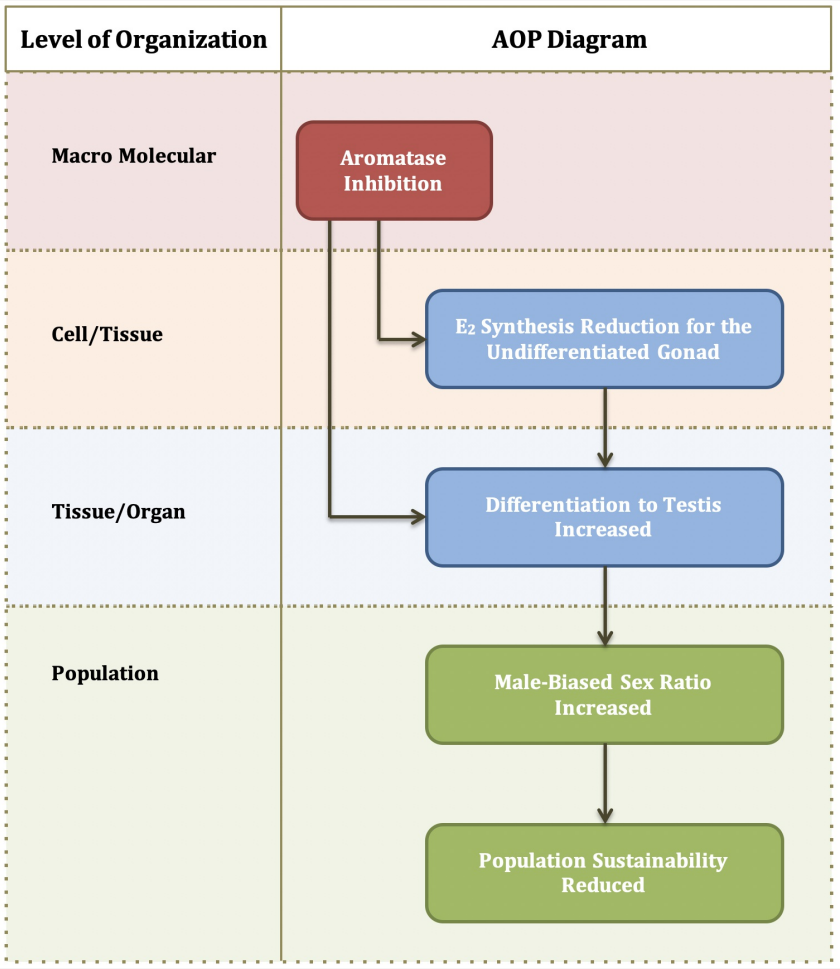
AOP ID and Title:

AOP 346: Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation
Short Title: Aromatase inhibition leads to male-biased sex ratio via impacts on gonad differentiation

Graphical Representation



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Status

Author status	OECD status	OECD project	SAAOP status
Under Development: Contributions and Comments Welcome			

Abstract

This adverse outcome pathway links inhibition of aromatase activity in teleost fish during gonadogenesis leading to a male-biased sex determination and successively, reduced population sustainability. Most gonochoristic fish species, develop either as males or females, and do not change sex throughout their entire life spans. However, there's a developmental window in which their sex determination can be sensitive to environmental conditions or chemical pollutants. Treatment with steroid hormones prior to sexual differentiation has shown to induce ovary or testis development according to the type of steroid that is administered. For most vertebrate taxa, aromatase (Cyp19a1) is the rate-limiting enzyme for the biosynthesis of 17β - estradiol from testosterone. Many endocrine disrupting chemicals such as fadrozole, letrozole and exemestane are well known chemicals that inhibit the activity aromatase. Exposure during the critical period of sex differentiation in gonochoristic teleost fish with an aromatase inhibitor that blocks estrogen biosynthesis can induce phenotypic males. Given that females carry the major reproductive production of the population, a male-biased sex ratio can result in a reduced population fitness, particularly for those species present in ecosystems that are heavily impacted by human activities.

Background

In fish, sexual differentiation occurs post hatching which makes them susceptible to the action of exogenous factors including hormones, temperature, pH, population density, social cues and more. As a result, the sex phenotype in most fish can be altered depending on the environmental conditions in which they are exposed during development, particularly during the critical period of sexual differentiation. At this stage, the bipotential gonad can be destined to take a testis or an ovary differentiation pathway that is reliant on both the genetic and environmental factors.

Sex steroid hormones are considered the natural inducers of sex differentiation for non-mammalian vertebrates where androgens and estrogens act, respectively, as testis and ovary inducers. In teleost fish, the hormonal balance between estrogens and androgens is essential during the sexual differentiation

period and this balance is in turn dependent on the availability and activity of steroid synthesizing enzymes such as aromatase[60].

Cytochrome P450 aromatase (CYP19) is the enzyme responsible for the conversion of C19 androgens to C18 estrogens in brain and gonadal tissues of vertebrates[52,70]. Therefore it a crucial enzyme for the female developmental pathway for many vertebrates. In fish, there are two isoforms of aromatase due to the teleost-specific whole-genome duplication. Cyp19a1a that is mostly expressed in the gonads and cyp19a1b that is expressed in the brain.

In recent years, there has been growing concern about the potential impacts of endocrine disrupting chemicals in the wildlife. Particularly of important concern, is the effects it can exert in early life stages that can lead to major impacts at the population level. Many EDC's are known to alter the sexual phenotype of fish by disrupting sex steroid synthesizing enzymes. Cyp1a1 can be a potential target for endocrine disrupting chemicals as it catalyzes the final step of estrogen biosynthesis which control crucial developmental and physiological processes.

Disruption of aromatase expression will alter the production rate of estrogens in the developing gonads, increasing an imbalance in the androgen-to-estrogen ratio leading to the disruption of estrogen related biological processes that lead to the determination and differentiation of the ovary. Therefore, as aromatase inhibitors block the synthesis of estrogens (by inhibiting the conversion of androgens to estrogens), the level of androgens in the developing organism increases, inducing testis differentiation and male maturation instead of a female developing pathway[7] . When the conditions that favor a male differentiation pathway persists, male biased sex ratios can occur. As a result, the number of breeding females can decrease over time and the population productivity can be affected. Therefore, altered sex ratios can have negative impacts on population growth and sustainability.

The present AOP provides the evidence framework of the negative impacts of aromatase inhibition at early developmental stage of teleost fish particularly during the critical period of sexual differentiation and how can this ongoing exposure on population can lead to a population dysfunction.

Summary of the AOP

Events

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

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

Key Event Relationships

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

Stressors

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

Stressor:292 Clotrimazole

Brown et al., 2015

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Development	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage

The life stage applicable to this AOP is developing embryos and juveniles prior to- or during the gonadal developmental stage. Since the sexually dimorphic expression of aromatase plays a crucial role in the differentiation to either testis or ovaries in the undifferentiated bipotential gonad, this key event relationship can be applicable to the exact stage of development at which the aromatase enzyme works to influence gonadal differentiation. This AOP is not applicable to sexually differentiated adults.

Studies with zebrafish have shown that both brain and gonadal aromatase expression can be observed at 20 days post-fertilization with and increase in expression at 25 days post-fertilization in zebrafish destined to become females which also coincided with onset of gonadal differentiation period (Lau et al. 2016). In tilapia, aromatase expression can be observed as early as 3-4 days post fertilization with and increase in expression starting at 11 days post-fertilization in genetic females (Kwon, J. et al. 2001). Additionally, it has been shown that the period of 7-14 days post-fertilization is the most sensitive towards an aromatase inhibitor and that a consecutive exposure of 2-3 weeks is sufficient for the masculinization of the majority of genetic female tilapia fish (Kwon, J. et al. 2000). This suggest that to redirect the sexual differentiation pathway from ovary to testis, an alteration of aromatase expression will be most effective during the early developmental stage prior and during the critical sex differentiation period.

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 among mammalian, amphibian, reptile, bird, and fish has shown that aromatase is well conserved among all vertebrates (Wilson JY et al., 2005). Additionally, CYP19 was detected in the amphioxus suggesting that it has possible origin in primitive chordates. 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 mind 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 by gene knockout of the cyp1a1 gene in zebrafish and tilapia. Teleost fish have two genes encoding for aromatase; *cyp1a1a* that is mainly expressed in the gonads and *cyp1a1b* expressed in the brain. Studies have demonstrated that mutant lines of *cyp1a1b* develop as females while *cypa1a* mutants develop as males suggesting that gonadal aromatase inhibition is crucial step for the subsequent key events to occur.

1. Lau et al. 2016[13] generated indel mutations in zebrafish *cyp19a1a* gene using TALEN and CRISPR/Cas9 approaches. All mutant *cyp19a1a*$^{-/-}$ developed as males. Histological examination (at 120 days post-fertilization) of the *cyp1a1a*$^{-/-}$ mutant showed that all exhibited normal spermatogenesis in the testis with no observable difference to the wild type (+/+) and heterozygous (+/-) males. However, to prove the role of E_2 synthesis for ovarian differentiation, they performed an experiment to rescue the phenotype of *cyp19a1a* mutant by E_2 treatment (0.05, 0.50 and 5.00 nM) over the time of gonadal differentiation (15–30 days port-fertilization). The result showed that exposure to E_2 caused normal ovarian formation with fully developed perinucleolar oocytes and little amount of stromal tissues, and the effect could be observed in some individuals even at the lowest concentration (0.05 nM). This supports the essentiality of aromatase inhibition relative to E_2 synthesis reduction as a critical step for testis differentiation.
2. On a similar study with zebrafish, Muth-Köhne et al. 2016[8] generated *cyp19a1a* and *cyp19a1b* gene mutant lines and a *cyp19a1a*;*cyp19a1b* double-knockout line in zebrafish using transcription activator-like effector nucleases (TALENs). All *cyp19a1a* mutants and *cyp19a1a;cyp19a1b* double mutants developed as males whereas *cyp1a1b* double mutant (-/-) had a 1:1 sex ratio similar to the wild type controls. This supports the essentiality of gonadal aromatase inhibition for testis differentiation leading to a male biased sex ratio population. Additionally, a rescue experiment was performed using 17 β-estradiol on all male mutant cyp1a1a$^{-/-}$ and the results suggested that treatment could rescue the sex ratio defect (9 females among 14 fish).
3. Similar support using Nile tilapia (Oreochromis niloticus) was provided in a study by Zhang et al. 2017[12]. Using genetic female mutant for *cypa1a* and *cyp1a1b*. Results showed that all *cyp19a1a*$^{+/-}$ XX and *cyp19a1a*$^{+/+}$ XX fish developed as females, whereas all *cyp19a1a*$^{-/-}$ XX and *cyp19a1a*-/- XY fish developed as males. The *cyp19a1a*$^{-/-}$ XX tilapia shifted to the male pathway at as early as 5 days after hatch (dah), as reflected by the gonadal expression and were fertile. This supports the essentiality of gonadal aromatase inhibition during early development for a testis differentiation pathway to be induced.

Key Event	Evidence	Essentiality/Assessment
Inhibition, Aromatase	strong	There is good evidence from gene knockout experiments of the two different isoforms of aromatase that support the specificity of gonadal aromatase inhibition for the subsequent key events to occur.
		There is evidence from a stop (by *cyp19a1*knockout) and recovery (through

E2 Synthesis by the undifferentiated gonad	moderate	compensation) experiment where E_2 can rescue the sex ratio altered due to the gonadal aromatase gene knockout suggesting that E_2 depletion is necessary for the subsequent key events to occur.
Differentiation to Testis	strong	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.

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Weight of Evidence Summary

Biological Plausibility

Aromatase is the key enzyme in the conversion of C19 androgens to C18 estrogens and the biological plausibility linking aromatase inhibition to E2 reduction is very solid. Additionally, the role of E2 as a major regulator for downstream estrogen-responsive genes necessary for proper female gonad development is well documented in literature (Gorelick et al. 2011; Guiguen et al. 2010). The link between E2 reduction for the undifferentiated gonad leading to an increased differentiation to testis is highly plausible. As the levels of estradiol are reduced, ER responsive genes required for proper ovarian differentiation will be downregulated in the bipotential gonad and instead allowing gene expression that leads to the morphological development of the testes due to an imbalance in the androgen to estrogen ratio (Shi et al., 2018; Yin et al. 2017; Zhang et al. 2017). Therefore, it is plausible that estradiol reduction in the undifferentiated gonad at the onset of sexual differentiation promotes testis differentiating in a concentration dependent manner (Baumann et al., 2015; Morthorst et al., 2010). 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, the aromatase inhibitor) overlap with the critical period of sex differentiation in a given population, it is reasonable that more male offspring will be produced (D'Cotta et al., 2001, Kwon et al., 2000; Luzio et al. 2016). Therefore, persistence of such conditions for repeated or prolong periods of times within the habitat of given 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 Relationship

Concentration dependence of the key events in response to the concentration of the aromatase inhibitor has been established for major key events in several teleost fish species using in vivo studies. The best supporting evidence would be studies that considered multiple key events in an in vivo study. However, in most of them there were exceptions. The differential sensitivity to inhibition of Cytochrome P450 Aromatase (CYP19) is best measured in vitro (Doering et al. 2019) but most studies that support this AOP are performed in vivo. There are cases in which the significant effect of reduced E2 was either not measured, measured at a time period outside the critical differentiation period, only one concentration of an aromatase inhibitor was used (Ruksana et al. 2010) or were gene knockout studies (Yin et al. 2017; Zhang et al. 2017) therefore these could not be considered for the dose-response relationship. Additionally, increased differentiation to testes is observed via histological examinations in which most studies using aromatase inhibitors only determined the general presence of male or female first and secondary characteristics but a degree of differentiation or differentiation stage of the gonads was not measured nor reported in most studies based on the doses. The most observable dose response relationship for this aop was for the non-adjacent relationship between aromatase inhibition and an increased male biased sex ratio in which several studies using multiple concentrations of an aromatase inhibitor leading to increased number of males in a dose-dependent way.

Concentration-dependent aromatase inhibition:

- Immunohistochemical analyses revealed that fish at 35 dah treated with higher concentrations of EM (500, 1000 and 2000 µg/g feed) had no reaction against P450arom but cells with strongly immunopositive responses against P450arom were evident in the lowest dose of EM (100 µg/g feed) similar to the differentiating ovaries of the control fish; these cells occurred as clusters in the vicinity of blood vessels (Ruksana et al. 2010)

Concentration dependent increased male biased sex ratio:

- Nile tilapia (Oreochromis niloticus), Fathead minnow (Pimephales promelas), Zebrafish (Danio rerio) exposed to different concentrations of aromatase inhibitors (Exemestane, Fadrozole, Prochloraz) lead to increased number of males in a dose-dependent way (Kwon et al., 2000; Uchida et al., 2004; Ruksana et al. 2010; Thorpe et al., 2011, Holbech et al., 2012).

Concentration dependent decline in population trajectory:

- Modeled population trajectories for male skews of zebrafish exposed to clotrimazole show a concentration-dependent reduction in projected population growth and viability (Brown et al. 2015). Population-level effects have not been measured directly.

Temporal Concordance

Temporal concordance of the AOP from aromatase inhibition to decreased E_2 production, increased differentiation to testes and increased male-biased sex ratio (e.g., (Ruksana et al., 2010; Yin et al. 2017; Zhang et al. 2017) has been established. However, beyond that key event, temporal concordance has not yet been established possibly due limiting capability to test and/or document particular population viability in situ. From the evidence gathered for this specific AOP, the best way to determine population viability is via multifactorial population viability analyses that generate the distribution of likely fates for a population exposed to endocrine disrupting chemicals that affect aromatase activity at the developmental stage.

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. 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. androgen receptor agonism, AOP 376) to male biased sex ratios.

Uncertainties, inconsistencies, and data gaps

Currently the major uncertainty in this AOP is the biological linkage between E2 synthesis reduction by the undifferentiated gonad leading to an increased, differentiation to testis. Biological plausibility connections have been established, but experimental measurements of E2 during the particular period of differentiation is lacking.

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 by screening for aromatase inhibitors.

References

Citation

Bannister, S. C., Smith, C. A., Roeszler, K. N., Doran, T. J., Sinclair, A. H., & Tizard, M. L. (2011). Manipulation of estrogen synthesis alters MIR202* expression in embryonic chicken gonads. Biology of reproduction, 85(1), 22–30. https://doi.org/10.1095/biolreprod.110.088476

Barakat, R., Oakley, O., Kim, H., Jin, J., & Ko, C. J. (2016). Extra-gonadal sites of estrogen biosynthesis and function. BMB reports, 49(9), 488–496. https://doi.org/10.5483/bmbrep.2016.49.9.141

Bogers, R., De Vries-Buitenweg, S., Van Gils, M., Baltussen, E., Hargreaves, A., van de Waart, B., De Roode, D., Legler, J., & Murk, A. (2006). Development of chronic tests for endocrine active chemicals. Part 2: An extended fish early-life stage test with an androgenic chemical in the fathead minnow (Pimephales promelas). *Aquatic Toxicology*, *80*(2), 119–130. https://doi.org/10.1016/j.aquatox.2006.07.020

Bondesson, M., Hao, R., Lin, C. Y., Williams, C., & Gustafsson, J. Å. (2015). Estrogen receptor signaling during vertebrate development. Biochimica et biophysica acta, 1849(2), 142–151. https://doi.org/10.1016/j.bbagrm.2014.06.005

Brown, A. R., Owen, S. F., Peters, J., Zhang, Y., Soffker, M., Paull, G. C., Hosken, D. J., Wahab, M. A., & Tyler, C. R. (2015). Climate change and pollution speed declines in zebrafish populations. Proceedings of the National Academy of Sciences of the United States of America, 112(11), E1237–E1246. https://doi.org/10.1073/pnas.1416269112

Butka, E. & Freedberg, S. (2018). Population structure leads to male-biased population sex ratios under environmental sex determination. Evolution. 73 (1), 99-110

Callard, G. V., Tarrant, A. M., Novillo, A., Yacci, P., Ciaccia, L., Vajda, S., Chuang, G. Y., Kozakov, D., Greytak, S. R., Sawyer, S., Hoover, C., & Cotter, K. A. (2011). Evolutionary origins of the estrogen signaling system: insights from amphioxus. The Journal of steroid biochemistry and molecular biology, 127(3-5), 176–188. https://doi.org/10.1016/j.jsbmb.2011.03.022

Canesini, G.; Ramos, J.G.; Muñoz de Toro, Monica, M.(2018) Determinación sexual y diferenciación gonadal en Yacaré overo. Genes involucrados en su regulación y efecto de la exposición a perturbadores endocrinos. (Unpublished Doctoral Thesis). Universidad Nacional Del Litoral

Capel, Blanche. (2017). Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. Nature Reviews Genetics. 18. 10.1038/nrg.2017.60.

Castro, L. F., Santos, M. M., & Reis-Henriques, M. A. (2005). The genomic environment around the Aromatase gene: evolutionary insights. BMC evolutionary biology, 5, 43. https://doi.org/10.1186/1471-2148-5-43

Cheshenko, K., Brion, F., Le Page, Y., Hinfray, N., Pakdel, F., Kah, O., Segner, H., & Eggen, R. I. (2007). Expression of zebra fish aromatase cyp19a and cyp19b genes in response to the ligands of estrogen receptor and aryl hydrocarbon receptor. *Toxicological sciences : an official journal of the Society of Toxicology*, *96*(2), 255–267. https://doi.org/10.1093/toxsci/kfm003

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., & Eggen, R. I. (2008). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. General and comparative endocrinology, 155(1), 31–62. https://doi.org/10.1016/j.ygcen.2007.03.005

Clout, M. & Elliott, G. & Robertson, B. (2002). Effects of supplementary feeding on the offspring sex ratio of Kakapo: a dilemma for the conservation of a polygynous parrot. Biological Conservation. 107. 13-18. 10.1016/S0006-3207(01)00267-1.

Cotton, S., & Wedekind, C. (2009). Population consequences of environmental sex reversal. Conservation biology : the journal of the Society for Conservation Biology, 23(1), 196–206. https://doi.org/10.1111/j.1523-1739.2008.01053.x

Coumailleau, P., Pellegrini, E., Adrio, F., Diotel, N., Cano-Nicolau, J., Nasri, A., Vaillant, C., & Kah, O. (2015). Aromatase, estrogen receptors and brain development in fish and amphibians. Biochimica et biophysica acta, 1849(2), 152–162. https://doi.org/10.1016/j.bbagrm.2014.07.002

Crews, D., & Bergeron, J. M. (1994). Role of reductase and aromatase in sex determination in the red-eared slider (Trachemys scripta), a turtle with temperature-dependent sex determination. The Journal of endocrinology, 143(2), 279–289. https://doi.org/10.1677/joe.0.1430279

Cutting, A., Chue, J., & Smith, C. A. (2013). Just how conserved is vertebrate sex determination?. Developmental dynamics : an official publication of the American Association of Anatomists, 242(4), 380–387. https://doi.org/10.1002/dvdy.23944

D'Cotta H, Fostier A, Guiguen Y, Govoroun M, Baroiller JF. Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia Oreochromis niloticus. Mol Reprod Dev. 2001;59(3):265-276. doi:10.1002/mrd.1031

D'Cotta, H., Fostier, A., Guiguen, Y., Govoroun, M., & Baroiller, J. F. (2001). Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia Oreochromis niloticus. Molecular reproduction and development, 59(3), 265–276. https://doi.org/10.1002/mrd.1031

Dang, Z. C., & Kienzler, A. (2019). Changes in fish sex ratio as a basis for regulating endocrine disruptors. *Environment International*, *130*(June), 104928. https://doi.org/10.1016/j.envint.2019.104928

DeFalco T, Capel B. Gonad morphogenesis in vertebrates: divergent means to a convergent end. Annu Rev Cell Dev Biol. 2009;25:457-482. doi:10.1146/annurev.cellbio.042308.13350

Eberhart-Phillips, Luke & Küpper, Clemens & Miller, Tom & Cruz-López, Medardo & Maher, Kathryn & dos Remedios, Natalie & Stoffel, Martin & Hoffman, Joseph & Krüger, Oliver & Székely, Tamás. (2017). Sex-specific early survival drives adult sex ratio bias in snowy plovers and impacts mating system and

population growth. Proceedings of the National Academy of Sciences. 114. 10.1073/pnas.1620043114.

Eick, G. N., & Thornton, J. W. (2011). Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. Molecular and cellular endocrinology, 334(1-2), 31–38. https://doi.org/10.1016/j.mce.2010.09.003

Fenske, M. & Segner, H. (2004). Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). Aquatic toxicology (Amsterdam, Netherlands). 67. 105-26. DOI 10.1016/j.aquatox.2003.10.008.

García-Cruz, E. L., Yamamoto, Y., Hattori, R. S., de Vasconcelos, L. M., Yokota, M., & Strüssmann, C. A. (2020). Crowding stress during the period of sex determination causes masculinization in pejerrey Odontesthes bonariensis, a fish with temperature-dependent sex determination. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology, 245, 110701. https://doi.org/10.1016/j.cbpa.2020.110701

Geffroy B, Wedekind C. Effects of global warming on sex ratios in fishes [published online ahead of print, 2020 Jun 10]. J Fish Biol. 2020;10.1111/jfb.14429. doi:10.1111/jfb.14429

Gerald T. Ankley, Michael D. Kahl, Kathleen M. Jensen, Michael W. Hornung, Joseph J. Korte, Elizabeth A. Makynen, Richard L. Leino, Evaluation of the Aromatase Inhibitor Fadrozole in a Short-Term Reproduction Assay with the Fathead Minnow (Pimephales promelas), Toxicological Sciences, Volume 67, Issue 1, May 2002, Pages 121–130, https://doi.org/10.1093/toxsci/67.1.121

Gorelick, D. A., & Halpern, M. E. (2011). Visualization of estrogen receptor transcriptional activation in zebrafish. Endocrinology, 152(7), 2690–2703. https://doi.org/10.1210/en.2010-1257

Grayson, K. L., Mitchell, N. J., Monks, J. M., Keall, S. N., Wilson, J. N., & Nelson, N. J. (2014). Sex ratio bias and extinction risk in an isolated population of Tuatara (Sphenodon punctatus). PloS one, 9(4), e94214. https://doi.org/10.1371/journal.pone.0094214

Guiguen, Y., Fostier, A., Piferrer, F., & Chang, C. F. (2010). Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. General and comparative endocrinology, 165(3), 352–366. https://doi.org/10.1016/j.ygcen.2009.03.002

Holbech, H., Kinnberg, K. L., Brande-Lavridsen, N., Bjerregaard, P., Petersen, G. I., Norrgren, L., Örn, S., Braunbeck, T., Baumann, L., Bomke, C., Dorgerloh, M., Bruns, E., Ruehl-Fehlert, C., Green, J. W., Springer, T. A., & Gourmelon, A. (2012). Comparison of zebrafish (Danio rerio) and fathead minnow (Pimephales promelas) as test species in the Fish Sexual Development Test (FSDT). *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *155*(2), 407–415. https://doi.org/10.1016/j.cbpc.2011.11.002

Hong, Y., Li, H., Yuan, Y. C., & Chen, S. (2009). Molecular characterization of aromatase. Annals of the New York Academy of Sciences, 1155, 112–120. https://doi.org/10.1111/j.1749-6632.2009.03703.x

Iwamatsu, Takashi & Kobayashi, Hirokuni & Sagegami, Reiko & Shuo, Takuya. (2006). Testosterone content of developing eggs and sex reversal in the medaka (Oryzias latipes). General and comparative endocrinology. 145. 67-74. 10.1016/j.ygcen.2005.07.003.

Jones, B. L., Walker, C., Azizi, B., Tolbert, L., Williams, L. D., & Snell, T. W. (2017). Conservation of estrogen receptor function in invertebrate reproduction. BMC evolutionary biology, 17(1), 65. https://doi.org/10.1186/s12862-017-0909-z

Kobayashi, Y., Nozu, R., & Nakamura, M. (2011). Role of estrogen in spermatogenesis in initial phase males of the three-spot wrasse (Halichoeres trimaculatus): effect of aromatase inhibitor on the testis. Developmental dynamics : an official publication of the American Association of Anatomists, 240(1), 116–121. https://doi.org/10.1002/dvdy.22507

Krovel, A. V. & Olsen, L. C. Sexual dimorphic expression pattern of a splice variant of zebrafish vasa during gonadal development. Dev. Biol. 271, 190–197 (2004).

Kwon, J. Y., Haghpanah, V., Kogson-Hurtado, L. M., McAndrew, B. J., & Penman, D. J. (2000). Masculinization of genetic female nile tilapia (Oreochromis niloticus) by dietary administration of an aromatase inhibitor during sexual differentiation. The Journal of experimental zoology, 287(1), 46–53.

Kwon, J. Y., McAndrew, B. J., & Penman, D. J. (2001). Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia Oreochromis niloticus. Molecular reproduction and development, 59(4), 359–370. https://doi.org/10.1002/mrd.1042

Lau, E. S., Zhang, Z., Qin, M., & Ge, W. (2016). Knockout of Zebrafish Ovarian Aromatase Gene (cyp19a1a) by TALEN and CRISPR/Cas9 Leads to All-male Offspring Due to Failed Ovarian Differentiation. Scientific reports, 6, 37357. https://doi.org/10.1038/srep37357

Le Galliard, J. F., Fitze, P. S., Ferrière, R., & Clobert, J. (2005). Sex ratio bias, male aggression, and population collapse in lizards. Proceedings of the National Academy of Sciences of the United States of America, 102(50), 18231–18236. https://doi.org/10.1073/pnas.0505172102

Li, M., Sun, L., & Wang, D. (2019). Roles of estrogens in fish sexual plasticity and sex differentiation. General and comparative endocrinology, 277, 9–16. https://doi.org/10.1016/j.ygcen.2018.11.015

Liao, P. H., Chu, S. H., Tu, T. Y., Wang, X. H., Lin, A. Y., & Chen, P. J. (2014). Persistent endocrine disruption effects in medaka fish with early life-stage exposure to a triazole-containing aromatase inhibitor (letrozole). Journal of hazardous materials, 277, 141–149. https://doi.org/10.1016/j.jhazmat.2014.02.013

Luzio, A., Matos, M., Santos, D., Fontaínhas-Fernandes, A. A., Monteiro, S. M., & Coimbra, A. M. (2016). Disruption of apoptosis pathways involved in zebrafish gonad differentiation by 17α-ethinylestradiol and fadrozole exposures. Aquatic toxicology (Amsterdam, Netherlands), 177, 269–284. https://doi.org/10.1016/j.aquatox.2016.05.029

Luzio, A., Monteiro, S. M., Rocha, E., Fontaínhas-Fernandes, A. A., & Coimbra, A. M. (2016). Development and recovery of histopathological alterations in the gonads of zebrafish (Danio rerio) after single and combined exposure to endocrine disruptors (17α-ethinylestradiol and fadrozole). Aquatic toxicology (Amsterdam, Netherlands), 175, 90–105. https://doi.org/10.1016/j.aquatox.2016.03.014

Luzio, A.,Monteiro, S., Garcia Santos, S., Rocha, E., Fontainhas-Fernandes, A.,& Coimbra, A. (2015). Zebrafish sex differentiation and gonad development after exposure to 17α-ethinylestradiol, fadrozole and their binary mixture: A stereological study. Aquatic Toxicology. 166. 83-95. DOI 10.1016/j.aquatox.2015.07.015.

Marshall Graves, J. A., & Peichel, C. L. (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options?. Genome biology, 11(4), 205. https://doi.org/10.1186/gb-2010-11-4-205

McLaren A. (1998). Gonad development: assembling the mammalian testis. Current biology : CB, 8(5), R175–R177. https://doi.org/10.1016/s0960-9822(98)70104-6

Mitchell, N. J., Kearney, M. R., Nelson, N. J., & Porter, W. P. (2008). Predicting the fate of a living fossil: how will global warming affect sex determination and hatching phenology in tuatara?. Proceedings. Biological sciences, 275(1648), 2185–2193. https://doi.org/10.1098/rspb.2008.0438

Miyata, S., & Kubo, T. (2000). In vitro effects of estradiol and aromatase inhibitor treatment on sex differentiation in Xenopus laevis gonads. General and comparative endocrinology, 119(1), 105–110. https://doi.org/10.1006/gcen.2000.7497

Miyoshi K, Hattori RS, Strüssmann CA, Yokota M, Yamamoto Y. Phenotypic/genotypic sex mismatches and temperature-dependent sex determination in a wild population of an Old World atherinid, the cobaltcap silverside Hypoatherina tsurugae. Mol Ecol. 2020;29(13):2349-2358. doi:10.1111/mec.15490

Muth-Köhne, E., Westphal-Settele, K., Brückner, J., Konradi, S., Schiller, V., Schäfers, C., Teigeler, M., & Fenske, M. (2016). Linking the response of endocrine regulated genes to adverse effects on sex differentiation improves comprehension of aromatase inhibition in a Fish Sexual Development Test. Aquatic toxicology (Amsterdam, Netherlands), 176, 116–127. https://doi.org/10.1016/j.aquatox.2016.04.018

Navarro-Martín, L., Blázquez, M., & Piferrer, F. (2009). Masculinization of the European sea bass (Dicentrarchus labrax) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. General and Comparative Endocrinology, 160(1), 3–11. https://doi.org/10.1016/J.YGCEN.2008.10.012

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., & Piferrer, F. (2011). DNA methylation of the gonadal aromatase (cyp19a)

promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS genetics, 7(12), e1002447. https://doi.org/10.1371/journal.pgen.1002447

Nef, S., & Parada, L. F. (2000). Hormones in male sexual development. Genes & development, 14(24), 3075–3086. https://doi.org/10.1101/gad.843800

Nishimura, T., & Tanaka, M. (2014). Gonadal development in fish. Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation, 8(5), 252–261. https://doi.org/10.1159/000364924

Nivelle, R., Gennotte, V., Kalala, E., Ngoc, N. B., Muller, M., Mélard, C., & Rougeot, C. (2019). Temperature preference of Nile tilapia (Oreochromis niloticus) juveniles induces spontaneous sex reversal. PloS one, 14(2), e0212504. https://doi.org/10.1371/journal.pone.0212504

Ospina-Alvarez, N., & Piferrer, F. (2008). Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PloS one, 3(7), e2837. https://doi.org/10.1371/journal.pone.0002837

Piferrer, F., & Blázquez, M. (2005). Aromatase distribution and regulation in fish. Fish physiology and biochemistry, 31(2-3), 215–226. https://doi.org/10.1007/s10695-006-0027-0

Piferrer, F., Zanuy, S., Carrillo, M., Solar, I. I., Devlin, R. H., & Donaldson, E. M. (1994). Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. Journal of Experimental Zoology, 270(3), 255–262. https://doi.org/10.1002/JEZ.1402700304

Robertson, B. C., Elliott, G. P., Eason, D. K., Clout, M. N., & Gemmell, N. J. (2006). Sex allocation theory aids species conservation. Biology letters, 2(2), 229–231. https://doi.org/10.1098/rsbl.2005.0430

Ruksana, S., Pandit, N. P., & Nakamura, M. (2010). Efficacy of exemestane, a new generation of aromatase inhibitor, on sex differentiation in a gonochoristic fish. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP, 152(1), 69–74. https://doi.org/10.1016/j.cbpc.2010.02.014

Santos, D., Luzio, A., & Coimbra, A. M. (2017). Zebrafish sex differentiation and gonad development: A review on the impact of environmental factors. Aquatic toxicology (Amsterdam, Netherlands), 191, 141–163. https://doi.org/10.1016/j.aquatox.2017.08.005

Schroeder, A. L., Ankley, G. T., Habib, T., Garcia-Reyero, N., Escalon, B. L., Jensen, K. M., Kahl, M. D., Durhan, E. J., Makynen, E. A., Cavallin, J. E., Martinovic-Weigelt, D., Perkins, E. J., & Villeneuve, D. L. (2017). Rapid effects of the aromatase inhibitor fadrozole on steroid production and gene expression in the ovary of female fathead minnows (Pimephales promelas). General and comparative endocrinology, 252, 79–87. https://doi.org/10.1016/j.ygcen.2017.07.022

Smirnov, Aleksandr & Trukhina, Antonina. (2019). Comparison of Sex Determination in Vertebrates (Nonmammals). 10.5772/intechopen.83831.

Stewart, K. R., & Dutton, P. H. (2014). Breeding sex ratios in adult leatherback turtles (Dermochelys coriacea) may compensate for female-biased hatchling sex ratios. PloS one, 9(2), e88138. https://doi.org/10.1371/journal.pone.0088138

Sun, L., Zha, J., Spear, P. A., & Wang, Z. (2007). Toxicity of the aromatase inhibitor letrozole to Japanese medaka (Oryzias latipes) eggs, larvae and breeding adults. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *145*(4), 533–541. https://doi.org/10.1016/j.cbpc.2007.01.017

Thornton J. W. (2001). Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proceedings of the National Academy of Sciences of the United States of America, 98(10), 5671–5676. https://doi.org/10.1073/pnas.091553298

Thorpe, K. L., Marca Pereira, M. L., Schiffer, H., Burkhardt-Holm, P., Weber, K., & Wheeler, J. R. (2011). Mode of sexual differentiation and its influence on the relative sensitivity of the fathead minnow and zebrafish in the fish sexual development test. Aquatic Toxicology, 105(3–4), 412–420. https://doi.org/10.1016/j.aquatox.2011.07.012

Trukhina, A. V., Lukina, N. A., Wackerow-Kouzova, N. D., & Smirnov, A. F. (2013). The variety of vertebrate mechanisms of sex determination. BioMed research international, 2013, 587460. https://doi.org/10.1155/2013/587460

Trukhina, Antonina & Lukina, Natalia & Smirnov, Aleksandr. (2016). Experimental Sex Inversion of Chicken Embryos at Aromatase Inhibition, Estrogen Receptor Modulation, DNA Demethylation and Progesterone Treatment. Natural Science. 08. 451-459. 10.4236/ns.2016.811047.

Uchida, D., Yamashita, M., Kitano, T., & Iguchi, T. (2004). An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology, 137(1), 11–20. https://doi.org/10.1016/s1095-6433(03)00178-8

Warner, D. A., Addis, E., Du, W. G., Wibbels, T., & Janzen, F. J. (2014). Exogenous application of estradiol to eggs unexpectedly induces male development in two turtle species with temperature-dependent sex determination. General and comparative endocrinology, 206, 16–23. https://doi.org/10.1016/j.ygcen.2014.06.008

Webster, K. A., Schach, U., Ordaz, A., Steinfeld, J. S., Draper, B. W., & Siegfried, K. R. (2017). Dmrt1 is necessary for male sexual development in zebrafish. Developmental biology, 422(1), 33–46. https://doi.org/10.1016/j.ydbio.2016.12.008

Wedekind C. (2017). Demographic and genetic consequences of disturbed sex determination. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 372(1729), 20160326. https://doi.org/10.1098/rstb.2016.0326

Wilson JY, McArthur AG, Stegeman JJ. Characterization of a cetacean aromatase (CYP19) and the phylogeny and functional conservation of vertebrate aromatase. Gen Comp Endocrinol. 2005;140(1):74-83. doi:10.1016/j.ygcen.2004.10.004

Yin, Y., Tang, H., Liu, Y., Chen, Y., Li, G., Liu, X., & Lin, H. (2017). Targeted Disruption of Aromatase Reveals Dual Functions of cyp19a1a During Sex Differentiation in Zebrafish. Endocrinology, 158(9), 3030–3041. https://doi.org/10.1210/en.2016-1865

Zhang, Xianbo & Li, Mengru & Ma, He & Liu, Xingyong & Shi, Hongjuan & Li, Minghui & Wang, Deshou. (2017). Mutation of foxl2 or cyp19a1a Results in Female to Male Sex Reversal in XX Nile Tilapia. Endocrinology. 158. 10.1210/en.2017-00127.

Appendix 1

List of MIEs in this AOP

Event: 36: Inhibition, Aromatase

Short Name: Inhibition, Aromatase

Key Event Component

Process	Object	Action
aromatase activity	aromatase	decreased

AOPs Including This Key Event

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

Stressors

Name
Fadrozole
Letrozole
Prochloraz

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
granulosa cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

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

Domain of Applicability

Taxonomic applicability: Aromatase (CYP19) orthologs are known to be present among most of the vertebrate lineage, at least down to the cartilaginous fishes. Orthologs have generally not been found in invertebrates, however, CYP19 was detected in the invertebrate chordate, amphioxus and analysis of conservation of gene order and content suggests a possible origin among primitive chordates (Castro et al. 2005). Fishes generally have two aromatase isoforms, cyp19a1a which is predominantly expressed in ovary and cyp19b, predominantly expressed in brain (Callard et al. 2001). Given that cyp19a1a is dominant isoform expressed in ovary and both isoforms appear to show similar sensitivity to aromatase inhibitors (Hinfray et al., 2006), for the purpose of this key event which focuses on gonadal aromatase activty, distinction of effects on one isoform versus the other are considered negligible. Total activity, without regard to isoform can be considered.

Key Event Description

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

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

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

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

How it is Measured or Detected

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

References

See Aromatase inhibition leading to reproductive dysfunction (in fish)

- Petkov PI, Temelkov S, Villeneuve DL, Ankley GT, Mekenyan OG. 2009. Mechanism-based categorization of aromatase inhibitors: a potential discovery and screening tool. SAR QSAR Environ Res 20(7-8): 657-678.
- Lephart ED, Simpson ER. 1991. Assay of aromatase activity. Methods Enzymol 206: 477-483.
- Letcher RJ, van Holsteijn I, Drenth H-J, Norstrom RJ, Bergman A, Safe S, et al. 1999. Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. Toxico App Pharm 160: 10-20.
- Sanderson J, Seinen W, Giesy J, van den Berg M. 2000. 2-chloro-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity. Toxicol Sci 54: 121-127.
- Villeneuve DL, Knoebl I, Kahl MD, Jensen KM, Hammermeister DE, Greene KJ, et al. 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (Pimephales promelas). Aquat Toxicol 76(3-4): 353-368.
- Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, et al. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (Pimephales promelas). Toxicol Sci 67: 121-130.
- Castro LF, Santos MM, Reis-Henriques MA. 2005. The genomic environment around the Aromatase gene: evolutionary insights. BMC Evol Biol 5: 43.
- Callard GV, Tchoudakova AV, Kishida M, Wood E. 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J Ster Biochem Mol Biol 79: 305-314.
- Cheshenko K, Pakdel F, Segner H, Kah O, Eggen RI. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen Comp Endocrinol. 2008 Jan 1;155(1):31-62.
- Hinfray N, Porcher JM, Brion F. Inhibition of rainbow trout (Oncorhynchus mykiss) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comp Biochem Physiol C Toxicol Pharmacol. 2006 Nov;144(3):252-62

List of Key Events in the AOP

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

Short Name: Reduction, E2 Synthesis by the undifferentiated gonad

Key Event Component

Process	Object	Action
estrogen biosynthetic process	17beta-estradiol	decreased

AOPs Including This Key Event

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

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
primordial germ cell

Organ term

Organ term
gonad

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Development	Moderate

Sex Applicability

Sex	Evidence

Unspecific Low

Sex **Evidence**

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

Key Event Description

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

References

Bondesson, M., Hao, R., Lin, C. Y., Williams, C., & Gustafsson, J. Å. (2015). Estrogen receptor signaling during vertebrate development. Biochimica et biophysica acta, 1849(2), 142–151.

Callard, G. V., Tarrant, A. M., Novillo, A., Yacci, P., Ciaccia, L., Vajda, S., Chuang, G. Y., Kozakov, D., Greytak, S. R., Sawyer, S., Hoover, C., & Cotter, K. A. (2011). Evolutionary origins of the estrogen signaling system: insights from amphioxus. The Journal of steroid biochemistry and molecular biology, 127(3-5), 176–188.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., & Eggen, R. I. (2008). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *General and comparative endocrinology*, *155*(1), 31–62.

Coumailleau, P., Pellegrini, E., Adrio, F., Diotel, N., Cano-Nicolau, J., Nasri, A., Vaillant, C., & Kah, O. (2015). Aromatase, estrogen receptors and brain development in fish and amphibians. *Biochimica et biophysica acta*, *1849*(2), 152–162.

Eick, G. N., & Thornton, J. W. (2011). Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. Molecular and cellular endocrinology, 334(1-2), 31–38.

Gorelick, D. A., & Halpern, M. E. (2011). Visualization of estrogen receptor transcriptional activation in zebrafish. Endocrinology, 152(7), 2690–2703. https://doi.org/10.1210/en.2010-1257

Guiguen, Y., Fostier, A., Piferrer, F., & Chang, C. F. (2010). Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *General and comparative endocrinology*, *165*(3), 352–366.

Jones, B. L., Walker, C., Azizi, B., Tolbert, L., Williams, L. D., & Snell, T. W. (2017). Conservation of estrogen receptor function in invertebrate reproduction. BMC evolutionary biology, 17(1), 65.

Li, M., Sun, L., & Wang, D. (2019). Roles of estrogens in fish sexual plasticity and sex differentiation. *General and comparative endocrinology*, *277*, 9–16. https://doi.org/10.1016/j.ygcen.2018.11.015

Ruksana, S., Pandit, N. P., & Nakamura, M. (2010). Efficacy of exemestane, a new generation of aromatase inhibitor, on sex differentiation in a gonochoristic fish. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP, 152(1), 69–74.

Schroeder, A. L., Ankley, G. T., Habib, T., Garcia-Reyero, N., Escalon, B. L., Jensen, K. M., Kahl, M. D., Durhan, E. J., Makynen, E. A., Cavallin, J. E., Martinovic-Weigelt, D., Perkins, E. J., & Villeneuve, D. L. (2017). Rapid effects of the aromatase inhibitor fadrozole on steroid production and gene expression in the ovary of female fathead minnows (Pimephales promelas). General and comparative endocrinology, 252, 79–87.

Thornton J. W. (2001). Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proceedings of the National Academy of Sciences of the United States of America, 98(10), 5671–5676.

Warner, D. A., Addis, E., Du, W. G., Wibbels, T., & Janzen, F. J. (2014). Exogenous application of estradiol to eggs unexpectedly induces male development in two turtle species with temperature-dependent sex determination. *General and comparative endocrinology*, *206*, 16–23.

Yin, Y., Tang, H., Liu, Y., Chen, Y., Li, G., Liu, X., & Lin, H. (2017). Targeted Disruption of Aromatase Reveals Dual Functions of cyp19a1a During Sex Differentiation in Zebrafish. *Endocrinology*, *158*(9), 3030–3041.

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 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).

References

Capel, Blanche. (2017). Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. Nature Reviews Genetics. 18. 10.1038/nrg.2017.60.

Cutting, A., Chue, J., & Smith, C. A. (2013). Just how conserved is vertebrate sex determination?. Developmental dynamics : an official publication of the American Association of Anatomists, 242(4), 380–387.

DeFalco T, Capel B. Gonad morphogenesis in vertebrates: divergent means to a convergent end. Annu Rev Cell Dev Biol. 2009;25:457-482. doi:10.1146/annurev.cellbio.042308.13350

Marshall Graves, J. A., & Peichel, C. L. (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options?. Genome biology, 11(4), 205. https://doi.org/10.1186/gb-2010-11-4-205

McLaren A. (1998). Gonad development: assembling the mammalian testis. *Current biology : CB*, *8*(5), R175–R177. https://doi.org/10.1016/s0960-9822(98)70104-6

Nishimura, T., & Tanaka, M. (2014). Gonadal development in fish. *Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation*, *8*(5), 252–261.

Santos, D., Luzio, A., & Coimbra, A. M. (2017). Zebrafish sex differentiation and gonad development: A review on the impact of environmental factors. *Aquatic toxicology (Amsterdam, Netherlands)*, *191*, 141–163.

Trukhina, A. V., Lukina, N. A., Wackerow-Kouzova, N. D., & Smirnov, A. F. (2013). The variety of vertebrate mechanisms of sex determination. BioMed research international, 2013, 587460. https://doi.org/10.1155/2013/587460

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).

References

Brown, A. R., Owen, S. F., Peters, J., Zhang, Y., Soffker, M., Paull, G. C., Hosken, D. J., Wahab, M. A., & Tyler, C. R. (2015). Climate change and pollution speed declines in zebrafish populations. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(11), E1237–E1246.

Canesini, G.; Ramos, J.G.; Muñoz de Toro, Monica, M.(2018) Determinación sexual y diferenciación gonadal en Yacaré overo. Genes involucrados en su regulación y efecto de la exposición a perturbadores endocrinos. (Unpublished Doctoral Thesis). Universidad Nacional Del Litoral

Ospina-Alvarez, N., & Piferrer, F. (2008). Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. *PloS one*, *3*(7), e2837.

Stewart, K. R., & Dutton, P. H. (2014). Breeding sex ratios in adult leatherback turtles (Dermochelys coriacea) may compensate for female-biased hatchling sex ratios. *PloS one*, *9*(2), e88138. https://doi.org/10.1371/journal.pone.0088138

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: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.

References

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

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Life Stage

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

Taxonomic Applicability

Phylogenetic analysis among mammalian, amphibian, reptile, bird, and fish has shown that aromatase is well conserved among all vertebrates (Wilson JY et al., 2005)[70]. Additionally, CYP19 was detected in the amphioxus suggesting that it has possible origin in primitive chordates. However this key event is only applicable to to vertebrates.

Evidence Supporting this KER

Empirical Evidence

- Tilapia (Oreochromis niloticus) reared at the standard 27°C showed that genetic males exhibited lower levels of aromatase gene expression and estradiol quantities during the critical period of sexual differentiation(18-26 days post fertilization) whereas a strong expression was detected for all genetic females for both aromatase gene expression and estradiol quantities. This correlation suggests that aromatase repression at the onset of sexual differentiation greatly reduces the biosynthesis of estradiol in the undifferentiated gonad. (D'Cotta et al., 2001)
- Generation of cyp19a1a and cyp19a1b gene mutant lines and a cyp19a1a;cyp19a1b double knockout line in zebrafish using transcription activator like effector nucleases (TALENs) showed that in both cyp19a1a-deficient and double knockout fish, the levels of estradiol were significantly lower than that in wild-type and cyp19a1b-deficient fish. [8]
- Control XY and cyp19a1a -/- (deficient and double knockout) XX nile tilapia fish had significantly lower levels of serum E2 when compared to the control XX and cyp19a1a+/- XX fish suggesting a decrease in E2 due to the cyp19a1a deficiency. [12]

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Development	Low

Key Event Relationship Description

Prior to sex determination, vertebrates have a bipotential gonad that can be fated to either sex depending on the genetic makeup of the embryo and the environmental conditions during development. This choice is governed by sets of genes that act to suppress each other, reinforcing one or the other cell fate.[17,43,42]

Once primary sex determination occurs, the production of hormones will influence the differentiation of male and female secondary sex characteristics. Endogenous steroid hormones are considered for most non-mammalian vertebrates the natural inducers of sex differentiation where estrogen and androgens act respectively as female and male inducers. The signaling actions of sex steroid hormones are mediated by their steroid nuclear receptors that bind to specific DNA sequences and activate gene transcription receptors that are crucial for gonad development.

Although both steroid hormones are required for proper development and/or maintenance of either gonad, it's the hormonal balance between the ratio of estrogens and androgens that seem to play a crucial role in the sex determination pathway the bipotenetial gonad will take.

Evidence Supporting this KER

Biological Plausibility

Among the different forms of estrogens, 17β-estradiol (estradiol) is considered the most fundamental in gonad differentiation in most vertebrates, as it is responsible for inducing and maintaining ovarian development (Bondesson et al., 2015; Li et al., 2019). Estrogens bind to estrogen receptors (ER), that regulate the transcription of downstream estrogen-responsive genes necessary for proper gonad development of for a female pathway (Guiguen et al., 2010; Gorelick et al., 2011). However, estradiol biosynthesis reduction during the critical period of sexual differentiation subsequently reduces estradiol plasma levels. As the levels of estradiol are reduced, ER responsive genes required for proper ovarian differentiation will not be expressed in the bipotential gonad and instead allowing gene expression that leads to the morphological development of the testis.Therefore, it is plausible that estradiol reduction in the undifferentiated gonad at the onset of sexual differentiation promotes testis differentiating in a concentration dependent manner.

Empirical Evidence

- During the critical period of sexual differentiation, repression or aromatase correlated with temperature-induced masculinization (35°F) of genetic male and females of Tilapia Oreochromis niloticus exposed from 10-40 days post fertilization suggesting that aromatase repression in the gonad is required to drive sexual differentiation to testis (D'cotta et al., 2001)
- Generation of cyp19a1a and cyp19a1b gene mutant lines and a cyp19a1a;cyp19a1b double knockout line in zebrafish using transcription activator like effector nucleases (TALENs) showed that all cyp19a1a-deficient and double knockout fish were all phenotypic males and the levels of estradiol were significantly lower than that in wild-type and cyp19a1b-deficient fish. [8]
- 17β-estradiol (estradiol) depletion due to the cyp19a1a deficiency correlated with and upregulation Dmrt1 and Sf1 in nile tilapia (12). Dmrt1 which is expressed in specifically in male gonads just after sex determination and has been linked to the determination and differentiation of testis in fish (53) and

many other vertebrates (42).

References

[42]Marshall Graves, J. A., & Peichel, C. L. (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options?. Genome biology, 11(4), 205. https://doi.org/10.1186/gb-2010-11-4-205

[43] Trukhina, A. V., Lukina, N. A., Wackerow-Kouzova, N. D., & Smirnov, A. F. (2013). The variety of vertebrate mechanisms of sex determination. BioMed research international, 2013, 587460. https://doi.org/10.1155/2013/587460

[53] Webster, K. A., Schach, U., Ordaz, A., Steinfeld, J. S., Draper, B. W., & Siegfried, K. R. (2017). Dmrt1 is necessary for male sexual development in zebrafish. Developmental biology, 422(1), 33–46. https://doi.org/10.1016/j.ydbio.2016.12.008

[60] Smirnov, Aleksandr & Trukhina, Antonina. (2019). Comparison of Sex Determination in Vertebrates (Nonmammals). 10.5772/intechopen.83831.

[61] D'Cotta, H., Fostier, A., Guiguen, Y., Govoroun, M., & Baroiller, J. F. (2001). Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia Oreochromis niloticus. Molecular reproduction and development, 59(3), 265–276. https://doi.org/10.1002/mrd.1031

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

AOPs Referencing Relationship

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

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
Development	High

Sex Applicability

Sex	Evidence
Unspecific	High

Taxonomic

Phylogenetic analysis among mammalian, amphibian, reptile, bird, and fish has shown that aromatase is well conserved among all vertebrates (Wilson JY et al., 2005)[70]. However in eutherian mammals (where sex determination is purely dependent on the chromosomal composition of the embryo) aromatase is expressed later in embryonic development and gonadal sex is formed independently of sex hormones [41, 43, 60]. Therefore, this key event relationship is only applicable to most non-mammalian vertebrates that do require sex steroid hormones for sex differentiation.

Life Stage

The life stage applicable to this key event relationship is developing embryos and juveniles prior to- or during the gonadal developmental stage. Since the sexually dimorphic expression of aromatase plays a crucial role in the differentiation to either testis or ovaries in the undifferentiated bipotential gonad, this key event relationship can be applicable to the exact stage of development at which the aromatase enzyme works to influence gonadal differentiation. This key event relationship is not applicable to sexually differentiated adults.

Key Event Relationship Description

Cytochrome P450 aromatase (CYP19) is the enzyme responsible for the conversion of C19 androgens to C18 estrogens in brain and gonadal tissues of vertebrates (Castro et al.,2005; Hong et al.,2009)[52,70]. During early developmental stages, the hormonal balance between estrogens and androgens is essential particularly durring the sexual differentiation period and this balance is in turn dependent on the availability and activity of steroid synthesizing

enzymes such as aromatase (Smirnov & Trukhina, 2019)[60]. For a bipotential gonad to differentiate into testis, an increase in the level of androgens is required to trigger the male differentiation pathway, while ovary differentiation requires increasing levels of estrogens (DeFalco 2019; Nef & Parada, 2000) [17, 69]. As aromatase inhibitors block the synthesis of estrogens (by inhibiting the conversion of androgens to estrogens), the level of androgens in the developing organism increases, inducing testis differentiation and male maturation (Muth-Kohne et al., 2016)[7].

Evidence Supporting this KER

Biological Plausibility

Inhibition of cytochrome P450 aromatase (CYP19) during the critical period of sexual differentiation of non-mammalian vertebrates can induce a male differentiation pathway due to an increasing imbalance in the androgen-to-estrogen ratio. Androgens have a critical physiological role in reproductive biology and sexual differentiation, particularly in the development of male first and secondary sex characteristics(DeFalco 2019) [17]. After sex has been determined, the increasing levels of androgens during the critical period of sexual differentiation will allow the morphological development of the testis, for which the early presence of three main differentiating cell types is fundamental; the gamete forming cells (spermatogonia), support cells (sertoli cells) and hormone secreting cells (leydig or interstitial cells) (Cotton & Wedekind, 2009)[44]. As gonads continue to differentiate into testes, the secretion of testicular hormones will be sufficient to promote the complete masculinization of the embryo (Nef & Parada, 2000) [69].

Empirical Evidence

Fish

- Well known aromatase inhibitor, fadrozole, has shown to cause concentration-dependent inhibition of aromatase activity in Zebrafish during the critical period of differentiation leading to a complete shift towards male development. [3,4, 6,7,9]
- Generation of cyp19a1a and cyp19a1b gene mutant lines and a cyp19a1a;cyp19a1b double knockout line in zebrafish using transcription activator like effector nucleases (TALENs) has shown that cyp19a1a mutants and cyp19a1a;cyp19a1b double mutants result in all male phenotypes [8,13] . This was characterized by high number of apoptotic cells and stromal cells by 29 days post fertilization and by 40 days post fertilization the typical testicular structure had appeared showing cystic spermatogeic cells[13].
- All nile tilapia fish treated with aromatase inhibitor Exemestane during the critical period of sexual differentiation (from 9 dah through 35 days after hatch) had well developed testes by 120 days after hatch.[5]
- Studies with Tilapia (*Oreochromis niloticus*), a species with genetic and environmental sex determination, have shown that aromatase repression in the gonad is required to drive sexual differentiation to testis. [61,71,72]

Birds

- Studies with chicken (*Gallus g. domesticus*) embryos using aromatase inhibitor (AI) letrozole on the first day of embryonic development has shown that the gonad of genetic females exposed to the AI had poorly developed seminiferous tubules suggesting that they had undergone testicular sexual differentiation pathway (Trukhina et al., 2016) [59]
- Female chicken (*Gallus g. domesticus*) gonads treated at embryonic day 3.5 with an aromatase inhibitor were masculinized by the embryonic day 9.5, and MIR202 expression was increased. Increased MIR202 expression correlated with up-regulation of DMRT1 and SOX9 which are required for proper testis development (Bannister et al., 2011)[62]

Reptiles

- Administration of aromatase inhibitors to red-eared slider turtle (*Trachemys scripta*) eggs incubated at female producing temperatures has shown to produce all male offspring (Crews & Bergeron, 1994)[56]

Amphibians

- Studies with in vitro *Xenopus laevis* gonads treated with aromatase inhibitor showed histological characteristics of the male phenotype. [58]

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)

References

[17] DeFalco T, Capel B. Gonad morphogenesis in vertebrates: divergent means to a convergent end. Annu Rev Cell Dev Biol. 2009;25:457-482.

[31] McLaren A. (1998). Gonad development: assembling the mammalian testis. Current biology : CB, 8(5), R175–R177.

[35] Butka, E. & Freedberg, S. (2018). Population structure leads to male-biased population sex ratios under environmental sex determination. Evolution. 73 (1), 99-110

[42] Marshall Graves, J. A., & Peichel, C. L. (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options?. Genome biology, 11(4), 205.

[43] Trukhina, A. V., Lukina, N. A., Wackerow-Kouzova, N. D., & Smirnov, A. F. (2013). The variety of vertebrate mechanisms of sex determination. BioMed research international, 2013, 587460. https://doi.org/10.1155/2013/587460

[44] Hill, M.A. (2020, August 1) Embryology Testis Development. Retrieved from https://embryology.med.unsw.edu.au/embryology/index.php/Testis_Development

[47] García-Cruz, E. L., Yamamoto, Y., Hattori, R. S., de Vasconcelos, L. M., Yokota, M., & Strüssmann, C. A. (2020). Crowding stress during the period of sex determination causes masculinization in pejerrey Odontesthes bonariensis, a fish with temperature-dependent sex determination. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology, 245, 110701. https://doi.org/10.1016/j.cbpa.2020.110701

[49] Miyoshi K, Hattori RS, Strüssmann CA, Yokota M, Yamamoto Y. Phenotypic/genotypic sex mismatches and temperature-dependent sex determination in a wild population of an Old World atherinid, the cobaltcap silverside Hypoatherina tsurugae. Mol Ecol. 2020;29(13):2349-2358. doi:10.1111/mec.15490

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].

References

[10] Brown, A. R., Owen, S. F., Peters, J., Zhang, Y., Soffker, M., Paull, G. C., Hosken, D. J., Wahab, M. A., & Tyler, C. R. (2015). Climate change and pollution speed declines in zebrafish populations. Proceedings of the National Academy of Sciences of the United States of America, 112(11), E1237–E1246.

[34]Le Galliard, J. F., Fitze, P. S., Ferrière, R., & Clobert, J. (2005). Sex ratio bias, male aggression, and population collapse in lizards. Proceedings of the National Academy of Sciences of the United States of America, 102(50), 18231–18236.

[35]Butka, E. & Freedberg, S. (2018). Population structure leads to male-biased population sex ratios under environmental sex determination. Evolution. 73 (1), 99-110 .

[36] Grayson, K. L., Mitchell, N. J., Monks, J. M., Keall, S. N., Wilson, J. N., & Nelson, N. J. (2014). Sex ratio bias and extinction risk in an isolated population of Tuatara (Sphenodon punctatus). *PloS one*, *9*(4), e94214.

[38] Wedekind C. (2017). Demographic and genetic consequences of disturbed sex determination. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 372(1729), 20160326.

[39] Robertson, B. C., Elliott, G. P., Eason, D. K., Clout, M. N., & Gemmell, N. J. (2006). Sex allocation theory aids species conservation. Biology letters, 2(2), 229–231.

[40] Clout, M. & Elliott, G. & Robertson, B. (2002). Effects of supplementary feeding on the offspring sex ratio of Kakapo: a dilemma for the conservation of a polygynous parrot. Biological Conservation. 107. 13-18. 10.1016/S0006-3207(01)00267-1.

[44]Cotton, S., & Wedekind, C. (2009). Population consequences of environmental sex reversal. Conservation biology : the journal of the Society for Conservation Biology, 23(1), 196–206.

[45]Eberhart-Phillips, Luke & Küpper, Clemens & Miller, Tom & Cruz-López, Medardo & Maher, Kathryn & dos Remedios, Natalie & Stoffel, Martin & Hoffman, Joseph & Krüger, Oliver & Székely, Tamás. (2017). Sex-specific early survival drives adult sex ratio bias in snowy plovers and impacts mating system and population growth. Proceedings of the National Academy of Sciences. 114. 10.1073/pnas.1620043114.

[46] Mitchell, N. J., Kearney, M. R., Nelson, N. J., & Porter, W. P. (2008). Predicting the fate of a living fossil: how will global warming affect sex determination and hatching phenology in tuatara?. *Proceedings. Biological sciences*, *275*(1648), 2185–2193.

[48] Geffroy B, Wedekind C. Effects of global warming on sex ratios in fishes [published online ahead of print, 2020 Jun 10]. *J Fish Biol*. 2020;10.1111/jfb.14429. doi:10.1111/jfb.14429

List of Non Adjacent Key Event Relationships

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

AOPs Referencing Relationship

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