Identifying Potential Key Events Linking Xenoestrogen Exposures in Female Fathead Minnows (*Pimephales promelas*) to Reproductive Impairment.

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Background

- Endocrine disrupting compounds (EDCs) are pervasive in the environment can adversely affect both people and the environment.[1]
- Estrogenic activity is one of the most common biological activities associated with surface waters and wastewater. [11,12]
- Screening methods for evaluating estrogen receptor binding and activation are well developed.
- There is strong evidence that estrogen receptor agonists can cause reproductive disruption in adult fish [9,10]
- Previously developed AOPs have linked exposure to some endocrine active substances to reproductive impairment in sexually mature fish. (https://aopwiki.org/aops/23, https://aopwiki.org/aops/25, https://aopwiki.org/aops/30)
- However, intermediate key events linking estrogen receptor activation (the molecular initiating event) to reproductive impairment (the adverse outcome) have not been extensively studied or defined.
- Identification of intermediate key events (KEs) in the pathway(s) linking ER activation to reproductive dysfunction in fish could support development of confirmatory assays, in vivo, that are more rapid and less resource intensive than a fish short term reproduction assay (OECD TG 229).

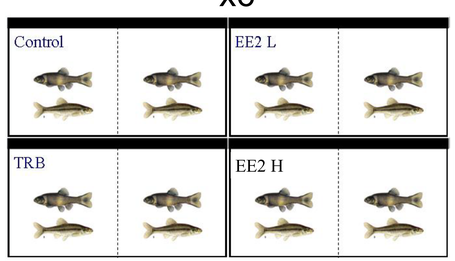
Overview

- The goal of the present study was to develop hypotheses for the mechanism(s) through which exogenous ER agonists lead to reproductive impairment in female fish.
- We examined changes in endocrine function in fathead minnows (*Pimephales promelas*) exposed for 14 d to 17α-ethynylestradiol (EE2), a potent estrogen receptor agonist of environmental concern.
- Effects of exposure to EE2 were compared with impact of exposure to 17β-trenbolone (TRB), a strong androgen receptor agonist (https://aopwiki.org/aops/23), by evaluating the associated key events.
- Microarray-based analysis of ovarian gene transcription between treatments was employed in an effort to help elucidate potential intermediate KEs in an AOP linking ER agonism to reproductive dysfunction in female fish.
- A follow-up analysis supported the conclusion that several of the transcripts that were differentially-expressed following EE2 exposure are maternally transferred to the unfertilized oocytes.

Experimental Design

- Sexually mature (~5-6 months) male and female fathead minnows
- 14-d continuous exposure without use of carrier solvents
- Fish were held in glass tanks containing 10 L of Lake Superior water (LSW) flow rate of 45 mL/min
- Fish were maintained at a temperature of 25°C±0.5°C and a 16:8 light to dark photoperiod
- Fish were fed twice per day
- Each experiment used four treatments

x6



- Four treatments with four replicates per treatment
- Tank separated into two chambers by a divider
- Each chamber housed one male and one female
- Control: LSW
- EE2 low (L) treatment: 1 ng/L EE2
- EE2 high (H) treatment: 10 ng/L EE2
- TRB treatment: 500 ng/L TRB

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Materials and Methods

Physiological end-points:
- Reproduction data collected (number of eggs laid per female)
- Fish were anesthetized in buffered MS222
- Collected plasma and gonad tissue
- Concentrations of vitellogenin protein in plasma were quantified using enzyme link immunosorbent assay (ELISA)
- Steroid concentrations (Estrogen (E2) from plasma and E2 and testosterone (T) released from gonad tissue held in culture of 12h) were quantified by radio immunoassay
- one-way analysis of variance followed by Dunnett's test used to determine significance (P-value ≤ 0.05)
- Ovary samples from 28 fish total (n=5 except 'EE2 low' and 'control' where n=4) were hybridized to a custom fathead minnow 15,000 oligonucleotide microarray
- DEGs determined relative to control. Examined pathway enrichment to infer possible KEs in ovary that lead to reproductive impairment.

Follow-up experiment:
- Oocytes were expressed from 10 sexually mature female fathead minnows
- RNA was extracted from expressed oocytes
- Quantitative polymerase chain reaction (qPCR) was used to measure target maternally-transferred genes in oocytes (Pou5f3, Npc1, Atg3, Gabarapa, Wdr45, Cfl1)

Results

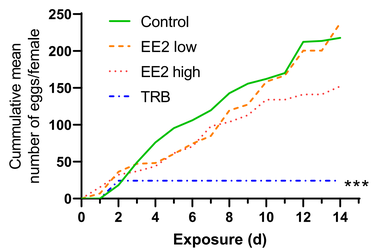

Figure 1: Fecundity of female fathead minnows in 14-day exposure to either low ethynylestradiol (EE2), high ethynylestradiol (EE2), or 17β-trenbolone (TRB). Data from females that died during the study were included until their death.

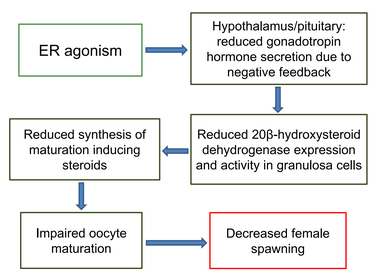

Figure 3: Hypothesized adverse outcome pathway for estrogen receptor agonism leading to impaired female reproduction.

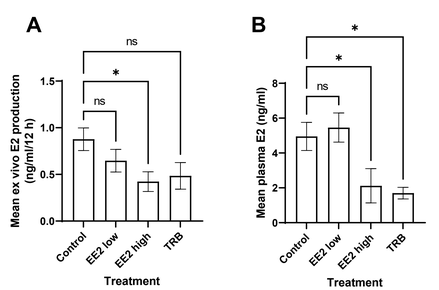

Figure 2: (**A**) 17β-estradiol (E2) and (**B**) testosterone (T) production were measured using ovary explants (ex vivo) were measured after a 14-day exposure to ethynylestradiol low (EE2 low), high (EE2 high) or 17β-trenbolone (TRB). Plasma concentration of (**C**) E2 and (**D**) vitellogenin (Vtg) were also measured after the same 14-day exposure.

Results

Table 1: Enriched pathways unique to 17α-ethynylestradiol exposure and pertinent to mechanistic understanding.

Pathway		Pathway P-value	Pathway fold enrichment
GO:0055113~epiboly involved in gastrulation with mouth forming second		0.005	2.750
Gene symbol	**Gene name**	**DEG P-value**	**DEG Fold Change**
Cdk5rap3	CDK5 regulatory subunit associated protein 3	0.039	-0.815
Npc1	Niemann-Pick disease, type C1	0.013	-1.208
Pou5f3	POU domain, class 5, transcription factor 3	0.010	-1.363
Cdh1	cadherin 1, type 1, E-cadherin (epithelial)	0.005	-1.230
Cacnb4b	calcium channel, voltage-dependent, beta 4b subunit	0.027	-1.162
Clf1	cofilin 1	0.000	-1.220
Chuk	conserved helix-loop-helix ubiquitous kinase	0.014	-1.171
Dsg2.1	desmoglein 2, tandem duplicate 1	0.016	-1.103
Pfn1	profilin 1	0.024	-1.152
Sdc4	syndecan 4	0.015	-1.239
Pathway		**Pathway P-value**	**Pathway fold enrichment**
GO:0006914~autophagy		0.017	2.210
Gene symbol	**Gene name**	**DEG P-value**	**DEG Fold Change**
Atg5	ATG5 autophagy related 5 homolog (S. cerevisiae)	0.004	-0.051
gabarapa	GABA(A) receptor-associated protein a	0.005	-1.211
Phf23a	PHD finger protein 23a	0.017	-1.142
Tbc1d5	TBC1 domain family, member 5	0.006	-1.163
Wdr45	WD repeat domain 45	0.001	-1.237
Wipi1	WD repeat domain, phosphoinositide interacting 1	0.041	-1.123
Atg3	autophagy related 3	0.005	-1.214
Ctsd	cathepsin D	0.496	-1.063
Rnf185	ring finger protein 185	0.009	-1.224
Tp53	tumor protein p53	0.005	-1.184
Zgc:77041	Zgc:77041	0.004	-0.830

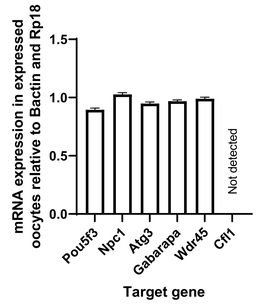

Figure 4: Relative expression levels of target gene mRNA determined by mean of C_T values divided by the geometric mean of C_T values of housekeeping genes Bactin and Rp18 (n=10).

Discussion

Physiological
- Although our present 14 d study did not show significant reductions of eggs produced per female per day (Fig. 1), there is strong evidence supporting that EE2 exposure leads to female reproductive dysfunction in fathead minnows.[13, 15-17]
- Like previously established AOPs for AR agonism and aromatase inhibition, EE2 exposure results in decreased ovarian *ex vivo* production and plasma concentrations in E2 (Fig. 2).
- Due to this decrease, AR agonism leads to a decrease in Vtg (Fig. 2c), in contrast, EE2 provides an external source of estrogen signaling causing an increase in plasma Vtg despite decrease in E2 (Fig. 2c).
- We hypothesize that although Vtg production proceeds, the predominantly negative feedback signal in the HPG axis caused by EE2 exposure impairs the production of maturation inducing steroids necessary for oocyte maturation and ovulation (Figure 3).

Transcriptomics
- None of the DEGs detected were changed by two-fold or more, suggesting overall gene expression changes in the ovary were relative minor.
- Two early developmental pathways were enriched in the EE2 high treatment that were not enriched in TRB treatment (Table 1).
- There is evidence to suggest that some of the DEGs in these pathways are maternally transferred mRNA. [18-22] QPCR performed on RNA extracted from unfertilized fathead minnow oocytes supports that (Figure 4).
- Current understanding of the regulation of maternal mRNA deposition is insufficient to determine whether this may be causally-related to the proposed AOP.

References

See appendix for reference list