DNA Methylation and Expression of Estrogen Receptor Alpha in Fathead minnows Exposed to 17α-Ethynylestradiol

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Introduction

Exposure to exogenous estrogens such as 17α-ethynylestradiol (EE2), is associated with reproductive problems in fish
- Decreased fecundity, sperm production, and feminization of males (1, 2)

Estrogen receptors genes have important roles in reproduction
- Encode nuclear receptors which allow binding of estrogens, or estrogen mimics, for transport to the nucleus where estrogens interact with estrogen response elements (EREs) in the upstream region of genes important for egg production

Transcriptional regulation via epigenetic mechanisms is one method by which organisms adapt to changing conditions (3)
- DNA methylation can dynamically respond to external environmental stimuli including toxins and allow alteration of gene function without changing the underlying DNA sequence (4)

Estrogen receptor alpha (*esr1*) is upregulated in male fathead minnows when fish are exposed to EE2. Since DNA methylation in the promoter region of genes is known to be associated with active transcription, we hypothesized that an upregulation of *esr1* would be inversely correlated with decreases of DNA methylation.

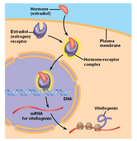


Image from Pearson Benjamin Cummings

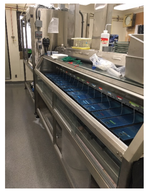
DNA methylation studies in fish are limited, so it is unknown whether EE2 affects DNA methylation level and/or pattern in the upstream region of *esr1* in fish and how that might be associated with reproductive pathways.

Objectives

- Characterize changes in the pattern and level of DNA methylation for *esr1* in fish exposed to EE2 and determine whether changes correlate with *esr1* gene expression levels from EE2 treated fish
- Determine whether methylation changes persist after the chemical challenge is withdrawn
- Determine if EE2-dependent methylation differences in treated males display a feminized pattern

Study Design

Reproductively mature male fathead minnows were exposed to low (1.4 ± 0.31 ng/L) and high (3.5 ± 1.34 ng/L) doses of EE2 as well as a control (0 ng/L EE2) in a flow-through diluter system for 48h. Unexposed females (n=8) were used for comparison. Following 48h (2d) exposure (n=27-29 individuals/group), a subset of fish were depurated in water for 7 (n=8-9 fish/group) and 14 (n=8-10 fish/group) days (Figure 1; Figure 2).



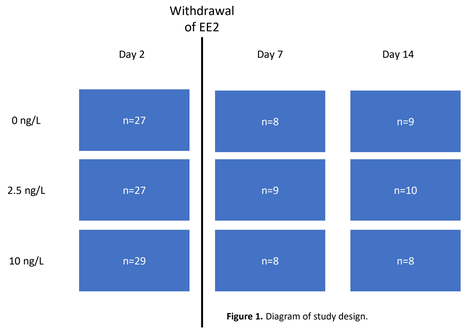


Figure 1. Diagram of study design.

Figure 2. Exposure tanks.

Methods and Materials

Water Chemistry
- Water samples were taken daily from all aquaria to quantify actual EE2 dose delivered to fish. Nominal doses of EE2 were 2.5 and 10 ng/L. Mean measured doses of EE2 for each treatment were 1.37 ± 0.31 and 3.47 ± 1.34 ng/L, respectively. Control group measured 0 ng/L EE2

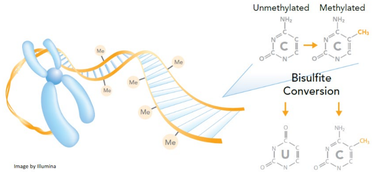
Necropsies
- Following exposure and at each time point, fish were anesthetized in 400mg/l tricaine methanesulfonate (MS-222) and brains and livers were dissected from each fish and flash frozen using liquid nitrogen

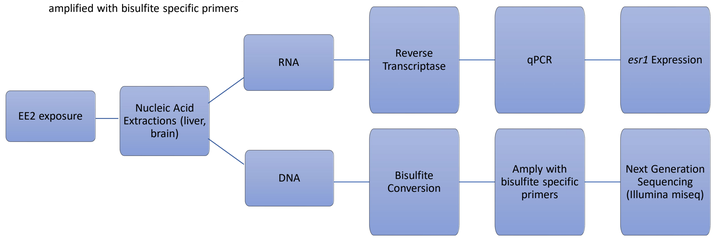
Nucleic Acid Extractions and Gene Expression
- Total RNA and DNA were extracted using the Allprep DNA/RNA Mini Kit (Qiagen) for each tissue type. 100 ng of total RNA per reaction was used in 20 µl reverse transcription (RT) reactions carried out in duplicate. No-template controls and no amplification controls (RT enzyme omitted) were included with each set of samples being processed. Resultant cDNA was used in qPCR reactions to quantify *esr1* expression

Bisulfite Conversion and Sequencing
- Total DNA was bisulfite-converted using EZ-96 DNA Methylation-Lightning MagPrep Kit (Zymo Research) according to manufacturer's protocol. Following bisulfite treatment, CpG enriched regions spanning 3kb (5' upstream through the second exon) targeted were amplified with bisulfite specific primers

Bioinformatics
- QC of sequencing data: FastQC (v0.11.8)
- Remove adapter sequences and primers: Cutadapt (v1.18)
- Read alignment: Bismark (v0.19.0), Bowtie2 (v2.3.1)

Statistics
- Bisulfite analysis: MethylKit in R 3.6.1, logistic regression to detect significantly differentiated CpG sites (5, 6)
- Gene expression: ANOVA, Tukey multiple comparisons of means





Discussion

Esr1 is upregulated when fish are exposed to EE2 in liver but not in brain tissues. A correlation analysis examined the relationship between *esr1* methylation gene expression in the 2d liver samples. Methylation of *esr1* in liver from male fish was found to have a moderate inverse relationship for individual CpG sites in both the promoter and gene body regions and gene expression (Pearson's correlation coefficient $r(81) = -.31$, $p < 0.01$).

We have demonstrated that EE2 exposure results in the differential methylation of CpG dinucleotides in the *esr1* gene in both brain and liver tissue. Promoter methylation patterns were largely similar between tissues in exposed males and, in liver, appeared to exhibit a dose-dependent shift toward a feminized pattern. Gene body methylation patterns of treated males differed between tissues. Lastly, some methylation effects of EE2 remained after chemical withdraw. Depuration resulted in relatively modest methylation changes with only singular CpG sites indicating significant change in the promoter region of *esr1*, and only 8 CpG sites in the gene body of *esr1* in brain at 7 days post-exposure. This lends some evidence to suggest that acute exposure to potent estrogens is sufficient to initiate a lasting biological cascade that continues to modify CpG methylation well after the cessation of exposure.

Though the current study has identified EE2-induced methylation changes in the proximal promoter and 5' intragenic regions of *esr1*, interpretation must be considered in the larger context of the complexity of *esr1* transcriptional regulation. Though preliminary, our results suggest the importance of further evaluation of EE2 effects in the brain on methylation, transcription, and downstream signaling with finer spatial resolution.

Results

- In liver, gene expression of treatment groups are significantly different from control at both doses of EE2 (Figure 3A, $p<0.001$). No significant gene expression is observed in brain (Figure 3B).

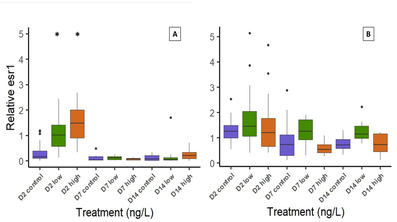


Figure 3. Relative expression of *esr1* in liver (A) and brain (B) tissues from male fathead minnows. X-axes indicate timepoint (D2, D7, and D14) and treatment group (control, low, high EE2 dose). At Day 2 (n=27-29 per group), both treatment groups differed significantly from control ($p<0.001$, ANOVA with Tukey test for multiple comparisons of means). There were no significant differences in treatment groups relative to control after depuration (D7 (n=8-9 per group), D14 (n=8-10 per group)). Significance is denoted by asterisks and outliers in the data are shown as black dots.

- 34 CpG sites were targeted in this study, 25 had enough sequence coverage for analysis (10 in the 5' upstream region of the gene, 15 sites within the gene body).
- In female fish liver, all 15 CpG sites located in the gene body of *esr1* display significantly different DNA methylation from males ($p < 0.001$) (Figure 4D). No significant difference is found in brain.
- In the 5' upstream gene region in liver, EE2-dependent methylation patterns in males were highly similar to the female pattern (CpG sites -463,-415,-298), suggesting EE2 induces a feminized pattern of methylation in the proximal promoter region (Figure 4, A-D).
- After 7 days depuration, 8 CpGs were differentially methylated in brain of fish originally exposed to the high EE2 dose, a response which was not observed at 14d post-exposure.
- Methylation of *esr1* is negatively correlated with gene expression in 2d liver samples. A moderate inverse relationship was observed for individual CpG sites in both the promoter and gene body regions and gene expression (Pearson's correlation coefficient $r(81) = -.31$, $p < 0.01$).

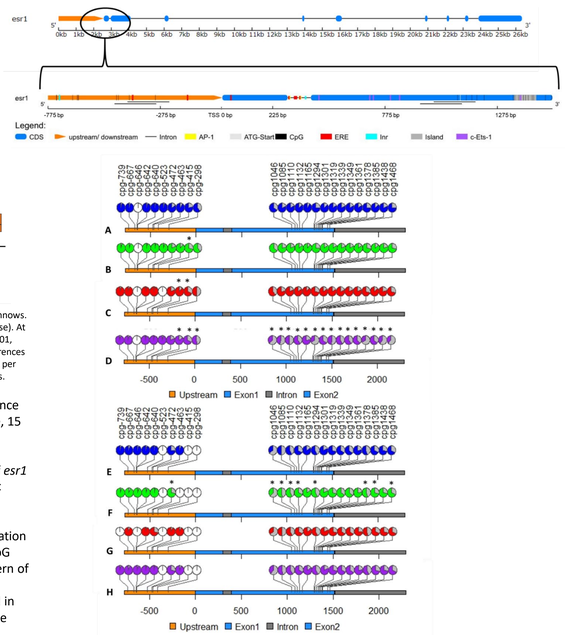


Figure 4. Putative esr1 gene model diagram (top) showing a targeted region including a 5' upstream region (orange), exons (blue), and intronic regions (black line), and putative transcription factor binding sites. CpG sites are shown with corresponding pie charts representing 2d liver tissues (A) control (water only), (B) low dose males (1.4 ± 0.31 ng/L EE2), (C) high dose males (3.5 ± 1.34 ng/L), (D) females (no exposure), and the same treatments in brain tissues (E) through (H), respectively (n=27-29 per group for males, n=8 for females). Mean methylation level for each CpG site is depicted by the colored region of each pie chart, and significance is denoted by asterisks (FDR=0.1, differential methylation calculated by logistic regression). CpG sites lacking sequence data are shown by empty pie charts.

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