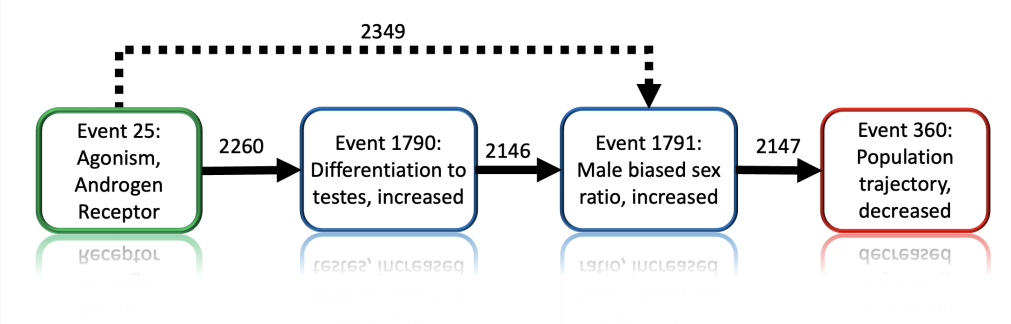
AOP ID and Title:

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

Graphical Representation



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Status

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Abstract

This adverse outcome pathway links androgen receptor agonism in teleost fish during gonadogenesis to male-biased sexual differentiation and successively, reduced population sustainability. Sex determination in teleost fishes is highly plastic; it can be both genetically and environmentally driven which can also make them very sensitive to environmental pollutants. Exogenous hormones are of ecological concern because they have the potential to alter gonad development and sex differentiation. Androgens play a crucial role in sex differentiation, sexual maturation, and spermatogenesis in vertebrates and their modes of action are mediated via androgen receptors (ARs). Like many steroid hormones, androgens acts by entering the cell and forming a complex with a its hormone receptor allowing it to enter the nucleus where it can bind to specific short DNA sequences (Androgen Responsive Elements) and serve as transcription factors of androgen mediated genes involved in the male differentiation pathway. Many studies have shown that administration during early development in some teleost species, sex steroids can induce complete gonadal sex inversion. This can result in male biased sex ratios which can heavily impact population fitness and survival given that the lack of females can reduce the reproductive production of the population.

Summary of the AOP

Events

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

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 trajectory	Decrease, Population trajectory

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 trajectory		
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
zebra fish	Danio rerio	High	NCBI
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

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

Taxonomic

The taxonomic applicability of this AOP is the class Osteichthyes. However phylogenetic analysis has shown that the *ar* genes appear to be specific to jawed vertebrates (Gnathostomata), since no *ar* gene has been reported from Agnatha (Thornton 2001; Hossain et al 2008). Therefore, because all key events in the present AOP can be applicable to most non-mammalian vertebrates, it is probable that this AOP could be relevant to amphibians, reptiles and birds as well. Though, the outcomes might differ due to species-specific differences.

Essentiality of the Key Events

Support for the essentiality of several of the Key Events in the AOP was provided mainly in combination of in vivo and in vitro studies of androgen receptor agonist and antagonist exposures during the critical period of sexual differentiation.

Golan & Levavi-Sivian 2014 exposed genetically female Nile tilapia (Oreochromis niloticus) to 17-a-methyltestosterone (MT) and dihydrotestosterone (DHT), two well-known androgen receptor agonisms that induces the male differentiation pathway and a dose dependent male biased sex ratio (downstream key events). However, when these are combined with androgen antagonist Flutamide, the sex inversion potency of androgen is reduced in a dose-dependent manner. The decrease in sex inversion efficiency caused by flutamide is due to the direct blocking of the androgen binding to its androgen receptor. Therefore, this suggest that androgen receptor agonism ligand binding is required for the subsequent key events to occur.

Crowder et al., 2018 generated zebrafish with a mutation in the ar gene ($ar^{uab105/105}$) and the resulting 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. This supports the essentiality for the androgen receptor agonism for the testis differentiation pathway to proceed.

In a similar study with zebrafish, Yu et al. 2018 generate ar gene mutant line using CRISPR/Cas9 technology. The resulting showed that 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 supports the essentiality for the ar agonism for the development of testis and subsequently 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.
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	weak	

Weight of Evidence Summary

Biological Plausibility

The biological plausibility linking androgen receptor agonism through the increased differentiation to testis is very solid. Actions of androgens are mediated by the androgen receptor. The Androgen Receptor (AR) is part of the nuclear receptor superfamily. ARs are ligand-dependent transcription factors and contain a highly conserved DNA-binding domain (DBD) and a moderately well conserved ligand-binding domain (LBD) (Hossain et al., 2008). Steroid hormones such as androgens acts by entering the cell and forming a complex with a its hormone receptor. After ligand binding, AR monomers undergo conformational change, dissociate from chaperone proteins, dimerize, and bind coactivator proteins (Bohen et al., 1995; Pratt and Toft, 1997). After this, the complex is translocated to the nucleus where it can bind to specific short DNA sequences (Androgen Responsive Elements) and serve as transcription factors of androgen mediated 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 also well supported by biological plausibility. If the conditions that favored a male producing phenotype (in this case, exposure to androgens) 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, persistence of androgen exposure for repeated or prolong periods of times within the habitat of given fish species, can result in a male-biased population. Empirical evidence supporting the direct link between male biased population and a reduced population sustainability in fish species is lacking. However, increasing or permanent biased sex ratios can definitely have significant effects in sustainable fish populations (Marty et al. 2017). A male-biased sex ratio already suggests that the number of breeding females is reduced. If the male-biased sex ratio persists and/or increases over time, the offspring production for such population could eventually decrease and consequently, population productivity would be reduced (Brown et al. 2015; Grayson et al. 2014).

Concordance of Dose Response

The concentration-dependence of the key event responses with regard to the concentration of androgens 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. Few studies that looked at multiple key events on this AOP were considered to be stronger support when evaluating the dose response relationship between key events. However, binding to the androgen receptor (the MIE) was not directly measured in any of the in vivo studies as this is often measured with in vitro studies. In fish, phenotypic masculinization of females has been used as an indirect measurement of in vivo androgen receptor agonism. However, in this case/aop, androgen receptor agonism is occurring prior to sexual differentiation and the resulting “phenotypic measurement” for the in vivo study is already considered as a separate downstream key event. Therefore, because support for the upstream key event is done in a different system (in vitro) than for the downstream key events (in vivo) , support for the dose concordance of the androgen receptor agonism (upstream) is done via in vitro studies that targeted the specific steroids used on the in vivo studies of the downstream key event.

1. Concentration depended 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 R188, 17*B*-trenbolone , and 17a-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 (Danio rerio) exposed to 17β-trenbolone and Japanese medaka (Oryzias latipes) resulted in masculinization at different biological effect levels 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 depended increased male biased sex ratio:

- Zebrafish (Danio rerio) exposed to different concentrations of 17β-trenbolone and Dihydrotestosterone lead to increased number of males in a dose-dependent way (Orn et al., 2003; Morthorst et al., 2010; Baumann et al., 2013; Baumann et al., 2014)

4. Concentration depended decline in population trajectory:
    - Modeled population trajectories show a concentration-dependent reduction in projected population size (Brown et al 2015, Miller et al. 2021?) based strongly on the ratio of male to female. Population-level effects have not been measured directly based on androgen receptor agonism.

Dose Concordance Table

Temporal concordance

Consistency

We are aware of no cases where the pattern of key events described was observed without also observing a significant impact on male sex ratios in teleost fish species. There are cases however, in which exposure to aromatizable androgens such as Methyltestosterone may lead to feminization of fish. This is due in part by excess aromatizable androgens available that can be converted to E2 and favor a female developmental pathway.

The adverse outcome is not specific to this AOP. Many of the key events included in this AOP overlap with AOPs linking other molecular initiating events during the period of development (ie. Aromatase inhibition, AOP 346) to male biased sex ratios.

Uncertainties, inconsistencies, and data gaps

It’s important to note that the use of aromatizable instead of non-aromatizable androgens for sex reversals exposures can lead to excess aromatizable androgens being converted to E2 and inducing a female developmental pathway. Chinook salmon (Oncorhynchus tshawytscha) exposed to Methyltestosterone showed a concentration dependent increase in percentage of males where 100% males were obtained at doses 400 ug/L. but at higher concentrations, the percentage of males decreased and at 10,000ug/L. the percentage of males reduced to 79.4%. (Peferrer & Donaldson, 1993).

However, there have been similar cases to the latter but with the use of non-aromatizable androgens such as Dihydrotestosterone in which the primary cause of this feminization is not clear yet. Bogers et al. 2006 exposed Fathead minnow (*Pimephales promelas*) to Dihydrotestosterone where fish exposed to 0.1 µg/L all had developed testes with one fish showing mixed sex (testis–ova). Exposure to 0.32 µg/L resulted in 80% males and 20% mixed gonads. But at 1.0ug/L the percentage of males reduced to 40% (40% female, 10% undifferentiated and 10% mixed gonad). Additionally, it has been reported that oral administration of non aromatizable androgen dihydrotestosterone to sexually undifferentiated Channel Catfish (*Ictalurus punctatus*) resulted in all female populations (Davis et al. 1992).

Quantitative Consideration

Based on the evidence reviewed during development of this AOP, quantitative understanding of the KERs is limited at this time.

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 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. Therefore, this AOP can provide additional support to the use of alternative measurements in this type of tests when screening for sex steroid hormones or any other type of EDC affecting androgen receptors.

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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
Organ term

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 gonad, the key genes for testicular differentiation and the structural morphology of the testes are highly conserved among vertebrates. Consequentially, this key even is applicable to most vertebrate taxa.

Key Event Description

Prior to sex determination in many vertebrates, the developing organism have a bipotential gonad that can be fated to either sex depending on the genetic makeup of the embryo (genetic sex determination), environmental conditions (environmental sex determination) or both.Among vertebrates, the primordial gonad and the structural morphology of the testes are highly conserved.

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 cells to differentiate into the different fetal gonad seminiferous cords surrounded by peritubular myoid cells and enclosing fetal germ cells. Sertoli cells can also differentiate into leydig cells. Successively, the interstitial Leydig cells differentiate and produce testosterone to induce masculinization (Fisher et al., 2003)

Although the timing and location of gene expression leading to this morphological development of the testis may differ among taxa, many vertebrate taxa share a common set of genes crucial for the testis differentiation pathway to be activated and be maintained. In most mammals, the autosomal gene SOX9 is first upregulated in the precursor Sertoli cells, which are important for proper testicular development and function. SOX9 works with fibroblast growth factor 9 (FGF9) in a feed-forward loop that represses female pathway genes such as the wnt family member 4 WNT4 an in turn maintaining the male pathway. After sex determination has been established, expression of DMRT1 (double- sex and mab-related transcription factor 1) in the developing gonads (during the downstream events of the testicular differentiation pathway) has been linked to proper development and maintenance of male gonads. For birds, it has been confirmed that DMRT1 is the bird sex- determining gene whereas for most mammals, the SRY gene initiates the testis determining molecular cascade (Marshall Graves et al., 2010; Trukhina et al., 2013).

How it is Measured or Detected

Histological examination by light microscopy are performed to identify the phenotypic sex characteristics. In general, phenotypic males in early development will show three main differentiating cell types; the gamete forming cells (spermatogonia), support cells (Sertoli cells) and hormone secreting cells (Leydig or interstitial cells).

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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

This key event is applicable to most non-mammalian vertebrates that exhibit environmental sex determination as their primary form of sex determination. Vertebrates with genetic sex determination as their primary form of sex determination but that often times exhibit sexual plasticity towards environmental conditions in their early sex determination stages resulting in a phenotypic sex different from the chromosomal and genetic make-up can be included in this key event.

Key Event Description

Animals that exhibit environmental sex determination (ESD) are often at risk of sex ratios being skewed toward a particular sex depending on the environmental conditions in which organisms are exposed during early developmental stages (Ospina-Alvarez et al., 2008;Stewart et al., 2014). This process is particular to every species with ESD as the conditions necessary for the development of either male or female gonads can vary among taxa. Exposure during the critical period of sex differentiation to environmentalconditions that lead offspring sex determination towards a male gonad differentiation pathway is capable of producing sex ratio alterations. Persistence of such male-producing environmental conditions for prolonged periods of times can result in a male-biased allocation among structured habitats for a given population (Brown et al., 2015).

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List of Adverse Outcomes in this AOP

Event: 360: Decrease, Population trajectory

Short Name: Decrease, Population trajectory

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: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 ID and Name	Event Type
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:299 - Excessive reactive oxygen species production leading to population decline via reduced fatty acid beta-oxidation	AdverseOutcome
Aop:311 - Excessive reactive oxygen species production leading to population decline via mitochondrial dysfunction	AdverseOutcome
Aop:216 - Excessive reactive oxygen species production leading to population decline via follicular atresia	AdverseOutcome
Aop:238 - Excessive reactive oxygen species production leading to population decline via lipid peroxidation	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 increased mortality via altered retinal layer structure	AdverseOutcome
Aop:349 - Inhibition of 11β-hydroxylase leading to decresed trajectory in fish	AdverseOutcome
Aop:348 - Inhibition of 11β-Hydroxysteroid Dehydrogenase leading to decreased trajectory in fish	AdverseOutcome
Aop:376 - Androgen receptor agonism leading to male-biased sex ratio	AdverseOutcome
Aop:386 - Increased reactive oxygen species production leading to population decline via inhibition of photosynthesis	AdverseOutcome
Aop:387 - Increased reactive oxygen species production leading to population decline via mitochondrial dysfunction	AdverseOutcome
Aop:388 - DNA damage 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 increased mortality via decreased eye size	AdverseOutcome
Aop:365 - Thyroperoxidase inhibition leading to increased mortality via altered photoreceptor patterning	AdverseOutcome
Aop:399 - Inhibition of Fyna leading to increased mortality via decreased eye size (Microphthalmos)	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

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

How it is Measured or Detected

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

Regulatory 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

Evidence Supporting this KER

Empirical Evidence

Fish

- Several studies with Zebrafish (Danio rerio) using different concentrations of well known androgen receptor agonism 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; Morthorst et al., 2010 Baumann et al., 2015).

- Additional studies with zebrafish exposed to Dihydrotestosterone, increased differentiation to testes was evidenced via the upregulation of dmrt1 (1.83-fold) and apoptosis-related genes but suppressed the transcription of cyp19a1a (3.16-fold) during the sex differentiation period (Shi et al., 2018).

- In similar studies using Japanese medaka (Oryzias latipes) exposed to androgen receptor agonists 17β-trenbolone and Dihydrotestosterone induced masculinization of both secondary sex characteristics and gonads when exposed during the period of development. (Seki et al., 2004; Orn et al., 2006)
-

Amphibians

- P. nigromaculatus tadpoles exposed to 17β-trenbolone (0.1, 1, 10 µg/L) from Gosner stage 24/25 to complete metamorphosis resulted in 85% males in all treatment groups. Like normal testes, the gonads with sex-ambiguous morphology (15b %) exhibited testicular histology, showing that the sex-ambiguous gonads were incomplete ovary-to-testis reversals. (Li et al. 2015).

Uncertainties and Inconsistencies

In three different frog species (*Xenopus laevis* (Pipidae) *Bufo*(*tes*) *viridis*(Bufonidae) and *Hyla arborea* (Hylidae)) exposed to three environmentally and/or physiologically relevant concentrations of Trenbolone (0.027 µg/L (10^{-10} M), 0.27 µg/L (10^{-9} M), 2.7 µg/L (10^{-8} M)) sex reversals nor masculinization of gonads was observed. Exposure only caused negative species-specific impacts on gonad morphology and differentiation after the completion of metamorphosis, independently of genetic sex. (Rozenblut-Kościsty et al., 2019).

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

Key Event Relationship Description

See biological plausibility.

Evidence Supporting this KER

Biological Plausibility

After sex has been determined, either by genetic and/or environmental factors, a cascade of molecular and cellular events will lead the pathway from which the phenotypic sex is build. For males, this involves the morphological development of the testis, for which the three main differentiating cell types are the gamete forming cells (spermatogonia), support cells (Sertoli cells) and hormone secreting cells (Leydig or interstitial cells).[44]

In species for which the environmental conditions during gonad development are capable of driving the phenotype towards a different pathway, altered sex ratios can occur. If the conditions that favor a male producing phenotype overlap withthe critical period of sex differentiation, it is plausible that more male offspring will be produced. Therefore, persistence of such conditions for repeated or prolong periods of times within the habitat of given species, will result in a male-biased allocation.

Empirical Evidence

- Crowding during the critical period of sex determination of the pejerrey (Odontesthes bonariensis) at 25 °C (a mixed-sex promoting temperature) has shown a higher cortisol and 11-KT titers, increased hsd11b2 transcription, and increased frequency of masculinization leading to a male-biased sex ratio (Garcia Cruz, E. et al., 2020)

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

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

Sex Applicability

Sex	Evidence
Male	High

Sex ratios considerations for population viability can be relevant to all living organisms.

Key Event Relationship Description

Sex ratio is a fundamental concept for population dynamics as sex skews can directly impact mating systems, genetic variation, population growth and sustainability. Many organisms, are often at risk of population dysfunction due to altered sex ratios, particularly for those present in habitats that are heavily impacted by human activities or climate change. For many vertebrae taxa, females carry the major reproductive production of the population. Consequentially, when male-biased sex ratio occurs, breeding female numbers decreases and population productivity is reduced. Thus, increasing male-biased sex ratios in populations of vulnerable species can put them at risk of extinction.

Evidence Supporting this KER

- Population viability analysis by Brown R. et al (2015) showed that male skews due to environmental stressors could lead to a sharp decline in zebrafish population and an increase risk of population extinction.

- Surveys and population viability analyses of the tuatara on the North Brother Island by Grayson, K. et al (2014) showed that the current population at 56% males at hatching result in a 12% probability of extinction within the 2000 year timeframe of the analysis (60 of 500 simulated populations become extinct, mean time to extinction = 1183.3 years 659.5 SE). With male biased sex ratio trends increasing though the years, the population is predicted to persist at hatchling sex ratios of up to 75% males. However, the study shows that probability of extinction becomes 100% when hatchling sex ratio is of 85% males (mean

time to extinction = 388.2 years 68.8 SE).

- On a behavioral approach, Le Galliard, J. F et al (2005) looked at how male-biased sex ratios on the common lizard (*Lacerta vivipara*)can negatively impact matting systems and further reduce population viability. The study showed that the presence of manycompeting males makes them more aggressive toward adult females causing fecundity drop, emigration and even reduced survival rates[34].

- For critically endangered species such as the Kakapo, male biased production results in a prolonged species recovery, which risks conservation efforts to build a sustainable population and prevent this species from going extinct [39,40].

Biological Plausibility

For any given population, a male-biased sex ratio already suggests that the number of breeding females is reduced. If the male-biased sex ratio persists and/or increases over time, the offspring production for such population could eventually decrease and consequently, population productivity would be reduced. Additionally, for certain species, an increasing number of males entail 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 affects genetic diversity, which can further reduce population viability due to possible fixation of deleterious alleles. Moreover, genetic-phenotypic mismatches in certain male-biased populations can also impact sex chromosomes as the reduced proportion of genetic males could lead to the loss of the Y chromosome [44, 48]. Consequentially, it is plausible that populations facing increasing male-biased sex ratios will be more vulnerable to population dysfunction and eventually reduced population sustainability. For some species with already critical habitats and population sizes, a male-biased sex ratio could make them more vulnerable to extinction.

Empirical Evidence

- Population viability analysis by Brown R. et al (2015) showed that male skews due to environmental stressors could lead to a sharp decline in zebrafish population and an increase risk of population extinction.

- Surveys and population viability analyses of the tuatara on the North Brother Island by Grayson, K. et al (2014) showed that the current population at 56% males at hatching, result in a 12% probability of extinction within the 2000 year timeframe of the analysis (60 of 500 simulated populations become extinct, mean time to extinction = 1183.3 years 659.5 SE). With male biased sex ratio trends increasing though the years, the population is predicted to persist at hatchling sex ratios of up to 75% males. However, the study shows that probability of extinction becomes 100% when hatchling sex ratio is of 85% males (mean time to extinction = 388.2 years 68.8 SE).

- On a behavioral approach, Le Galliard, J. F et al (2005) looked at how male-biased sex ratios on the common lizard (*Lacerta vivipara*) can negatively impact matting systems and further reduce population viability. The study showed that the presence of many competing males makes them more aggressive toward adult females causing fecundity drop, emigration and even reduced survival rates[34].

- For critically endangered species such as the Kakapo, male-biased production results in a prolonged species recovery, which risks conservation efforts to build a sustainable population and prevent this species from going extinct [39,40].

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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

Life Stage Applicability

Life Stage	Evidence
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High