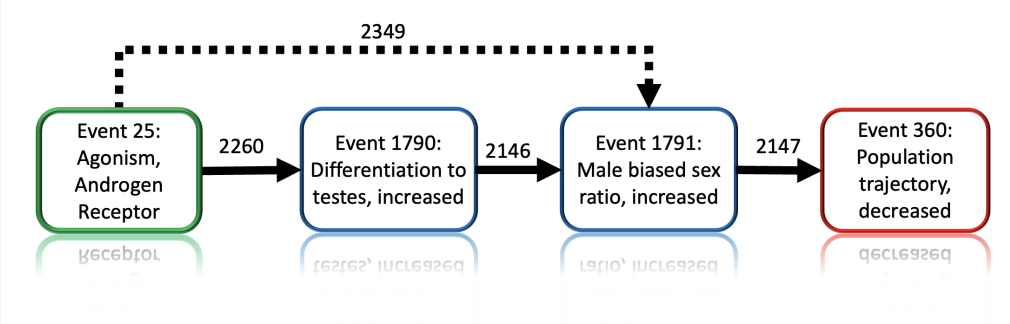
AOP ID and Title:

AOP 376: Androgen receptor agonism leading to male-biased sex ratio
Short Title: AR agonism leading to male-biased sex ratio

Graphical Representation



Authors

Kelvin J. Santana Rodriguez, Oak Ridge Institute for Science and Education, U.S. Environmental Protection Agency, Great Lakes Ecology Divison, Duluth, MN

Daniel L. Villeneuve, Kathleen M. Jensen, and Gerald Ankley, US Environmental Protection Agency, Great Lakes Toxicology and Ecology Division, Duluth, MN

David H. Miller, US Environmental Protection Agency, Great Lakes Toxicology and Ecology Division, Ann Arbor, MI.

Status

Author status	OECD status	OECD project	SAAOP status
Open for citation & comment			

Abstract

This adverse outcome pathway links androgen receptor agonism in teleost fish during gonadogenesis to male-biased sexual differentiation and consequently, reduced population growth rate. Sex determination in teleost fishes is highly plastic; it can be genetically or environmentally influenced. Species with environmentally-based sex determination in particular can be very sensitive to exogenous chemicals during the period of differentiation. Exogenous hormones are of ecological concern because they have the potential to alter gonad development and sex differentiation. Activation of the androgen receptor (AR) by endogenous androgens plays a crucial role in normal sex differentiation, sexual maturation, and spermatogenesis in vertebrates and inappropriate signaling by exogenous AR agonists can disrupt theses processes. For example, studies have shown that during early development in some teleost species, exposure to androgenic steroids can induce complete gonadal sex inversion, resuting in male-biased sex ratios. This can lead to impacts on population growth rates due to the decreased number of reproductively viable females in the population.

Background

This AOP shares multiple KEs and KERs with AOP 346 which links aromatase inhibition to male-biased sex ratios in vertebrates with environmental sex determination.

Summary of the AOP

Events

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

Sequence	Type	Event ID	Title	Short name

Sequence	Type	Event ID	Title	Short name
1	MIE	25	Agonism, Androgen receptor	Agonism, Androgen receptor
2	KE	1790	Increased, Differentiation to Testis	Increased, Differentiation to Testis
3	KE	1791	Increased, Male Biased Sex Ratio	Increased, Male Biased Sex Ratio
4	AO	360	Decrease, Population growth rate	Decrease, Population growth rate

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Agonism, Androgen receptor	adjacent	Increased, Differentiation to Testis		
Increased, Differentiation to Testis	adjacent	Increased, Male Biased Sex Ratio		
Increased, Male Biased Sex Ratio	adjacent	Decrease, Population growth rate		
Agonism, Androgen receptor	non-adjacent	Increased, Male Biased Sex Ratio		

Stressors

Name	Evidence
17beta-Trenbolone	High
Chemical:33664 17-Methyltestosterone	Moderate
5alpha-Dihydrotestosterone	Moderate
Methyldihydrotestosterone	Moderate
11-Keto-testosterone	Low

Overall Assessment of the AOP

See details below.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Development	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
medaka	Oryzias latipes	Low	NCBI
fathead minnow	Pimephales promelas	Low	NCBI
channel catfish	Ictalurus punctatus	Low	NCBI
Oreochromis niloticus	Oreochromis niloticus	Low	NCBI
Chinook salmon	Oncorhynchus tshawytscha	Low	NCBI
zebrafish	Danio rerio	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage

The life stage applicable to this AOP is developing embryos and juveniles prior to- or during the gonadal developmental stage. This AOP is not applicable to sexually differentiated adults.

Sex

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

Taxonomic

Most evidence for this AOP is derived from fish in the class Osteichthyes. Both phylogenetic analysis and evaluation of protein sequence conservation via SeqAPASS (https://seqapass.epa.gov/seqapass/) has shown that the structure of the AR is well conserved among most jawed vertebrates (Gnathostomata). This AOP is not expected to apply to mammals, birds, or other jawed vertebrates with genetic sex determination. However, it may be applicable to fishes, amphibians, and reptiles with environmentally-dependent sex determination, although outcomes may differ across physiologically different taxa. The present AOP is not considered relevant to Agnathans since the AR appears not to be present in jawless fishes (Thornton 2001; Hossain et al 2008).

Essentiality of the Key Events

Support for the essentiality of several of the key events in the AOP is provided by both in vivo and in vitro studies with chemicals acting as AR agonists and antagonists. Important in vivo studies typically have been conducted during the critical period of sexual differentiation.

Golan & Levavi-Sivian (2014) exposed genetic females of Nile tilapia (*Oreochromis niloticus*) to 17α-methyltestosterone (MT) and dihydrotestosterone (DHT), and showed that the two AR agonists induced a male biased sex ratio in a dose-dependent manner. However, in combined exposures with the AR antagonist flutamide (a pharmaceutical), the sex inversion potency of MT and DHT was reduced in a dose-dependent manner. This provides direct evidence that activation of the AR is required for the subsequent key events to occur.

Crowder et al. (2018) generated zebrafish (*Danio rerio*) with a mutation in the AR gene ($ar^{uab105/105}$). Most mutants developed ovaries and displayed female secondary sexual characteristics. The small percentage of mutants that developed as males displayed female secondary sexual characteristics with structurally disorganized testes, and were unable to produce normal levels of sperm. This demonstrates that the AR is required for proper testis development and fertility and supports the essentiality of AR activation in testis differentiation.

In a similar study with zebrafish, Yu et al. (2018) generated an AR gene mutant line using CRISPR/Cas9 technology. The number of female offspring was increased and the resulting AR-null males had female secondary sex characteristics and were infertile due to defective spermatogenesis. This study again supports the essentiality of AR agonism for the development of testis and how inappropriate (increased) signaling in the pathway could result in a male biased sex ratio.

Key Event	Evidence	Essentiality/Assessment
Agonism, Androgen	moderate	There is good evidence from a sex inversion treatment via the direct blocking of AR using androgen antagonist that support the specificity of androgen receptor agonism for the subsequent key events to occur.
Differentiation to Testis	moderate	Biological plausibility provides strong support for the essentiality of this event for the subsequent key events to occur. By definition, males have testis.
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, due to reduced numbers of females in the population, and consequentially reduced population growth rates.
Population Sustainability	weak	Full life-cycle and even multi-generation tests would be the ideal method for the detection of population-relevant endpoints. Modeling, however, supports this outcome from a conceptual perspective.

Weight of Evidence Summary

Biological Plausibility

The biological plausibility linking AR activation to increased differentiation to testis is very strong. Actions of androgens are mediated by the AR which is part of the nuclear receptor superfamily. ARs are ligand-dependent transcription factors (Hossain et al., 2008). Steroidal androgens act by entering the cell and forming a complex with the AR, resulting in conformational change (Bohen et al., 1995; Pratt and Toft, 1997). The ligand-AR complex is translocated to the nucleus where it binds to specific short DNA sequences (Androgen Responsive Elements), thereby activating transcripton of androgen regulated genes (Harbott et al., 2009). During sexual development, endogenous androgen can therefore induce the upregulation of many genes involved in the

male developmental pathway.

The direct link between increased differentiation to testis leading to a male biased sex ratio is highly plausible. If the conditions that favored a male producing phenotype (in this case, exposure to AR agonsts) overlap with the critical period of sex differentiation in a given population, it is reasonable that more phenotypic males will be produced (Orn et al., 2003; Seki et al., 2004; Bogers et al., 2006; Morthorst et al., 2010; Baumann et al., 2014; Golan & Levavi-Sivian 2014). Therefore, androgen exposure for repeated or prolonged periods of time conceptually will result in a male-biased population. Empirical evidence supporting the direct link between male-biased sex ratio and reduced population growth rate in fish species is lacking. However, altered sex ratios have the potential to affect fish populations (Marty et al. 2017). For example, a male-biased sex ratio suggests that the number of breeding females would be reduced. If the male-biased sex ratio persists and/or increases over time, the offspring produced per reproductive cycle would decrease, eventually leading to a diminished population size, assuming other demongraphic parameters remained largely constant (Brown et al. 2015; Grayson et al. 2014; Miller et al. 2022).

Concordance of Dose Response Relationships

The concentration-dependence of the key event responses with regard to the concentration of exogenous AR agonists has been established in vivo for some key events in the AOP. In general, effects on downstream key events occurred at concentrations equal to or greater than those at which upstream events occurred. However, binding to the androgen receptor (the MIE) was not directly measured in any of the in vivo studies. Nonetheless, AR binding of several of the agonists tested in vivo has been documented with in vitro studies with fish ARs (e.g., Wilson et al. 2004). In fish, phenotypic masculinization of female secondary sex characteristics has been used as an indirect measurement of in vivo AR agonism. However, in the of case this AOP specifically, AR agonism is occurring prior to sexual differentiation and the resultant “phenotypic measurement” for the in vivo study (gonad differentiation) is a discrete downstream key event. Consequently, in vitro evidence can reliably be used to identify specific chemicals as AR agonists (i.e., able to activate the MIE). That is, dependence of the severity of the downstream in vivo responses on concentration and potency of chemicals activating the AR in vitro can be used as indirect evidence of dose-response concordance between the MIE and downstream key events.

1. Concentration dependent androgen receptor agonism (in vitro)

- COS Whole Cell Binding Assay with fathead minnow AR (fhAR) were used in competitive binding experiments testing several natural and synthetic steroids, some of which are environmental contaminants, such as R1881, 17*beta*-trenbolone , and 17alpha-trenbolone. All showed a concentration dependent displacement of [3H]R1881 binding proving to be high affinity ligands for the fmAR. (Wilson et al., 2004).
    - The synthetic steroids, R1881 and methytestosterone, had the highest affinities of all the chemicals tested with IC50 values of about 1.6 nM, followed by the synthetic steroids 17α- and 17β-trenbolone with IC50 values of about 8 and 16 nM, respectively.
    - Of the natural steroids, dihydrotestosterone was the strongest competitor with an IC50 of about 20 nM. The IC50 for the fish specific androgen, 11- ketotestosterone, was approximately 40 nM, followed by both testosterone and androstenedione at about 100 nM
- Important to note that all of the above steroids tested were used in the in vivo studies that were selected to support this AOP demonstrating that all bound to the fhAR with a higher affinity than 11- ketotestosterone.

2. Concentration dependent increased differentiation to testes:

- Studies with zebrafish and Japanese medaka (*Oryzias latipes*) exposed to 17β-trenbolone during development resulted in masculinization in a concentration-dependent manner as evidenced from a significantly increased maturity of testes (Orn et al., 2006; Morthorst et al., 2010; Baumann et al., 2014) for some studies, this was determined ether by the abundance of spermatozoa and/or by the area of the testis.

3. Concentration dependent increased male biased sex ratio:

    - Exposure of developing zebrafish to different concentrations of 17β-trenbolone and dihydrotestosterone led to an increased number of males in a dose-dependent fashion (Orn et al., 2003; Holbech et al.,2006; Morthorst et al., 2010; Baumann et al., 2013; Baumann et al., 2014)

4. Concentration dependent decline in population trajectory:
    - Modeled population trajectories show a concentration-dependent reduction in projected population size (Brown et al 2015, Miller et al. 2022) based on the ratio of male to female. Population-level effects exposure of fish to AR agonists have not been measured directly.

Dose Concordance Table

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

We are aware of no cases where substantial exposure of susceptible teleost fish species during sexual differentiation to known AR agonists did not impact male sex ratios (for reviews see Pandian and Sheela 1995 and Leet et al. 2011). There are cases however, in which exposure to aromatizable androgens such as methyltestosterone can lead both to masculinization and feminization of fish (e.g., Piferrer et al. 1993; Orn et al., 2003); this most likely is due to conversion of the androgen to its corresponding estrogen analogue (i.e., methylestradiol; e.g., Hornung et al. 2004). In other instances, non-aromatizable androgens (e.g., dihydrotestosterone) have been reported to feminize fish exposed during early development (e.g., Davis et al. 1992; Bogers et al. 2006). The mechanism underlying this is uncertain, but plausibly could involve binding to the estrogen receptor which is known to interact with a variety of steroids, including androgens at comparatively test concentrations.

The adverse outcome is not wholly specific to this AOP. Key events included overlap with AOPs linking other MIEs (e.g., aromatase inhibition, AOP 346) that can lead to male biased sex ratios.

Uncertainties, inconsistencies, and data gaps

Overall, there is strong empirical support for the proposed AOP. We did not find notable inconsistencies in the literature reviewed as part of this AOP development. However, there were several notable data gaps which could potentially be addressed through further research:

(1) The detailed gene expression and signaling mechanisms via with AR activation induces differentiation to testes is not well understood or defined. If key steps in this process could be defined, one or more additional key events could potentially be inserted between Event 25 (agonism, androgen receptor) and Event 1790 (differentiation to testes, increased).

(2) Population-level consequences of a male biased sex ratio are based on modeling. At present, we found no empirical studies that establish the effect of a male-biased sex ratio on population growth rates. Current models assume that other demographic variables such as predation rates, prey availability, habitat availability, etc. are unaffected by sex ratio. This may or may not be the case in real-world populations.

Quantitative Consideration

At this time available data are insufficient to develop a quantitative AOP linking AR activation to male biased fish populations.

Considerations for Potential Applications of the AOP (optional)

Sex ratios can be a useful endpoint in risk and hazard assessment of chemicals. In July 2011, the Fish Sexual Development Test (FSDT) has officially been adopted as OECD test guideline no. 234 for the detection of EDCs within the OECD conceptual framework at level 4 (OECD, 2011b). The Fish Sexual Development Test (FSDT; OECD TG 234, OECD, 2011) covers endocrine disruption during the developmental period of sexual differentiation of particularly zebrafish and uses gonadal differentiation and sex ratio as endocrine disruption-associated endpoints for indicating EAS (estrogen, androgen and steroidogenesis) activity (Dang & Kienzler 2019). Therefore, this AOP can provide additional support to the use of alternative measurements in this type of tests by screening for androgen receptor agonists.

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Appendix 1

List of MIEs in this AOP

Event: 25: Agonism, Androgen receptor

Short Name: Agonism, Androgen receptor

Key Event Component

Process	Object	Action
androgen receptor activity	androgen receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:23 - Androgen receptor agonism leading to reproductive dysfunction (in repeat-spawning fish)	MolecularInitiatingEvent
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	MolecularInitiatingEvent

Stressors

Name
17beta-Trenbolone
Spironolactone
5alpha-Dihydrotestosterone

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

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

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

5alpha-Dihydrotestosterone

Chemical is a non-aromatizable androgen.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
fathead minnow	Pimephales promelas	High	NCBI
medaka	Oryzias latipes	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Female	High

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

Key Event Description

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

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

AR paralogs:

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

How it is Measured or Detected

Measurement/detection:

- **In vitro methods:**
  - OECD Test No. 458: Stably transfected human androgen receptor transcriptional activation assay for detection of androgen agonists and antagonists has been reviewed and validated by OECD and is well suited for detection of this key event (OECD 2016).
  - Binding to the androgen receptor can be directly measured in cell free systems based on displacement of a radio-labeled standard (generally testosterone or DHT) in a competitive binding assay (e.g., (Olsson et al. 2005; Sperry and Thomas 1999; Wilson et al. 2007; Tilley et al. 1989; Kim et al. 2010).
  - Cell based transcriptional activation assays are typically required to differentiate agonists from antagonists, in vitro. A number of reporter gene assays have been developed and used to screen chemicals for AR agonist and/or antagonist

activity (e.g., (Wilson et al. 2002; van der Burg et al. 2010; Mak et al. 1999; Araki et al. 2005).
    - Expression of androgen responsive proteins like spiggin in primary cell cultures has also been used to detect AR agonist activity (Jolly et al. 2006).
- **In vivo methods**:
    - In fish, phenotypic masculinization of females has frequently been used as an indirect measurement of in vivo androgen receptor agonism.
        - Development of nuptial tubercles, a dorsal fatpad, and a characteristic banding pattern has been observed in female fathead minnows exposed to androgen agonists (Ankley et al. 2003; Jensen et al. 2006; Ankley et al. 2010; LaLone et al. 2013; OECD 2012).
        - Anal fin elongation in female western mosquitofish (*Gambusia affinis*) has similarly been viewed as evidence of AR activation (Raut et al. 2011; Sone et al. 2005).
        - In medaka, development of papillary processes, which normally only appear on the second to seventh or eighth fin aray of the anal fin, has also been used as an indirect measure of androgen receptor agonism (OECD 2012).
        - Production of the nest building glue, spiggin, in three female 3-spined sticklebacks (Gasterosteus aculeatus) has also been well documented as an indicator of androgen receptor agonism (Jakobsson et al. 1999; Hahlbeck et al. 2004). Quantification of the spiggin protein in exposed female 3-spined stickleback or green fluorescence protein expression in a transgenic spg1-gfp medaka line (Sébillot et al. 2014) can be used to detect androgen receptor agonism.
- **High Throughput Screening**
    - Measures of AR agonism have been included in high throughput screening programs, such as US EPA's Toxcast program. Toxcast assays relevant for screening chemicals for their ability to bind and/or activate the AR include:
        - ATG_AR_TRANS A cell based assay that can differentiate agonism from antagonism
        - NVS_NR_hAR A cell free assay using recombinant human AR. Can detect binding, but cannot distinguish agonism from antagonism.
        - NVS_NR_rAR A cell free assay using recombinant rat AR. Can detect binding, but cannot distinguish agonism from antagonism.
        - OT_AR_ARELUC_AG_1440 A cell based assay that measures expression of a reporter gene under control of androgen-responsive elements. Can distinguish agonism from antagonism.
        - Tox21_AR_BLA_Agonist_ratio A cell based assay with an inducible reporter. Can distinguish agonists from antagonists.
        - Tox21_AR_LUC_MDAKB2_agonist A cell based assay with an inducible reporter. Can distinguish agonists from antagonists.
    - Assay descriptions

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List of Key Events in the AOP

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	KeyEvent
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Organ term

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	KeyEvent
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	KeyEvent

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)	AdverseOutcome
Aop:25 - Aromatase inhibition leading to reproductive dysfunction	AdverseOutcome
Aop:29 - Estrogen receptor agonism leading to reproductive dysfunction	AdverseOutcome
Aop:30 - Estrogen receptor antagonism leading to reproductive dysfunction	AdverseOutcome
Aop:100 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of female spawning behavior	AdverseOutcome
Aop:122 - Prolyl hydroxylase inhibition leading to reproductive dysfunction via increased HIF1 heterodimer formation	AdverseOutcome
Aop:123 - Unknown MIE leading to reproductive dysfunction via increased HIF-1alpha transcription	AdverseOutcome
Aop:155 - Deiodinase 2 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome
Aop:156 - Deiodinase 2 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:157 - Deiodinase 1 inhibition leading to increased mortality via reduced posterior swim bladder inflation	AdverseOutcome

AOP ID and Name	Event Type
Aop:158 - Deiodinase 1 inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:159 - Thyroperoxidase inhibition leading to increased mortality via reduced anterior swim bladder inflation	AdverseOutcome
Aop:101 - Cyclooxygenase inhibition leading to reproductive dysfunction via inhibition of pheromone release	AdverseOutcome
Aop:102 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with meiotic prophase I /metaphase I transition	AdverseOutcome
Aop:63 - Cyclooxygenase inhibition leading to reproductive dysfunction	AdverseOutcome
Aop:103 - Cyclooxygenase inhibition leading to reproductive dysfunction via interference with spindle assembly checkpoint	AdverseOutcome
Aop:292 - Inhibition of tyrosinase leads to decreased population in fish	AdverseOutcome
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	AdverseOutcome
Aop:16 - Acetylcholinesterase inhibition leading to acute mortality	AdverseOutcome
Aop:312 - Acetylcholinesterase Inhibition leading to Acute Mortality via Impaired Coordination & Movement	AdverseOutcome
Aop:334 - Glucocorticoid Receptor Agonism Leading to Impaired Fin Regeneration	AdverseOutcome
Aop:336 - DNA methyltransferase inhibition leading to population decline (1)	AdverseOutcome
Aop:337 - DNA methyltransferase inhibition leading to population decline (2)	AdverseOutcome
Aop:338 - DNA methyltransferase inhibition leading to population decline (3)	AdverseOutcome
Aop:339 - DNA methyltransferase inhibition leading to population decline (4)	AdverseOutcome
Aop:340 - DNA methyltransferase inhibition leading to transgenerational effects (1)	AdverseOutcome
Aop:341 - DNA methyltransferase inhibition leading to transgenerational effects (2)	AdverseOutcome
Aop:289 - Inhibition of 5α-reductase leading to impaired fecundity in female fish	AdverseOutcome
Aop:297 - Inhibition of retinaldehyde dehydrogenase leads to population decline	AdverseOutcome
Aop:346 - Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation	AdverseOutcome
Aop:326 - Thermal stress leading to population decline (3)	AdverseOutcome
Aop:325 - Thermal stress leading to population decline (2)	AdverseOutcome
Aop:324 - Thermal stress leading to population decline (1)	AdverseOutcome
Aop:363 - Thyroperoxidase inhibition leading to altered visual function via altered retinal layer structure	AdverseOutcome
Aop:349 - Inhibition of 11β-hydroxylase leading to decresed population trajectory	AdverseOutcome
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased population trajectory	AdverseOutcome
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	AdverseOutcome
Aop:386 - Deposition of ionizing energy leads to leading to population decline via inhibition of photosynthesis	AdverseOutcome
Aop:387 - Deposition of ionising energy leading to population decline via mitochondrial dysfunction	AdverseOutcome
Aop:388 - Deposition of ionising energy leading to population decline via programmed cell death	AdverseOutcome
Aop:389 - Oxygen-evolving complex damage leading to population decline via inhibition of photosynthesis	AdverseOutcome
Aop:364 - Thyroperoxidase inhibition leading to altered visual function via decreased eye size	AdverseOutcome
Aop:365 - Thyroperoxidase inhibition leading to altered visual function via altered photoreceptor patterning	AdverseOutcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	AdverseOutcome
Aop:410 - GSK3beta inactivation leading to increased mortality via defects in developing inner ear	AdverseOutcome
Aop:216 - Deposition of energy leading to population decline via DNA strand breaks and follicular atresia	AdverseOutcome
Aop:238 - Deposition of energy leading to population decline via DNA strand breaks and oocyte apoptosis	AdverseOutcome
Aop:299 - Deposition of energy leading to population decline via DNA oxidation and follicular atresia	AdverseOutcome
Aop:311 - Deposition of energy leading to population decline via DNA oxidation and oocyte apoptosis	AdverseOutcome
Aop:444 - Ionizing radiation leads to reduced reproduction in Eisenia fetida via reduced spermatogenesis and cocoon hatchability	AdverseOutcome

AOP ID and Name	Event Type
Aop:138 - Organic anion transporter (OAT1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:177 - Cyclooxygenase 1 (COX1) inhibition leading to renal failure and mortality	AdverseOutcome
Aop:97 - 5-hydroxytryptamine transporter (5-HTT; SERT) inhibition leading to population decline	AdverseOutcome
Aop:203 - 5-hydroxytryptamine transporter inhibition leading to decreased reproductive success and population decline	AdverseOutcome
Aop:218 - Inhibition of CYP7B activity leads to decreased reproductive success via decreased locomotor activity	AdverseOutcome
Aop:219 - Inhibition of CYP7B activity leads to decreased reproductive success via decreased sexual behavior	AdverseOutcome
Aop:323 - PPARalpha Agonism Impairs Fish Reproduction	AdverseOutcome

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

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2260: Agonism, Androgen receptor leads to Increased, Differentiation to Testis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
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	High	NCBI
medaka	Oryzias latipes		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 prior to- or during the gonadal developmental stage. This KER is not applicable to sexually differentiated adults.

Sex

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

Taxonomic

Most evidence for this KER is derived from fish in the class Osteichthyes. Both phylogenetic analysis and evaluation of protein sequence conservation via SeqAPASS (https://seqapass.epa.gov/seqapass/) has shown that the structure of the AR is well conserved among most jawed vertebrates (LaLone et al. 2018). This KER is not expected to apply to mammals, birds, or other jawed vertebrates with genetic sex determination. However, it may be applicable to fishes, amphibians, and reptiles with environmentally-dependent sex determination, although outcomes may differ across physiologically different taxa. The present KER is not considered relevant to Agnathans since the AR appears not to be present in jawless fishes (Thornton 2001; Hossain et al 2008).

Key Event Relationship Description

This key event relationship links androgen receptor agonism in teleost fish during gonadogenesis to increased differentiation to testis. Sex determination in teleost fishes is highly plastic; it can be genetically or environmentally influenced. Species with environmentally-based sex determination in particular can be very sensitive to exogenous chemicals during the period of differentiation. Exogenous hormones are of ecological concern because they have the potential to alter gonad development and sex differentiation. Activation of the androgen receptor (AR) by endogenous androgens plays a crucial role in normal sex differentiation, sexual maturation, and spermatogenesis in vertebrates and inappropriate signaling by exogenous AR agonists can disrupt theses processes. For example, studies have shown that during early development in some teleost species, exposure to androgenic steroids can induce complete gonadal sex inversion, resuting in increased differentiation to testis.

Evidence Supporting this KER

See below.

Biological Plausibility

The biological plausibility linking AR activation to increased differentiation to testis is very strong. Actions of androgens are mediated by the AR, a ligand-dependent transcription factors (Hossain et al., 2008). Steroidal androgens act by entering the cell and forming a complex with the AR, resulting in conformational change (Bohen et al., 1995; Pratt and Toft, 1997). The ligand-AR complex is translocated to the nucleus where it binds to specific short DNA sequences thereby activating transcripton of androgen regulated genes (Harbott et al., 2009). During sexual development, endogenous androgen can therefore induce the upregulation of many genes involved in the male developmental pathway, including gonad development/differentiation.

Empirical Evidence

Several studies with zebrafish (*Danio rerio*) with the known AR agonist 17β-trenbolone has shown to cause a concentration dependent increased differentiation to testis when exposed during the critical period of differentiation. This was evidenced via histological examinations to determine gonad maturation and sperm stage (Orn et al., 2006; Holbech et al. 2006; Morthorst et al., 2010; Baumann et al., 2015).

Additional studies with zebrafish exposed to dihydrotestosterone, another well established AR agonist, increased differentiation to testes was evidenced via the upregulation of dmrt1 and apoptosis-related genes but suppressed the transcription of cyp19a1a (aromtatase) during the sex differentiation period (Shi et al., 2018).

In similar studies using Japanese medaka (*Oryzias latipes*) exposure to 17β-trenbolone and dihydrotestosterone induced masculinization of both secondary sex characteristics and gonads when fish were exposed during development (Seki et al., 2004; Orn et al., 2006).

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 quatitative understanding of the linkage between AR activation 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.

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)

Known Feedforward/Feedback loops influencing this KER

None known.

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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

Term	Scientific Term	Evidence	Links
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

See below.

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 habitiat (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: 2349: Agonism, Androgen receptor leads to Increased, Male Biased Sex Ratio

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Androgen receptor agonism leading to male-biased sex ratio	non-adjacent		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebrafish	Danio rerio	High	NCBI
medaka	Oryzias latipes	Moderate	NCBI
Chinook salmon	Oncorhynchus tshawytscha	Moderate	NCBI
fathead minnow	Pimephales promelas	Moderate	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 prior to- or during the gonadal developmental stage. This KER is not applicable to sexually differentiated adults.

Sex

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

Taxonomic

Most evidence for this KER is derived from fish in the class Osteichthyes. Both phylogenetic analysis and evaluation of protein sequence conservation via SeqAPASS (https://seqapass.epa.gov/seqapass/) has shown that the structure of the AR is well conserved among most vertebrates (e.g., LaLone et al. 2018). This KER is not expected to apply to mammals, birds, or other vertebrates with genetic sex determination. However, it may be applicable to fishes, amphibians, and reptiles with environmentally-dependent sex determination, although outcomes may differ across physiologically different taxa. The present KER is not considered relevant to Agnathans since the AR appears not to be present in jawless fishes (Thornton 2001; Hossain et al 2008).

Key Event Relationship Description

This key event relationship (KER) links androgen receptor agonism in teleost fish during gonadogenesis to a male-biased sex ratio in a population. Sex determination in teleost fishes is highly plastic; it can be genetically or environmentally influenced. Species with environmentally-based sex determination in particular can be very sensitive to some steroid hormones during the period of differentiation. Exogenous hormones are of ecological concern because they have the potential to alter gonad development and sex differentiation. Activation of the androgen receptor (AR) by endogenous androgens plays a crucial role in normal sex differentiation, sexual maturation, and spermatogenesis in vertebrates and inappropriate signaling by exogenous AR agonists can disrupt theses processes. For example, studies have shown that during early development in some teleost species, exposure to androgenic steroids can induce complete gonadal sex inversion, resuting in increased differentiation to testis. This will result in a male-biased sex ratio in a population.

Evidence Supporting this KER

See below.

Biological Plausibility

The biological plausibility linking AR activation to a male-biased sex ratio in a population is very strong. Actions of androgens are mediated by the AR, a ligand-dependent transcription factors (Hossain et al., 2008). Steroidal androgens act by entering the cell and forming a complex with the AR, resulting in conformational change (Bohen et al., 1995; Pratt and Toft, 1997). The ligand-AR complex is translocated to the nucleus where it binds to specific short DNA sequences thereby activating transcripton of androgen regulated genes (Harbott et al., 2009). During sexual development, endogenous androgen can therefore induce the upregulation of many genes involved in the male developmental pathway, including gonad development/differentiation.

If the conditions that favor a male developmental pathway (in this case, exposure to AR agonsts) overlap with the critical period of sex differentiation in a given population, it is reasonable that more phenotypic males will be produced (Orn et al., 2003; Seki et al., 2004; Bogers et al., 2006; Morthorst et al., 2010; Baumann et al., 2014; Golan & Levavi-Sivian 2014). Therefore, androgen exposure for repeated or prolonged periods of time conceptually will result in a male-biased population.

Empirical Evidence

There have been several studies with teleost fish exposed to known androgen receptor agonists during early development that have documented a consequent occurrence of male-biased sex ratios.

- Exposure of fish to androgens during early development has been used as a technique to preferentially produce male-biased populations in aquaculture for decades. Pandian and Sheela (1995) provided a comprehensive overview of effects of hormones on sex inversion in the context of aquacultural practices. They reported, for example, that the synthetic androgen 17alpha-methyltestosterone had been used to successfully produce male-biased sex ratios in 25 different teleost species.
- Controlled expoure of zebrafish (*Danio rerio*) to the synthetic androgen 17β-trenbolone during development has been shown to result in male biased sex ratios (Holbech et al., 2006; Orn et al., 2006; Larsen & Baatrup, 2010; Morthorst et al., 2010; Baumann et al., 2013, 2015; Golan & Levavi-Sivian 2014). Zebrafish studies using binary mixtures of 17β-trenbolone with 17alpha-ethynylestradiol administered via the water also reported an elevated occurrence of males even when the estrogen was present (Orn et al., 2016)
- Exposure to methyltestosterone resulted in male biased cohorts in zebrafish, fathead minnows (*Pimephales promela*s), and Japanese medaka (*Oryzias latipes*) (Bogers et al., 2006; Orn et al., 2003; Seki et al., 2004)
- Exposure of zebrafish to dihydrotestosterone, an endogenous AR agonist, during early development reulted in a male biased sex ratio (Baumann et al., 2013; Shi et al., 2018).
- Two-hour immersion of newly hatched, homogametic female Chinook salmon (*Oncorhynchus tshawytscha*) in different synthetic and natural androgens (methyltestosterone, methyldihydrotestosterone and 11-ketotestosterone) reulted in a concentration dependent increase in male sex ratio in the treated fish (Piferrer & Donaldson, 1993)
- A concentration-dependent increase in percentage of males was observed in channel catfish (*Ictalurus punctatus*) that were orally

administered trenbolone acetate for 60 days starting with swim-up fry (Galvez et al., 1995)

Uncertainties and Inconsistencies

Some studies with sexually undifferentiated channel catfish have demonstrated that oral administration of androgens (methyltestosterone, 17a-ethynyltestosterone, dihydrotestosterone) during development can produce all female populations (Goudie et al., 1983; Davis et al., 1990, 1992). In some instances this could be due to the use of aromatizable androgens such as methyltestosterone that can lead both to masculinization and feminization of fish (e.g., Piferrer et al. 1993), due to conversion of the androgen to its corresponding estrogen analogue (i.e., methylestradiol; Hornung et al. 2004). In the cases of non-aromatizable androgens (e.g., dihydrotestosterone) that have been reported to feminize fish exposed during early development, the mechanism underlying this is uncertain, but plausibly could involve activation of the estrogen receptor, which is known to interact with a variety of steroids, including androgens at comparatively high test concentrations.

Also, as noted below, it is uncertain as to the full range of species this key event relationship might be applicable due to susbtantial taxonomic variation in the role that steroid signaling plays in gonadal differentiation.

Quantitative Understanding of the Linkage

There are too few data to develop a quatitative understanding of the linkage between AR activation and male biased sex ratio in fish.

Response-response relationship

Not applicable.

Time-scale

The timeframe for differentiation of the bipotential gonad and subsequent phenotypic expression of sex 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.

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)

Known Feedforward/Feedback loops influencing this KER

None known.

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