can vary across taxa (Cutting et al. 2013), actual structural morphology of the testes is similar across vertebrates (DeFalco and Capel 2009; McLaren 1998). Consequentially, this KE is applicable to most vertebrate taxa.

Key Event Description

Prior to gonadal sex determination in vertebrates, the developing organism has a primordial bipotential gonad that can be fated to either sex depending on the genetic makeup of the embryo (genetic sex determination) or environmental conditions (environmental sex determination) or a combination of both factors.

During male development, the embryonic stem cells can differentiate to primordial germ cells, which in turn proliferate and differentiate into precursor spermatogonia stem cells. Sertoli cells are the first to differentiate into the different fetal gonad seminiferous cords surrounded by peritubular myoid cells enclosing fetal germ cells. Sertoli cells can also differentiate into Leydig cells. Successively, the interstitial Leydig cells differentiate and produce sex steroids such as testosterone to maintain the testis and control aspects of masculinization including secondary sex characteristics (McLaren 1998; DeFalco and Capel 2009; Trukina et al. 2013).

Although the timing and location of gene expression leading to the morphological development of the testis may differ among vertebrate taxa, the basic molecular machinery and pathways involved are well conserved (Cutting et al. 2013). Similarly, the cell types and basic morphological structure of the testis across vertebrates are well-conserved (McLaren 1998; DeFalco and Capel 2009).

How it is Measured or Detected

Depending upon the size of the test organism and life stage it may be possible to identify the presence of developed testes versus ovaries visually or with low-power magnification without a need for gonad removal, fixation and processing. This would require, of course, experienced personnel well-versed in the biology of the species of interest.

In instances where organisms are small, at early life-stages and/or have poorly differentiated gonads, it will be necessary to employ histological examination by light microscopy to identify nature of the gonad. In all vertebrates, the gonads of phenotypic males in early development have three main differentiating cell types; the gamete forming germ cells (spermatogonia), support cells (Sertoli cells), and hormone-secreting Leydig or interstitial cells (DeFalco and Capel 2009; McLaren 1998).

There are many standardized techniques available for fixation, processing and staining of tissues of concern, including gonads (e.g., Carson and Cappellano 2014). There also are species-specific resources available to aid interpretation of histological images; for example, the National Toxicology Program maintains an on-line Atlas of Non-Neoplastic lesions for a variety of organs, including gonads, in rodents (https://ntp.niehs.nih.gov/nnl/index.htm).

Although there are fewer publicly-accessible resources available for interpretation of histological images in other vertebrate classes, there is often published reference material suitable for this purpose (e.g., Spitsbergen et al. 2009).

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Event: 1791: Increased, Male Biased Sex Ratio

Short Name: Increased, Male Biased Sex Ratio

Key Event Component

Process	Object	Action
male sex differentiation	population of organisms	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	Key Event
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	Key Event

Biological context

Level of Biological Organization
Population

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High

Any sexually reproducing organism can theoretically experience a male-biased population, although the phenomenon certainly has not been demonstrated empirically in all species of potential concern.

Key Event Description

Sex ratio is the ratio of males to females in a population. A male-biased sex ratio for a given species is defined as a significant increase in the number of males, relative to the average ratio found in most populations of that species.

While simple in concept, the “normal” sex ratio for a given species can be challenging to define.

- In organisms with genetic sex determination (GSD) such as mammals and birds, as well as many poikilothermic vertebrates, the male to female ratio often is 1:1. In these instances it is easy to define a deviation from normal in terms of either a relatively greater number of males or females.
- When considering organisms with environmental sex determination (ESD), such as many reptiles and some amphibians and fish, deviations from a 1:1 relationship can and do occur that nonetheless may be normal in the context of the organism’s life history. For example, some reptile species have temperature-dependent sex

determination where differentiation of developing organisms to males versus females predominates at different temperatures (Norris and Carr 2020).

- Further complicating a generalized definition of normal sex ratios are situations where sexual differentiation is determined by a combination of genetic and environmental variables, such is the case in many fish species.

Even in species potentially requiring fewer males than females to maintain a viable population, at some point a female-biased population could become problematic in terms of having an adequate number of males to fertilize eggs produced by females or, in the longer term, ensure a robust level of genetic diversity in a population. Further, in situations where a population is male-biased relative to conditions considered normal for a given species, overall productivity may be negatively impacted due to fewer females being available to produce eggs.

A variety of external factors can produce populations that would be characterized as abnormally male-biased based on analysis of phenotypic sex ratios (examples, not comprehensive):

- Differential mortality can occur in males versus females. This might include situations where predation or harvest techniques geared toward larger individuals, which could be either males or females depending upon species may effectively skew the apparent male to female ratio higher.
- Endocrine disruption during early development, most prominently, during gonadal differentiation. For example, in some fish species, exposure during gonadal differentiation to androgen receptor agonists or inhibitors of cytochrome P450 19a1 (aromatase), an enzyme involved in the synthesis of 17β-estradiol, can caused male-biased populations (Delbes et al. 2022).

How it is Measured or Detected

Fundamentally, determination of sex ratio (and consequently male-biased sex ratio) is based on counts of the number of males and/or non males in a population, or some statistically representative sub-sample of a population.

- For mature animals that are sexually dimorphic, direct observation of phenotypic secondary sex characteristics is a common method for assessing sex ratios.
- In animals that are not sexually dimorphic or those in pubertal/juvenile stages examination of the gonad, either via gross observation or histological examination is required to determine phenotypic sex.
- There can be instances where gonads cannot be clearly identified histologically as either testis or ovary because cell types indicative of both are simultaneously present. This type of intersex condition has been observed in some amphibians and fish, and may require a third classification category (Abdul-moneim et al. 2015).
- For animals with GSD, genotyping or the use of genetic markers can also be employed to determine genotypic sex ratio. However, it is noted that there are cases where genotypic sex ratio and phenotypic sex ratio may not be equivalent.

Considerations when evaluating measurements of sex ratio:

- Care needs to be taken to collect an adequate number of animals to ensure that statistical power of the sex ratio point estimates is sufficient to address whether true deviations from normal conditions exist. It is not uncommon for published papers to

report skewed sex ratios based on sample sizes far too small to result in environmentally meaningful conclusions.

- Determination of sex ratios is generally straight-forward in a laboratory environment where all (or a defined proportion of) animals from a particular experimental treatment of interest can be collected and examined. Under such conditions, determination of a male bias relative to normal is a simple matter of a statistical comparison between the treated and control groups.
- Determination of sex ratios in the field/wild can often be quite challenging as variables such as sampling gear used, or time and location of collection could bias samples toward one sex versus another. Additionally, often more difficult than ascertaining phenotypic male to female ratio is determining whether observations deviate from what would be considered normal for a particular species of interest. As discussed above (*Key Event Description*), the relative number of males normally expected will be taxa-dependent, and in some cases may also vary by region and/or environmental conditions. In cases where a male bias is being proposed for a population in the field, compelling scientific support for the "normal" sex ratio expected in the field and for the unbiased nature of the sampling should be made.

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List of Adverse Outcomes in this AOP

Event: 360: Decrease, Population growth rate

Short Name: Decrease, Population growth rate

Key Event Component

Process	Object	Action
population growth rate	population of organisms	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)	Adverse Outcome
Aop:25 - Aromatase inhibition leading to reproductive dysfunction	Adverse Outcome
Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction	Adverse Outcome
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	Adverse Outcome
Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior	Adverse Outcome
Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	Adverse Outcome
Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	Adverse Outcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	Adverse Outcome
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	Adverse Outcome
Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release	Adverse Outcome
Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I /metaphase I transition	Adverse Outcome
Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction	Adverse Outcome
Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint	Adverse Outcome
Aop:292 - Inhibition of tyrosinase leads to decreased population in fish	Adverse Outcome
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	Adverse Outcome
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	Adverse Outcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	Adverse Outcome
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	Adverse Outcome
Aop:336 - DNA methyltransferase inhibition leading to population decline (1)	Adverse Outcome

AOP ID and Name	Event Type
Aop:337 - DNA methyltransferase inhibition leading to population decline (2)	Adverse Outcome
Aop:338 - DNA methyltransferase inhibition leading to population decline (3)	Adverse Outcome
Aop:339 - DNA methyltransferase inhibition leading to population decline (4)	Adverse Outcome
Aop:340 - DNA methyltransferase inhibition leading to transgenerational effects (1)	Adverse Outcome
Aop:341 - DNA methyltransferase inhibition leading to transgenerational effects (2)	Adverse Outcome
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	Adverse Outcome
Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline	Adverse Outcome
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	Adverse Outcome
Aop:326 - Thermal stress leading to population decline (3)	Adverse Outcome
Aop:325 - Thermal stress leading to population decline (2)	Adverse Outcome
Aop:324 - Thermal stress leading to population decline (1)	Adverse Outcome
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	Adverse Outcome
Aop:349 - Inhibition of 11β-hydroxylase leading to decresed population trajectory	Adverse Outcome
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory	Adverse Outcome
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	Adverse Outcome
Aop:386 - Deposition of ionizing energy leads to leading to population decline via inhibition of photosynthesis	Adverse Outcome
Aop:387 - Deposition of ionising energy leading to population decline via mitochondrial dysfunction	Adverse Outcome
Aop:388 - Deposition of ionising energy leading to population decline via programmed cell death	Adverse Outcome
Aop:389 - Oxygen-evolving complex damage leading to population decline via inhibition of photosynthesis	Adverse Outcome
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	Adverse Outcome
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	Adverse Outcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	Adverse Outcome
Aop:410 - GSK3beta inactivation leading to increased mortality via defects in developing inner ear	Adverse Outcome
Aop:216 - Deposition of energy leading to population decline via DNA strand breaks and follicular atresia	Adverse Outcome
Aop:238 - Deposition of energy leading to population decline via DNA strand breaks and oocyte apoptosis	Adverse Outcome
Aop:299 - Deposition of energy leading to population decline via DNA oxidation and follicular atresia	Adverse Outcome
Aop:311 - Deposition of energy leading to population decline via DNA oxidation and oocyte apoptosis	Adverse Outcome
Aop:444 - Ionizing radiation leads to reduced reproduction in Eisenia fetida via reduced spermatogenesis and cocoon hatchability	Adverse Outcome
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	Adverse Outcome
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	Adverse Outcome

AOP ID and Name	Event Type
Aop:97 - 5-hydroxytryptamine transporter (5-HTT; SERT) inhibition leading to population decline	Adverse Outcome
Aop:203 - 5-hydroxytryptamine transporter inhibition leading to decreased reproductive success and population decline	Adverse Outcome
Aop:218 - Inhibition of CYP7B activity leads to decreased reproductive success via decreased locomotor activity	Adverse Outcome
Aop:219 - Inhibition of CYP7B activity leads to decreased reproductive success via decreased sexual behavior	Adverse Outcome
Aop:323 - PPARalpha Agonism Impairs Fish Reproduction	Adverse Outcome

Biological context

Level of Biological Organization
Population

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
all species	all species	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

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

Key Event Description

A population can be defined as a group of interbreeding organisms, all of the same species, occupying a specific space during a specific time (Vandermeer and Goldberg 2003, Gotelli 2008). As the population is the biological level of organization that is often the focus of ecological risk assessments, population growth rate (and hence population size over time) is important to consider within the context of applied conservation practices.

If N is the size of the population and t is time, then the population growth rate (dN/dt) is proportional to the instantaneous rate of increase, r, which measures the per capita rate of population increase over a short time interval. Therefore, r, is a difference between the instantaneous birth rate (number of births per individual per unit of time; b) and the instantaneous death rate (number of deaths per individual per unit of time; d) [Equation 1]. Because r is an instantaneous rate, its units can be changed via division. For example, as there are 24 hours in a day, an r of 24 individuals/(individual x day) is equal to an r of 1

individual/(individual/hour) (Caswell 2001, Vandermeer and Goldberg 2003, Gotelli 2008, Murray and Sandercock 2020).

Equation 1: $r = b - d$

This key event refers to scenarios where $r < 0$ (instantaneous death rate exceeds instantaneous birth rate).

Examining r in the context of population growth rate:

- A population will decrease to extinction when the instantaneous death rate exceeds the instantaneous birth rate ($r < 0$).
- The smaller the value of r below 1, the faster the population will decrease to zero.
- A population will increase when resources are available and the instantaneous birth rate exceeds the instantaneous death rate ($r > 0$)
- The larger the value that r exceeds 1, the faster the population can increase over time
- A population will neither increase or decrease when the population growth rate equals 0 (either due to $N = 0$, or if the per capita birth and death rates are exactly balanced). For example, the per capita birth and death rates could become exactly balanced due to density dependence and/or to the effect of a stressor that reduces survival and/or reproduction (Caswell 2001, Vandermeer and Goldberg 2003, Gotelli 2008, Murray and Sandercock 2020).

Effects incurred on a population from a chemical or non-chemical stressor could have an impact directly upon birth rate (reproduction) and/or death rate (survival), thereby causing a decline in population growth rate.

- Example of direct effect on r: Exposure to 17b-trenbolone reduced reproduction (i.e., reduced b) in the fathead minnow over 21 days at water concentrations ranging from 0.0015 to about 41 mg/L (Ankley et al. 2001; Miller and Ankley 2004).

Alternatively, a stressor could indirectly impact survival and/or reproduction.

- Example of indirect effect on r: Exposure of non-sexually differentiated early life stage fathead minnow to the fungicide prochloraz has been shown to produce male-biased sex ratios based on gonad differentiation, and resulted in projected change in population growth rate (decrease in reproduction due to a decrease in females and thus recruitment) using a population model. (Holbech et al., 2012; Miller et al. 2022)

Density dependence can be an important consideration:

- The effect of density dependence depends upon the quantity of resources present within a landscape. A change in available resources could increase or decrease the effect of density dependence and therefore cause a change in population growth rate via indirectly impacting survival and/or reproduction.
- This concept could be thought of in terms of community level interactions whereby one species is not impacted but a competitor species is impacted by a chemical stressor resulting in a greater availability of resources for the unimpacted species. In this scenario, the impacted species would experience a decline in population growth rate. The unimpacted species would experience an increase in

population growth rate (due to a smaller density dependent effect upon population growth rate for that species).

Closed versus open systems:

- The above discussion relates to closed systems (there is no movement of individuals between population sites) and thus a declining population growth rate cannot be augmented by immigration.
- When individuals depart (emigrate out of a population) the loss will diminish population growth rate.

Population growth rate applies to all organisms, both sexes, and all life stages.

How it is Measured or Detected

Population growth rate (instantaneous growth rate) can be measured by sampling a population over an interval of time (i.e. from time t = 0 to time t = 1). The interval of time should be selected to correspond to the life history of the species of interest (i.e. will be different for rapidly growing versus slow growing populations). The population growth rate, r, can be determined by taking the difference (subtracting) between the initial population size, $N_{t=0}$ (population size at time t=0), and the population size at the end of the interval, $N_{t=1}$ (population size at time t = 1), and then subsequently dividing by the initial population size.

Equation 2: $r = (N_{t=1} - N_{t=0}) / N_{t=0}$

The diversity of forms, sizes, and life histories among species has led to the development of a vast number of field techniques for estimation of population size and thus population growth over time (Bookhout 1994, McComb et al. 2021).

- For stationary species an observational strategy may involve dividing a habitat into units. After setting up the units, samples are performed throughout the habitat at a select number of units (determined using a statistical sampling design) over a time interval (at time t = 0 and again at time t = 1), and the total number of organisms within each unit are counted. The numbers recorded are assumed to be representative for the habitat overall, and can be used to estimate the population growth rate within the entire habitat over the time interval.
- For species that are mobile throughout a large range, a strategy such as using a mark-recapture method may be employed (i.e. tags, bands, transmitters) to determine a count over a time interval (at time = 0 and again at time =1).

Population growth rate can also be estimated using mathematical model constructs (for example, ranging from simple differential equations to complex age or stage structured matrix projection models and individual based modeling approaches), and may assume a linear or nonlinear population increase over time (Caswell 2001, Vandermeer and Goldberg 2003, Gotelli 2008, Murray and Sandercock 2020). The AOP framework can be used to support the translation of pathway-specific mechanistic data into responses relevant to population models and output from the population models, such as changing (declining) population growth rate, can be used to assess and manage risks of chemicals (Kramer et al. 2011). As such, this translational capability can increase the capacity and efficiency of safety assessments both for single chemicals and chemical mixtures (Kramer et al. 2011).

Some examples of modeling constructs used to investigate population growth rate:

● A modeling construct could be based upon laboratory toxicity tests to determine effect(s) that are then linked to the population model and used to estimate decline in population growth rate. Miller et al. (2007) used concentration–response data from short term reproductive assays with fathead minnow (*Pimephales promelas*) exposed to endocrine disrupting chemicals in combination with a population model to examine projected alterations in population growth rate.

● A model construct could be based upon a combination of effects-based monitoring at field sites (informed by an AOP) and a population model. Miller et al. (2015) applied a population model informed by an AOP to project declines in population growth rate for white suckers (Catostomus commersoni) using observed changes in sex steroid synthesis in fish exposed to a complex pulp and paper mill effluent in Jackfish Bay, Ontario, Canada. Furthermore, a model construct could be comprised of a series of quantitative models using KERs that culminates in the estimation of change (decline) in population growth rate.

● A quantitative adverse outcome pathway (qAOP) has been defined as a mathematical construct that models the dose–response or response–response relationships of all KERs described in an AOP (Conolly et al. 2017, Perkins et al. 2019). Conolly et al. (2017) developed a qAOP using data generated with the aromatase inhibitor fadrozole as a stressor and then used it to predict potential population-level impacts (including decline in population growth rate). The qAOP modeled aromatase inhibition (the molecular initiating event) leading to reproductive dysfunction in fathead minnow (Pimephales promelas) using 3 computational models: a hypothalamus–pituitary–gonadal axis model (based on ordinary differential equations) of aromatase inhibition leading to decreased vitellogenin production (Cheng et al. 2016), a stochastic model of oocyte growth dynamics relating vitellogenin levels to clutch size and spawning intervals (Watanabe et al. 2016), and a population model (Miller et al. 2007).

● Dynamic energy budget (DEB) models offer a methodology that reverse engineers stressor effects on growth, reproduction, and/or survival into modular characterizations related to the acquisition and processing of energy resources (Nisbet et al. 2000, Nisbet et al. 2011). Murphy et al. (2018) developed a conceptual model to link DEB and AOP models by interpreting AOP key events as measures of damage-inducing processes affecting DEB variables and rates.

● Endogenous Lifecycle Models (ELMs), capture the endogenous lifecycle processes of growth, development, survival, and reproduction and integrate these to estimate and predict expected fitness (Etterson and Ankley, 2021). AOPs can be used to inform ELMs of effects of chemical stressors on the vital rates that determine fitness, and to decide what hierarchical models of endogenous systems should be included within an ELM (Etterson and Ankley, 2021).

Regulatory Significance of the AO

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.

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Appendix 2 – Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2144: Inhibition, Aromatase leads to Reduction, E2 Synthesis by the undifferentiated gonad

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Oreochromis niloticus	Oreochromis niloticus	Low	NCBI
zebrafish	Danio rerio	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
before or during gonadal sex differentiation	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Life Stage

The life stage applicable to this KER is developing embryos and juveniles during the gonadal differentiation. This KER is not applicable to sexually differentiated adults.

Sex

Because this KER occurs during differentiation, the relationship is relevant to animals with an undetermined (non-specific) sex.

Taxonomic Applicability

Sequencing studies with mammalian, amphibian, reptile, bird, and fish species have shown that aromatase is well conserved among all vertebrates (Wilson et al. 2005; LaLone et al. 2018).

However, it is difficult to predict the biological domain of applicability of this KER based on phylogenetic characteristics. There is considerable within class variability, for example, among both fish and reptile species as to the role of aromatase expression and estrogen signaling in determining gonadal sex (Angelopoulou et al. 2012; Sarre et al. 2004). Thus susceptibility and relative sensitivities may vary considerably between species.

Key Event Relationship Description

Aromatase (cyp191a) is a cytochrome P450-based enzyme that is rate limiting for the synthesis of 17ß-estradiol (E2) from testosterone in vertebrates (Simpson et al. 1994; Miller 1988; Payne and Hale 2004). The expression and activity of aromatase in the bipotential gonad of developing organisms, and subsequent autocrine and/or paracrine signaling mediated by E2 interactions with the estrogen receptor (or lack thereof), are thought to be key regulators of sex determination and gonadal differentiation in vertebrates (Angelopoulou et al. 2012; Nakamura 2010).

Evidence Supporting this KER

Biological Plausibility

There is little direct evidence of E2 production by the bipotential gonad, or that inhibition of aromatase decreases in E2 production in same. However, given the well-established role of aromatase in E2 production (Simpson et al. 1994; Payne and Hale, 2004) and the close association between aromatase expression and activity and gonadal sex determination/differentiation (Angelopoulou et al. 2012; Nakamura 2010), it is highly plausible that local estrogen production in the bipotential gonad plays a significant role in gonadal differentiation. However, particularly for species with genetic sex determination, it is just one of multiple determinants that ultimately influences differentiation of the gonad (Angelopoulou et al. 2012).

Empirical Evidence

Multiple lines of empirical evidence support a link between aromatase inhibition and decreased E2 synthesis in bipotential gonads of developing fish.

- In Nile Tilapia (*Oreochromis niloticus*) reared at the 27°C, genetic males exhibited lower levels of aromatase gene expression and E2 levels during the critical period of sexual differentiation (18-26 days post fertilization) than genetic females. This correlation suggests that aromatase repression at the onset of sexual differentiation reduces the biosynthesis of E2 in the undifferentiated gonad. (D'Cotta et al. 2001)
- Generation of *cyp19a1a* and *cyp19a1b* (gonadal and brain forms of aromatase, respectively) gene mutant lines and a *cyp19a1a;cyp19a1b* double knockout line in zebrafish using transcription activator like effector nucleases (TALENs) showed that in both *cyp19a1a*-deficient and double knockout fish, E2 levels were significantly lower than in wild-type and *cyp19a1b*-deficient fish (Yin et al. 2017).
- Control XY and *cyp19a1a* -/- (deficient and double knockout) XX Nile tilapia had significantly lower levels of serum E2 when compared to the control XX and *cyp19a1a*+/- XX fish suggesting a decrease in E2 due to the *cyp19a1a* deficiency. (Zhang et al. 2017)

Uncertainties and Inconsistencies

As noted below it is difficult to predict the full suite of vertebrate species this KER might apply to. In addition, studies directly examining synthesis of E2 by bipotential gonads in organisms exposed to aromatase inhibitors are lacking.

Quantitative Understanding of the Linkage

Quantitative understanding of this linkage is currently weak.

Response-response relationship

To date, none of the studies reviewed have offered insights into the quantitative relationship between the degree of aromatase inhibition and E2 synthesis by the undifferentiated, bipotential gonad.

Time-scale

- Based on studies in mature adult fish (fathead minnows, *Pimephales promelas*) effects of model aromatase inhibitors on E2 production (e.g., plasma concentrations) can be detected within a few hours of exposure in vivo (Schroeder et al. 2017; Skolness et al. 2011).
- Based on in vitro studies, significant reductions in aromatase activity and associated E2 synthesis can be detected in 90 min or less (Villeneuve et al. 2006).

Known modulating factors

Aromatase expression during gonadal differentiation is subject to both environmental and genetic controls to various degrees depending on species (Angelopoulou et al. 2012, Sarre et al. 2004). However, generalizable relationships that account for effects of specific parameters in the response-response relationships underlying this KER are currently unknown.

Known Feedforward/Feedback loops influencing this KER

Aromatase expression and E2 synthesis in adult fish of several species are subject to feedback regulation via the brain-pituitary-gonadal axis (e.g., Villeneuve et al. 2009; 2013; Ankley et al. 2009; Yu et al. 2020; Norris 1997; Miller 1988; Callard et al. 2001).

However, it is unclear whether these feedback mechanisms are active during gonadal differentiation.

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Relationship: 2145: Reduction, E2 Synthesis by the undifferentiated gonad leads to Increased, Differentiation to Testis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	adjacent	Moderate	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
Oreochromis niloticus	Oreochromis niloticus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Development	Low

Sex Applicability

Sex	Evidence
Mixed	Moderate

Life stage

The upstream event in for this KER is associated with the undifferentiated bipotential gonad. Therefore, this relationship is relevant to early life-stages prior to sexual development/differentiation.

Sex

Because the upstream event in this relationship pertains to the undifferentiated gonad, the sex applicability of this relationship is non-specific.

Taxonomic applicability

This relationship is most applicable to vertebrates subject to environmental sex determination. The relevance to species with predominantly genetic sex determination is less clear, likely depending on species-specific plasticity.

Key Event Relationship Description

Prior to sex determination, vertebrates have a bipotential gonad that can develop into testis or ovary depending on genetic makeup (genetic sex determination), environmental conditions during development (environmental sex determination) or a combination of both (Graves et al. 2010; Trukhina et al. 2013).

A key variable influencing gonad differentiation is the production of sex steroids such as 17ß-estradiol (E2) and testosterone (T). In many vertebrates, including a variety of fish species, the "default" gonadal sex is male, with the presence of E2 (or perhaps the relative

relationship between E2 and T production/levels) controlling the alternative path to development of ovaries.

Evidence Supporting this KER

Biological Plausibility

Among the different forms of estrogens, E2 is considered the most fundamental to gonad differentiation in most vertebrates, as it is responsible for inducing and maintaining ovarian development (Bondesson et al., 2015; Li et al., 2019). Estrogens bind to estrogen receptors (ER), that regulate the transcription of estrogen-responsive genes necessary for proper gonad development of for a female pathway (Guiguen et al., 2010; Gorelick et al., 2011). However, reductions in E2 biosynthesis during the critical period of sexual differentiation of the bipotential gonad would logically lead to decreased E2 signaling necessary for ovarian development, thereby leading to morphological development of testis. Therefore, it is plausible that E2 reduction in the undifferentiated gonad at the onset of sexual differentiation promotes the preferential occurrence of testis.

Empirical Evidence

There are multiple lines of indirect empirical evidence for this KER.

- During sexual differentiation (10-40 days post-fertilization), depression of E2 production through inhibition of aromatase (cytochrome P450 19a [*cyp 19a1*]] was associated with temperature-induced masculinization (35°C**)** of genetic male and female Nile tilapia (*Oreochromis niloticus*) indicating the critical role of estrogen synthesis in causing sexual differentiation to testis (D'cotta et al. 2001).
- Zhang et al. (2017) found that control XY and cyp19a1a -/- (deficient and double knockout) XX Nile tilapia had significantly lower levels of serum E2 compared to the control XX and cyp19a1a+/- XX fish, which corresponded with increased differentiation to testis.
- Rucksana et al. (2010) treated early life stage Nile tilapia with the aromatase inhibitor exemestane and found at 120 days posthatch in exposed fish complete testes differentiation with efferent ducts and with all stages of spermatogenic germ cells, from spermatogonia to spermatozoa.
- In zebrafish (*Danio rerio*), generation of *cyp19a1a* and *cyp19a1b* (gonad and brain aromatase isoforms, respectively) gene mutant lines and a *cyp19a1a;cyp19a1b* double knockout using transcription activator like effector nucleases (TALENs) showed that all *cyp19a1a*-deficient and double knockout fish were phenotypic males, corresponding with significantly lower levels of E2 than in wild-type and *cyp19a1b*-deficient fish (Yin et al. 2017).
- Rashid et al. (2007) reported that dietary exposure to fadrozole decreased ovary cavity formation and increased testicular differentiation in fugu (*Takifugu rubripes*).

Uncertainties and Inconsistencies

Even for vertebrate classes known to be subject to environmental sex determination, the relative importance of genetic versus environmental factors in terms of influencing local production of steroids by the bipotential gonad is not well characterized, nor readily predicted based on phylogeny (Angelopoulou et al. 2012, Sarre et al. 2004). Consequently, both the occurrence and importance of this relationship may vary considerably among species.

Quantitative Understanding of the Linkage

At present, the quantitative understanding of this relationship is weak.

Response-response relationship

There are not sufficient data to support derivation of a generalizable relationship between levels of E2 in differentiating gonad tissue and development to a testis phenotype.

Time-scale

The timeframe for differentiation of the bipotential gonad is species-dependent occurring, for example, over the course of days to weeks in most fishes.

Known modulating factors

Various environmental and genetic factors are known to influence differentiation of the bipotential gonad. However, quantitative understanding of this relationship is inadequate to precisely define the effect of such factors on the concentrations of E2 required to support differentiation to testis versus ovary, particularly in a manner that could be generalized across multiple species.

Known Feedforward/Feedback loops influencing this KER

Undefined at present.

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Relationship: 2146: Increased, Differentiation to Testis leads to Increased, Male Biased Sex Ratio

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	adjacent	High	
Androgen receptor agonism leading to male-biased sex ratio	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Odontesthes bonariensis	Odontesthes bonariensis	Low	NCBI
Oreochromis niloticus	Oreochromis niloticus		NCBI
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Juvenile	Moderate
Development	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate

This KER is applicable to any species in which males are defined by the occurrence of testis and/or associated male secondary sexual characteristics.

Key Event Relationship Description

Prior to gonadal sex determination in vertebrates, the developing organism has a primordial bipotential gonad that can be fated to either sex depending on the genetic makeup of the embryo (genetic sex determination; GSD) or environmental conditions (environmental sex determination; ESD) or a combination of both factors.

Regardless of whether gonadal development is controlled via GSD or ESD (or both), the operational definition of male versus female in terms of function usually is defined by the presence, respectively, of testes versus ovaries. For species exhibiting sex-specific secondary sexual characteristics preferential differentiation to testis can be accompanied by easily discerned external phenotypic changes as well. If there is increased differentiation to testis in individuals of a population of organisms this will by default produce a male biased sex ratio as defined by what would be considered normal for that species.

Evidence Supporting this KER

Biological Plausibility

It is highly plausible that as a gonadal phenotype increases toward testis formation, male-biased sex ratios in a defined cohort of organisms will occur. If this condition persists for repeated or prolonged periods of times within the habitat of given species, this will result in a male-biased sex ratio.

Empirical Evidence

There are a variety of examples in multiple fish species where histological evidence of increased gonad differentiation/development to testis results in male-biased sex ratios. These studies in many instances employed chemical inhibitors of aromatase, a key enzyme involved in estrogen synthesis (Simpson et al. 1994), to intentionally alter gonad development.

Zebrafish (*Danio rerio*) exposed to dietary fadrozole (500 ug/g) from 35-71 days posthatch (dph) were 100% masculinized, consistent with histological documentation of gonad tissue containing prominent numbers of testicular cells (Fenske et al. 2004).

Histological evidence in zebrafish of gonadal transition from ovary-type tissue (early default state in this species) to testis at 29-31 dph was observed in fish exposed via the diet to fadrozole from 15-45 dph. By the end of the experiment, exposure to 10, 100 or 1000 ug fadrozole/g diet resulted in male-biased sex ratios of 62.5, 100 and 100%, respectively (Uchida et al. 2004).

Luzio et al. (2015; 2016a; 2016b) conducted a series of studies in which zebrafish were exposed to fadrozole for varying periods of time starting at 2 hours post-hatch up to 90 dph. In all studies fadrozole caused enhanced histological evidence of testis development, with a greater than 90% occurrence of males by test conclusion, a condition that persisted up to 150 dph.

Nile tilapia (*Oreochromis niloticus*) exposed to dietary exemestane (500, 1000, 2000 ug/g) from 9-35 dph exhibited histological evidence of complete differentiation to testis in 100% of the animals classified as males in the 1000 and 2000 ug/g treatments (Ruksana et al. 2010).

Histological evidence of testis development in yellow catfish (*Pelteobagrus fulvidraco*) exposed to letrozole for 45 dph was associated with male-biased sex ratios (Shen et al. 2013).

Gonadal development in zebrafish exposed to fadrozole (10, 32, 100 ug/L water) from 0-63 dph exhibited accelerated differentiation to testis, resulting in male-biased sex ratios at all test concentrations (Muth-Kohne et al. 2016).

Uncertainties and Inconsistencies

A major uncertainty for this KER involves what would be defined as "normal" for degree of testis differentiation and by extension sex ratio. There needs to be knowledge as to baseline expectations for testis differentiation for a given species in a given habitat (or lab setting) to ascertain whether increases are occurring. Baseline information of this type is available or can be inferred for some species but certainly not for all that might be considered.

A second significant uncertainty involves situations where the gonad cannot be clearly defined as either testis or ovary. This can occur in some fish and amphibian species, where the gonad has cell types indicative of both testes and ovaries (Abdul-moneim et al. 2015). In these instances classification of individuals as male versus female may not be possible,

requiring a third category related to an intersex condition. There are seemingly multiple underlying causes of intersex, one of which appears to be exposure to estrogenic chemicals during gonad differentiation (Jobling et al. 1998; Norris et al. 2018; Grim et al. 2020).

A third uncertainty involves whether all individuals defined as males based on gonad phenotype will have the same degree of function in terms of producing viable gametes. It is possible, for example, that genotypic females which develop a male phenotype due to an environmental factor such as exposure to an endocrine-active chemical may not be functionally equivalent to a genetic male relative to sperm production/viability. This could be an important consideration relative to the types of predictions attempted based on a male-biased sex ratio in a population.

Quantitative Understanding of the Linkage

Because the degree of testis occurrence in a given population dictates the relative number of organisms defined as males, there is a direct quantitative relationship between the two KEs.

Response-response relationship

Not applicable.

Time-scale

Timescales will vary based on species-specific developmental rates, but since one KE often will define the second (i.e., an animal is defined as a male based on the presence of testis) timescale may not be a relevant consideration.

Known modulating factors

Not applicable.

Known Feedforward/Feedback loops influencing this KER

Not applicable.

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Relationship: 2147: Increased, Male Biased Sex Ratio leads to Decrease, Population growth rate

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	adjacent	Low	
Androgen receptor agonism leading to male-biased sex ratio	adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	Low	NCBI
Sphenodon punctatus	Sphenodon punctatus	High	NCBI
Strigops habroptilus	Strigops habroptilus	High	NCBI
Lacerta vivipara	Zootoca vivipara	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High

Any sexually-reproducing species theoretically could experience male-biased sex ratios and consequent population-level effects.

Key Event Relationship Description

Long-term maintenance of viable populations is dependent on the nature of interactions between males and females. One commonly used metric for capturing these interactions is evaluation of deviations from normal of the relative number of males versus females in a population. The ratio of males versus females needed for successful sexual reproduction varies by taxa, with some species requiring a one-to-one relationship, while in other species far fewer males than females may suffice in terms of producing an adequate number of fertile embryos to maintain a population. However, even in species potentially requiring fewer males than females to maintain a viable population, at some point a male-biased population could become problematic in terms of having an adequate number of males to fertilize eggs produced by females or, in the longer term, ensure a robust level of genetic diversity in a population. Further, in situations where a population is male-biased relative to conditions considered normal for a given species, overall productivity may be negatively impacted due to fewer females being available to produce eggs.

Evidence Supporting this KER

As described below there are both empirical data and population modeling/simulation approaches that provide evidence for this KER.

Biological Plausibility

The plausibility that a male-biased sex ratio would affect population status of different species is strong. For any given population, a male-biased sex ratio suggests that the number of available breeding females is reduced. If the male-biased sex ratio persists and/or increases over time, the offspring production will decrease and population size would be reduced. Additionally, for certain species, an increasing number of males could cause negative behavioral responses, for example, a higher competition for mating leading to more aggressive behaviors that can result in reduced adult survival rates for both male and females. A reduced effective population also affects genetic diversity, which can further reduce population viability.

Empirical Evidence

There have been limited examples of field evaluation of the consequences of male-biased sex ratios on population status, as well as several modeling efforts focused on aspects of population viability in situations where a male-skewed situation could occur. These analyses have focused on avian, reptile or fish species, several of which undergo at least some degree of environmental sex determination.

- Surveys and viability analyses of a Tuatara (*Sphenodon punctatus*) population by Grayson et al. (2014) showed that a current population of 56% males at hatching would result in a 12% probability of extinction within the timeframe of the analysis (60 of 500 simulated populations become extinct, mean time to extinction=1183.3 years).
- Using a behavioral approach Le Galliard et al. (2005) looked at how male-biased sex ratios in the common lizard (*Lacerta vivipara*) can negatively impacted mating to reduce population viability.
- In Kakapo (*Strigops habroptilus*), an endangered parrot species, male-biased production was shown to result in a prolonged species recovery, which risks conservation efforts to build a sustainable population and prevent the species from going extinct (Clout et al 2002; Robertson et al. 2006).
- A model-based viability analysis by Brown et al. (2015) showed that a male-biased population due to environmental stressors could lead to a sharp decline in zebrafish (*Danio rerio*) population levels.
- Miller et al. (2022) developed a matrix model for fathead minnow (*Pimephales promelas*) that demonstrated how even minor increases in the proportion of males in this species could substantially affect population status over time due to a loss of breeding females.

Uncertainties and Inconsistencies

Studies at the population level can be quite challenging in terms of required resources and, given the number of variables that might simultaneously influence a population, interpretation of results. Consequently, evaluation of population status in the context of adverse outcome pathways often relies upon model predictions that almost always are applicable only to a limited number of--sometimes one--species because of requirements associated with model parameterization. Given this, although it is entirely reasonable from an evolutionary perspective that male-biased sex ratios will negatively impact populations of a given species, it can be difficult to fully assess what this impact may be.

Quantitative Understanding of the Linkage

For a given species the linkage between a male-biased population and impacts on overall status of that population can be highly quantitative. For example, the model described by Miller et al. (2022) is designed specifically to provide quantitative forecasts of the effects of different male:female sex ratios on population status in fathead minnows. However, parameterization of any population model for vital rates (survival, reproductive output) is necessarily species-specific so, even if a given model construct is potentially suitable for a wide range of species, a significant amount of taxa-specific biological information might be needed to produce reliable quantitative predictions of effects.

Response-response relationship

Brown et al. (2015) and Miller et al. (2022) provide examples for zebrafish and fathead minnows, respectively, of approaches used to establish quantitative response-response relationships between male-biased sex ratios and population size/trends. In general, however, population models almost always rely on female productivity rather than male contributions to forecast population status.

Time-scale

The time-scale for this KER is entirely dependent on the life-cyle of the organism of interest. Small, short-lived animal species could experience population-level alterations due to biased sex ratios in days to weeks, while impacts on larger, long-lived species may take years to decades.

Known modulating factors

Population status can be impacted by a multitude of interacting biotic and abiotic variables, some of which could entirely supersede the effects of a male-biased sex ratio. For example, under conditions of severe food limitations or a regime of extreme temperature there may be no production of young irrespective of male:female sex ratios.

Known Feedforward/Feedback loops influencing this KER

It is difficult to define what form a feedforward/feedback loop might take for this KER. This would likely largely be a function of the stressor causing a male-biased population. If the stressor was short-term (e.g., affecting one age cohort) the situation might be self-correcting, as opposed to a longer-term stressor that continually causes a male-biased sex ratio, which theoretically should usually result in population extirpation.

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List of Non Adjacent Key Event Relationships

Relationship: 2167: Inhibition, Aromatase leads to Increased, Differentiation to Testis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	non-adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
Oreochromis niloticus	Oreochromis niloticus	High	NCBI
red-eared slider	Trachemys scripta	Low	NCBI
African clawed frog	Xenopus laevis	Low	NCBI
Gallus gallus	Gallus gallus	Low	NCBI

Life Stage Applicability

Life Stage	Evidence
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage

The life stage applicable to this KER is developing embryos and juveniles during the gonadal differentiation. This KER is not applicable to sexually differentiated adults.

Sex

Because this KER occurs during differentiation, the relationship is relevant to animals with an undetermined (non-specific) sex.

Taxonomic Applicability

Sequencing studies with mammalian, amphibian, reptile, bird, and fish species have shown that aromatase is well conserved among all vertebrates (Wilson et al. 2005; LaLone et al. 2018).

However, it is difficult to predict the biological domain of applicability of this KER based on phylogenetic characteristics. There is considerable within class variability, for example, among both fish and reptile species as to the role of aromatase expression and estrogen signaling in determining gonadal sex (Angelopoulou et al. 2012; Sarre et al. 2004). Thus susceptibility and relative sensitivities may vary considerably among species.

Key Event Relationship Description

Prior to sex determination, many vertebrates have a bipotential gonad that can develop into testis or ovary depending on genetic makeup (genetic sex determination), environmental conditions during development (environmental sex determination) or a combination of both (Trukhina et al. 2013).

A key variable influencing gonad differentiation is the production of sex steroids such as 17ß-estradiol (E2) and testosterone (T). In many vertebrates, including a variety of fish species, the "default" gonadal sex is male, with the presence of E2 (or perhaps the relative relationship between E2 and T production/levels) controlling the alternative path to development of ovaries (Angelopoulou et al. 2012).

Cytochrome P450 aromatase (CYP191a) is the enzyme responsible for the conversion of T to E2 in gonadal tissues of vertebrates (Miller 1988; Simpson et al. 1994). Consequently, inhibition of CYP191a expression/activity during gonadal differentiation can lead to an increased occurrence of testis.

Evidence Supporting this KER

Biological Plausibility

Plausibility is high. CYP19a1 aromatase is rate-limiting for the synthesis of E2 in vertebrates (Simpson et al. 1994; Payne et al. 2004), so inhibition of the enzyme reduces E2 levels. Gonadal differentiation of many non-mammalian vertebrates, including a number of fish species, is dependent upon signaling associated with the sex steroids T and E2 (Guiguen et al. 2010; Nakamura 2010). In many of these species there exists a bipotential gonad during early development that, based on steroidal signaling, can differentiate into either testis of ovary. When the "default" differentiation pathway is to testis, as is often the case (Angelopoulou et al. 2012), decreases in E2 plausibly favor the development of testis.

Empirical Evidence

There is empirical evidence in several species representing different vertebrate classes that aromatase inhibition leads to increased differentiation to testis.

Fish

- An established chemical inhibitor of CYP19a1, fadrozole, has been shown to cause a concentration-dependent inhibition of aromatase activity in zebrafish (*Danio rerio*) during gonadal differentiation resulting in a shift towards male development (Fenske et al. 2004; Luzio et al. 2015; Luzio et al. 2016; Muth-Köhne et al. 2016; Luzio et al. 2016)
- Generation of cyp19a1a (brain form of aromatase) and cyp19a1b (gondadal form of aromatase) gene mutant lines and a cyp19a1a;cyp19a1b double knockout line in zebrafish using transcription activator like effector nucleases (TALENs) has shown that cyp19a1a mutants and cyp19a1a;cyp19a1b double mutants result in all male gonadal phenotypes (Lau et al. 2016; Yin et al. 2017) . This was characterized by a high number of apoptotic cells and stromal cells by 29 days post fertilization (dpf) and by 40 dpf a typical testicular structure had appeared showing cystic spermatogeic cells.
- Nile tilapia (*Oreochromis niloticus*) treated with the aromatase inhibitor exemestane during sexual differentiation (from 9 through 35 dph) all had well developed testes by 120 dph (Ruksana et al., 2010)

- Additional studies with the Nile tilapia have shown that aromatase repression in the gonad is required to favor sexual differentiation to testis (Kwon et al., 2000; D'Cotta et al., 2001; Kwon et al. 2001)

Birds

- Studies with chicken (*Gallus g. domesticus*) embryos with the aromatase inhibitor letrozole on the first day of embryonic development has shown that the gonad of genetic females had poorly developed seminiferous tubules suggesting that they had undergone testicular sexual differentiation (Trukhina et al. 2016).
- Gonads of genetic female chickens treated at embryonic day 3.5 with the aromatase inhibitor Fadrozole were masculinized by the embryonic day 9.5 (Bannister et al., 2011).

Reptiles

- Administration of aromatase inhibitors CGS16949A and CGS20267 to red-eared slider turtle (*Trachemys scripta*) eggs incubated at female producing temperatures resulted in all male offspring (Crews and Bergeron 1994).

Amphibians

- In vitro exposure of *Xenopus laevis* (African clawed frog) gonads treated with the aromatase inhibitor CGS 16949A resulted in histological characteristics indicative of a male phenotype (Miyata and Kubo 2000).

Uncertainties and Inconsistencies

Due to substantial taxonomic variation in the role that steroid signaling plays in gonadal differentiation, the range of species that this key event relationship applies to is uncertain.

Quantitative Understanding of the Linkage

There are too few data to develop a quantitative understanding of the linkage between aromatase inhibition and increased differentiation to testis.

Response-response relationship

Not applicable.

Time-scale

The timeframe for differentiation of the bipotential gonad is species-dependent occurring, for example, over the course of days to weeks in most fishes. However, this period of time could be substantially longer in long-lived species.

Known modulating factors

There are almost certainly many factors that could modulate this KER, but a systematic description of these is not currently possible.

Known Feedforward/Feedback loops influencing this KER

None known.

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Relationship: 2350: Inhibition, Aromatase leads to Increased, Male Biased Sex Ratio

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	non-adjacent	Moderate	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI
Oreochromis niloticus	Oreochromis niloticus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
before or during gonadal sex differentiation	High

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage

The life stage applicable to this KER is developing embryos and juveniles during the gonadal differentiation. This KER is not applicable to sexually differentiated adults.

Sex

Because this KER occurs during differentiation, the relationship is relevant to animals with an undetermined (non-specific) sex.

Taxonomic Applicability

Sequencing studies with mammalian, amphibian, reptile, bird, and fish species have shown that aromatase is well conserved among all vertebrates (Wilson et al. 2005; LaLone et al. 2018).

However, it is difficult to predict the biological domain of applicability of this KER based on phylogenetic characteristics. There is considerable within class variability, for example, among both fish and reptile species as to the role of aromatase expression and estrogen signaling in determining gonadal sex (Angelopoulou et al. 2012; Sarre et al. 2004). Thus susceptibility and relative sensitivities may vary considerably among species.

Key Event Relationship Description

Prior to sex determination, many vertebrates have a bipotential gonad that can develop into testis or ovary depending on genetic makeup (genetic sex determination), environmental conditions during development (environmental sex determination) or a combination of both (Trukhina et al. 2013).

A key variable influencing gonad differentiation is the production of sex steroids such as 17ß-estradiol (E2) and testosterone (T). In many vertebrates, including a variety of fish species, the "default" gonadal sex is male, with the presence of E2 (or perhaps the relative relationship between E2 and T production/levels) controlling the alternative path to development of ovaries (Angelopoulou et al. 2012).

Cytochrome P450 aromatase (CYP19a1a) is the enzyme responsible for the conversion of T to E2 in gonadal tissues of vertebrates (Miller 1988; Simpson et al. 1994). Consequently, inhibition of CYPa191a expression/activity during gonadal differentiation can lead to an increased occurrence of testis. This can subsequently result in a male-biased sex ratio in the population of interest.

Evidence Supporting this KER

Biological Plausibility

This key event relationship is highly plausible. If inhibition of aromatase (E2 production) overlaps with the critical period of sex differentiation in a susceptible species there will be an increase in the number of organisms developing testes, which would produce a male-biased population.

Empirical Evidence

Studies with fish deficient in aromatase (knock-out experiments) as well as studies with known inhibitors of aromatase activity have shown increased occurrence of males.

- Several studies with zebrafish (*Danio rerio*) using the model aromatase inhibitor fadrozole administered via the diet during early development resulted in a predominant male population (Fenske et al. 2004; Uchida et al. 2004; Thresher et al. 2011).
- Other studies exposing early life-stage zebrafish via water to fadrozole also resulted in male-skewed populations (Luzio et al. 2015; Luzio et al. 2016; Luzio et al. 2016; Muth-Köhne et al. 2016).
- Dietary exposure of Nile tilapia (*Oreochromis niloticus*) to the aromatase inhibitor exemetane during early development resulted in 100% males in treated fish (Ruksana et al., 2010)
- In knockout studies of the aromatase gene using Nile tilapia and zebrafish, all *cyp19a1a*-deficient fish developed as males (Lau et al. 2016; Yin et al. 2017; Zhang et al. 2017)
- Exposure of zebrafish to the aromatase inhibitor clotrimazole induced male-skewed sex ratios (Brown et al. 2015)
- Exposure of fathead minnows (*Pimephales promelas*) and zebrafish (*Danio rerio*) to the aromatase inhibitor prochloraz skewed sex-ratios to males in a dose-dependent manner (Thorpe et al. 2011; Holbech et al. 2012).

Uncertainties and Inconsistencies

Due to substantial taxonomic variation in the role that steroid signaling plays in gonadal differentiation, the range of species that this key event relationship applies to is uncertain.

Quantitative Understanding of the Linkage

There are too few data to develop a quantitative understanding of the linkage between aromatase inhibition and increased relative number of males in populations.

Response-response relationship

Not applicable.

Time-scale

The timeframe for differentiation of the bipotential gonad to testis and, consequently, to a male phenotype is species-dependent occurring, for example, over the course of days to weeks in most fishes. However, this period of time could be substantially longer in long-lived species.

Known modulating factors

There are almost certainly many factors that could modulate this KER, but a systematic description of these is not currently possible.

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