A Key Event Relationship by Key Event Relationship Approach to Adverse Outcome Pathway Development: Peroxisome Proliferator-Activated Receptor Alpha Agonism Impairs Fish Fertility

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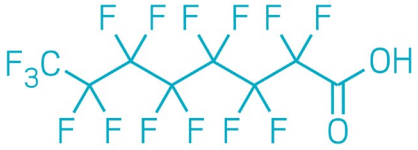
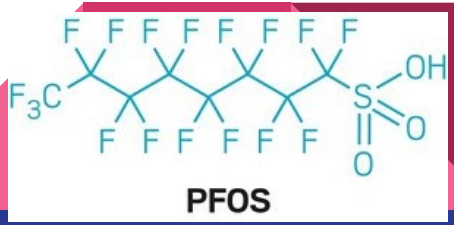
Introduction & Purpose of Development



PFOA

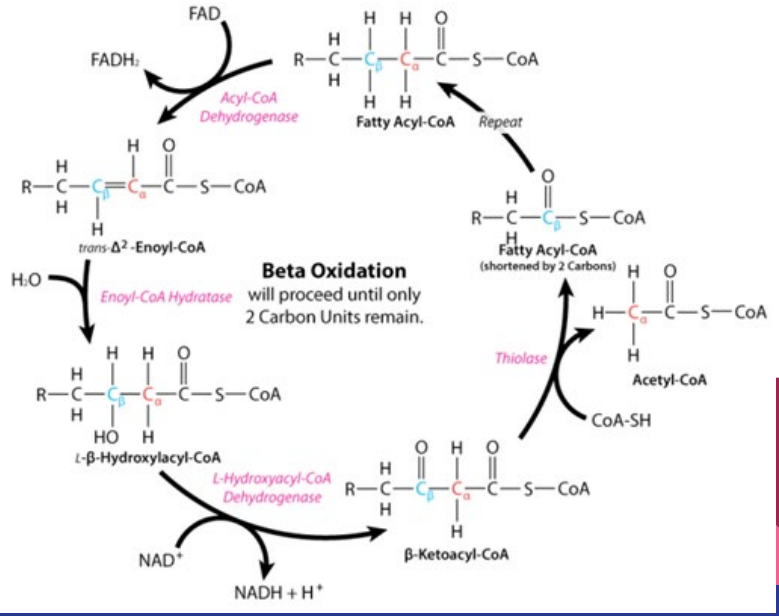
- In recent days, per- and polyfluoroalkyl substances (PFAS) has led to widespread concern due to their increasing prevalence within the environment and humans
  - PFAS - class of man-made chemicals used in a variety of industries and daily use
- One of EPA's focuses is to better understand the biological effects and targets that PFAS have
  - https://www.epa.gov/system/files/documents/2021-10/pfas-roadmap_final-508.pdf
- Recent high-throughput screening of over 140 PFAS demonstrated peroxisome proliferator-activated receptor alpha (PPARα) activity in multiple PFAS
- As a result, development of an adverse outcome pathway relating to PPARα agonism became of interest.



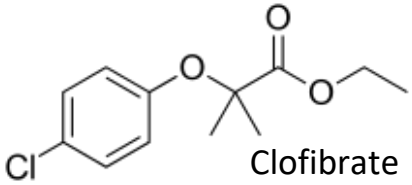
PFOS

What is PPARα?

- Ligand-activated nuclear receptor that dimerizes with Retinoid X Receptor
- When heterodimerized, the complex binds to regions of DNA known as peroxisome proliferator response elements (PPRE)
  - Promotes transcription of Fatty Acid β-oxidation genes
- Endogenous fatty acids are primary ligands
- Located in peroxisomes, mitochondria

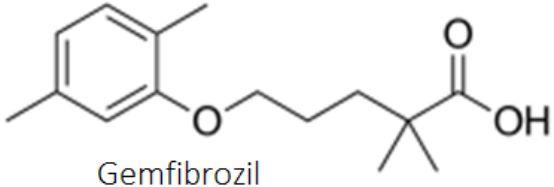


Early Development Stages/Strategy



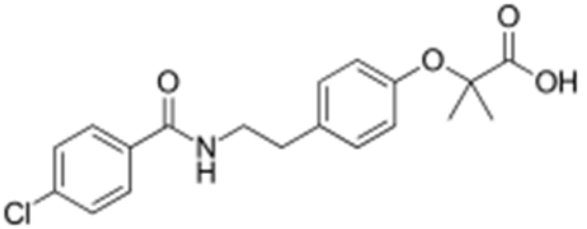
Clofibrate

- Fibrates drug studies on fish were used to develop AOP
  - Lack of studies involving PFAS and model organisms
  - Fibrates are a class of drugs designed to activate PPARα which lowers cholesterol



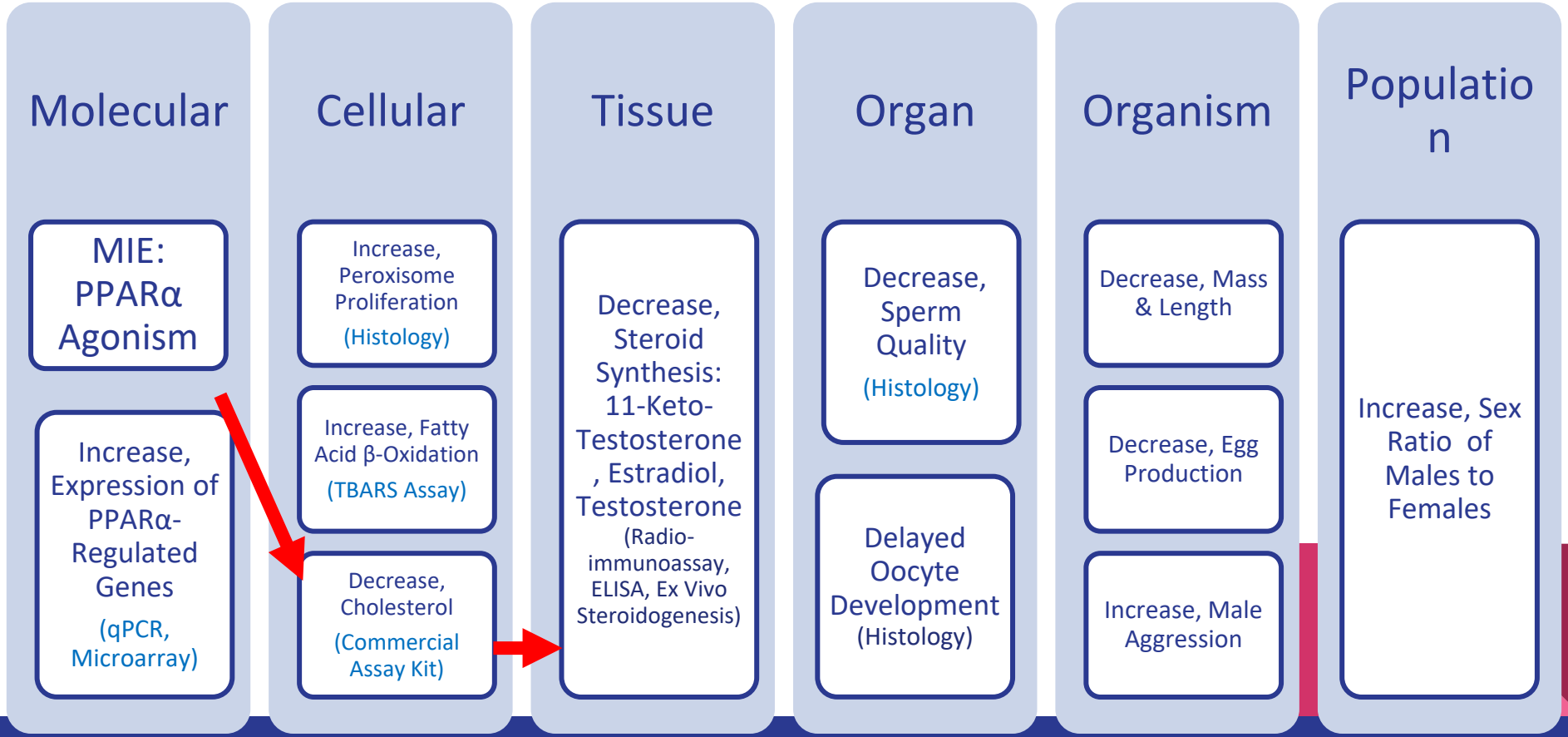
Gemfibrozil

- Initial development work also included
  - Genes regulated by PPARα in fish
  - Sources via google scholar searches and ECOTOX queries

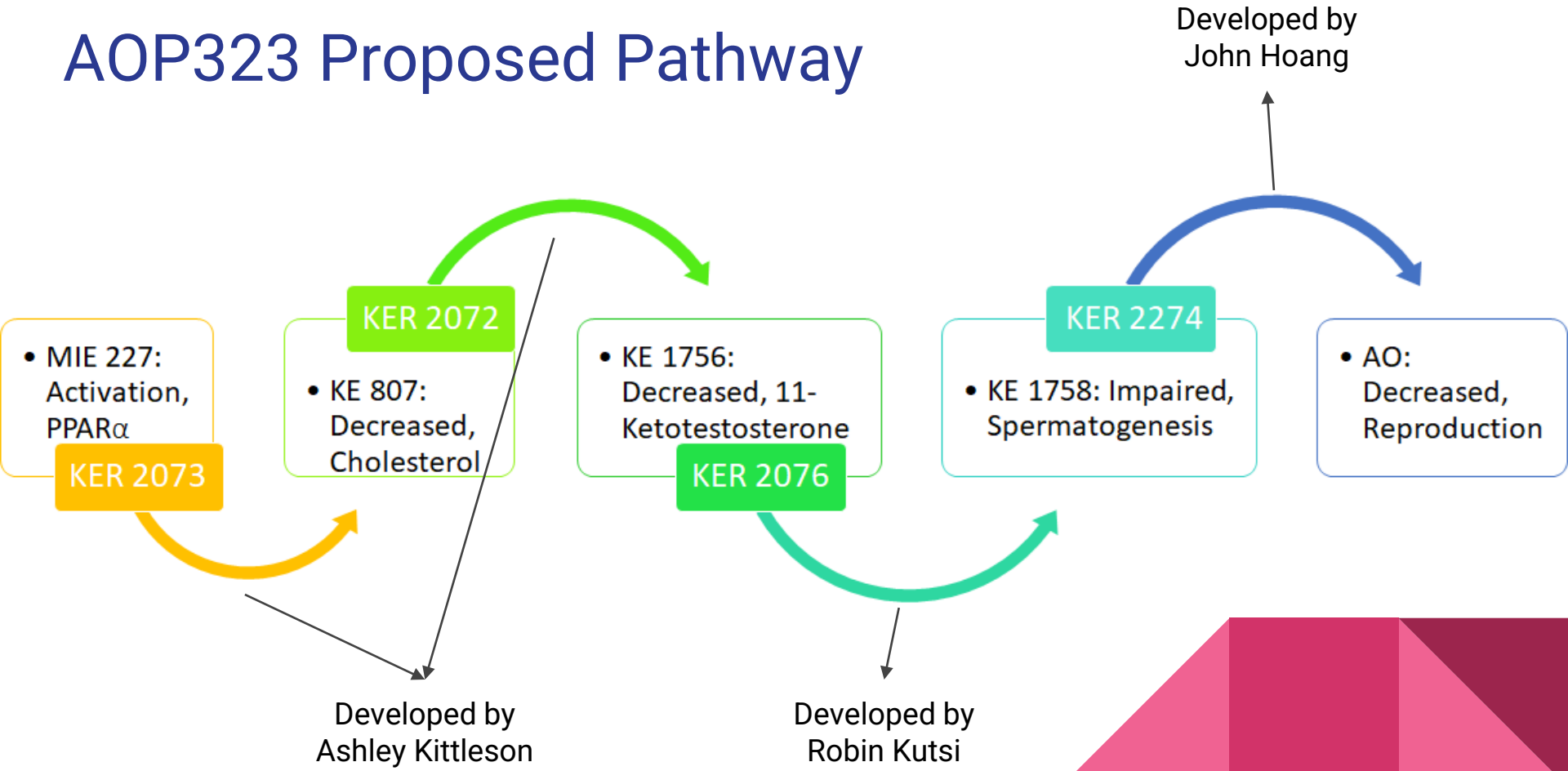


Bezafibrate

AOP323 Draft

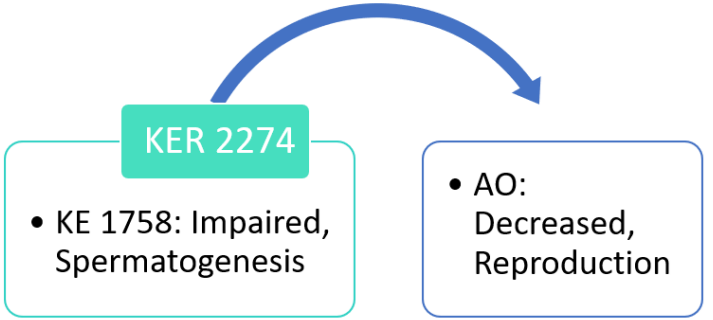


AOP323 Proposed Pathway



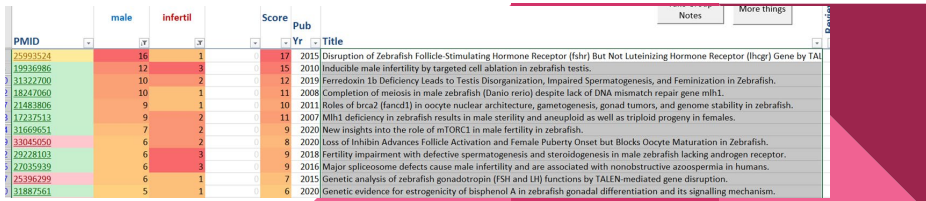
KER by KER Development Strategy Taken

- Google Scholar search for initial background information/key terms curation
- Literature Search via Abstract Sifter (https://doi.org/10.12688/f1000research.12865.1)
  - Objectivity
- Initial scan of abstracts to determine relevance
- Thorough read of relevant papers
- Organized relevant papers into concordance tables
- Evaluated by 3rd party
- Uploaded to AOP wiki



Literature Search & Other Sources

- Search Engine: Google Scholar
  - Search Terms: Impaired spermatogenesis male infertility & ISMI in Fish
    - 41600 Search Results but looked at the first page only to gain familiarity with spermatogenesis
- Search Engine: AbstractSifter
  - Search Terms: Spermatogenesis AND Fish
    - 1587 Initial Results → 9 when filtered with male, infertility, and reduced
    - 11 papers with 1 overlap when filtered with male, infertility, and impaired
  - Search Terms: Spermatogenesis AND Zebrafish
    - 192 Initial Results → 25 papers with 4 overlap when filtered with male and infertil



	male	infertil	Score	Pub	
PMID				Yr	Title
25993524	16	1	17	2015	Disruption of Zebrafish Follicle-Stimulating Hormone Receptor (fshr) But Not Luteinizing Hormone Receptor (lhcgr) Gene by TAL
19936986	12	3	15	2010	Inducible male infertility by targeted cell ablation in zebrafish testis.
31322700	10	2	12	2019	Ferredoxin 1b Deficiency Leads to Testis Disorganization, Impaired Spermatogenesis, and Feminization in Zebrafish.
18247060	10	1	11	2008	Completion of meiosis in male zebrafish (Danio rerio) despite lack of DNA mismatch repair gene mlh1.
21483806	9	1	10	2011	Roles of brca2 (fancd1) in oocyte nuclear architecture, gametogenesis, gonad tumors, and genome stability in zebrafish.
17237513	9	2	11	2007	Mlh1 deficiency in zebrafish results in male sterility and aneuploid as well as triploid progeny in females.
31669651	7	2	9	2020	New insights into the role of mTORC1 in male fertility in zebrafish.
33045050	6	2	8	2020	Loss of Inhibin Advances Follicle Activation and Female Puberty Onset but Blocks Oocyte Maturation in Zebrafish.
29228103	6	3	9	2018	Fertility impairment with defective spermatogenesis and steroidogenesis in male zebrafish lacking androgen receptor.
27035939	6	3	9	2016	Major spliceosome defects cause male infertility and are associated with nonobstructive azoospermia in humans.
25396299	6	1	7	2015	Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption.
31887561	5	1	6	2020	Genetic evidence for estrogenicity of bisphenol A in zebrafish gonadal differentiation and its signalling mechanism.

Other Sources & Example of Initial Organization

- Additional sources were used towards the creation of the weight of evidence for this KER including:
  - Papers recommended by colleagues
  - “Breadcrumb” papers
- Reasons for this included:
  - Papers to provide more information regarding spermatogenesis
  - Lack of papers involving chemical stressors

Spermatogenesis Summary Table(Reduced)

Spermatogenesis Effect
Fertility Effect
Red Text - Red Flag

Paper	Exposure/What tests	Effects
Uhrin et al., 2000 Disruption	-mPCI gene targeted by embryonic stem cells using a pPNT vector to disrupt gene function -Used F1 generation of heterozygous mice to create F2 -in vitro fertilization test	- Knockout male mice were infertile; no pregnancy despite normal sexual activity as revealed by # of copulation plugs - More than 95% of sperm from epididymis were morphologically abnormal; most lacked tails and degenerated, and some also had malformed heads - 12.5 motile sperm vs 50.5% and 51.5 % motility from heterozygous and normal sperm - Reduced fertility from in vivo fertilization experiments, 2 oocytes out of 416 (0.5%) recovered from wild-type females were fertilized - 92% (n=415) and 94% (n=420); were recovered from normal and heterozygous - In vitro fertilization experiments in PCL-/- animals are sufficient to explain infertility, even w/o possible additional effects caused by absence of PCI from secretion of sexual glands - Female knockouts reproduced normally and exhibited normal ovaries - Possibly due to abnormal spermatogenesis due to destruction of Sertoli cell barrier, perhaps due to unopposed proteolytic activity - This malfunction or lack of function of Sertoli cells would lead to partially apoptotic spermatocytes, which in turn would lead to malformed sperm accumulating in the seminiferous tubules and in the epididymal duct
Wang et al., 2016 Knockout	-Cre/loxP flp/FRT recombination systems to exons 3 and 4 of BRD7 -breeding assay -sperm counts -sexual hormone assay - normal breeding test -PAS staining	- Causes a complete arrest of spermatogenesis at step 13 of condensing spermatids when looking with periodic acid-schiff staining - post meiotic development of elongating spermatids was disrupted and characterized by abnormal morphology in round spermatids (S1–8) and elongating spermatids (S9–11) (Fig. 4A), and massive degeneration was observed in condensing (S12–13) and condensed spermatids - abnormal spermatids are characterized by an irregular head shape in the CS and CDS, an absent or deformed acrosome - Took sperm from azoospermia males(basically absence of motile sperm in semen) who suffered from spermatogenesis arrest and found a lack of BRD7 present - When mated KO male mice with WT, no pups were produced or were there any signs of pregnancy - No epididymal sperm was observed in KO mice - Increased proportions of abnormal spermatids - Downregulation of various markers for condensing and condensed spermatids - Association of male infertility resulting from BRD7 disruption with human idiopathic azoospermia where collected 58 samples from azoospermia patients and 35 normal; BRD7 associated in primary

Concordance Table

Concordance Table

Experimental design	Species	Signs of Impaired Spermatogenesis(IS)	Signs of Reduced Reproduction(RR)	IS observed?	RR observed?	Citation	Notes
-Disruption of Protein C inhibitor (PCI) through combining mutant embryonic stem cells with swiss morula embryos to create mutants. -F1 heterozygous mice were then bred to create an F2 that was subsequently used in the study.	Adult Mice (Mus musculus)	-morphologically abnormal sperm -reduced motility(12.5%) compared to control (51.5%) -apoptotic spermatocytes	-reduced in vitro fertilization rate (n=416 blastocysts) (0.5%) vs control (n= 420 blastocysts) (94%) -infertile under standard breeding despite showing signs of normal sexual activity	Yes	Yes	Uhrin et al., 2000	-PCI - inhibitor of anticoagulant serine protease activated protein C and a variety of proteases -PCI is largely present in seminal plasma and is responsible for inhibiting acrosin
Knockout of BRD7 was done through Cre/loxP and flp/FRT recombination and embryonic cells to create a positive clone that was then used to create BRD7-deficient mice	Adult Mice (Mus musculus)	-irregular head shape -deformed acrosome -post meiotic development of elongating spermatids disruption -increased proportion of abnormal spermatids(49.95 ± 7.13% of round spermatids, 67.84 ± 3.51% of elongating spermatids, 80.65 ± 5.8 % of condensing spermatids and 100% of condensed spermatids) -downregulation of various spermatogenic markers	-infertile under standard breeding despite showing signs of normal sexual activity	Yes	Yes	Wang et al., 2016	-BRD7 is a bromodomain gene that inhibits cell growth and cell cycle progression and is a co-factor for p53 -BRD7 has high expression in mice testes
Targeted genetic disruption of fdx1b using a TALEN	Adult Zebrafish (Danio	-reduced sperm count compared to control (p=0.0097%)	-infertile under standard breeding despite being able to cause spawning of eggs(0%	Yes	Yes	Oakes et al., 2019	-fdx1b is an electron-providing cofactor for steroidogenic

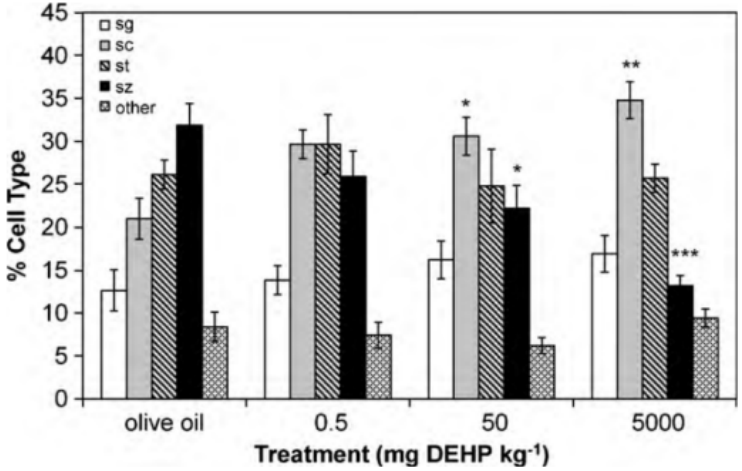
Types of Support Found

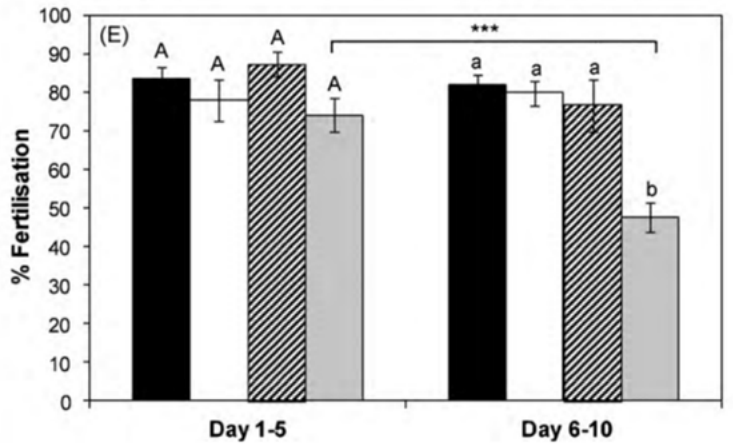
- Empirical evidence
  - Dose concordance
    - KEupstream impacted @ doses equal to or lower than KEdownstream
  - Temporal Concordance
    - KEupstream observed earlier in a time-course than KEdownstream

- Quantitative Understanding
  - Response-response Relationship
    - Information regarding response-response relationship between the two KEs.
  - Time-scale
    - approximate time-scale changes of KEdownstream from KEupstream changes.
  - Known modulating factors
    - factors that alter the shape of the response-response function

Example of Dose Concordance

- Example: When exposed to 50 mg DEHP kg-1 via intraperitoneal injection for 10 days, zebrafish (*Danio rerio*) experienced a reduction in the proportion of spermatozoa present compared to controls. However, the zebrafish did not experience a significant impact in fertilization success. Whereas when exposed to 5000 mg of DEHP kg-1 the same method, they experienced both a reduction in spermatozoa and fertilization success (Uren-Webster et al., 2010).
- Fertilization Graph Key: Black - olive oil; white - 0.5 mg/kg; hatched - 50 mg/kg; 5000 mg/kg





Conclusions

- Despite a lack of chemical stressors papers extracted, this approach was effective in establishing strong weight of evidence for the relevant key event relationship while remaining objective.
- Overall, a KER by KER approach taken here was effective for collaborative AOP development
- AOP323 is now under review by the Organisation for Economic Co-operation and Development(OECD) for a KER by KER approach case study.

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Thank you!
Any questions, comments, or concerns, please email me @ hoang.john@epa.gov