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Rapid Effects of the Aromatase Inhibitor Fadrozole on Steroid Production and Gene Expression in the Ovary of Female Fathead Minnows (*Pimephales promelas*)

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Abstract

Cytochrome P450 aromatase catalyzes conversion of C19 androgens to C18 estrogens and is critical for normal reproduction in female vertebrates. Fadrozole is a model aromatase inhibitor that has been shown to suppress estrogen production in the ovaries of fish. However, little is known about the early impacts of aromatase inhibition on steroid production and gene expression in fish. Adult female fathead minnows (*Pimephales promelas*) were exposed via water to 0, 5, or 50 µg fadrozole/L for a time-course of 0.5, 1, 2, 4, and 6 h, or 0 or 50 µg fadrozole/L for a time-course of 6, 12, and 24 h. We examined *ex vivo* ovarian 17β-estradiol (E2) and testosterone (T) production, and plasma E2 concentrations from each study. Expression profiles of genes known or hypothesized to be impacted by fadrozole including aromatase (cytochrome P450 [*cyp*] 19a1a), steroidogenic acute regulatory protein (*star*), cytochrome P450 side-chain cleavage (*cyp11a*), cytochrome P450 17 alpha hydroxylase/17,20 lyase (*cyp17*), and follicle stimulating hormone receptor (*fshr*) were measured in the ovaries by quantitative real-time polymerase chain reaction (QPCR). In addition, broader ovarian gene expression was examined using a 15k fathead minnow microarray. The 5 µg/L exposure significantly reduced *ex vivo* E2 production by 6 h. In the 50 µg/L treatment, *ex vivo* E2 production was significantly reduced after just 2 h of exposure and remained depressed at all time-points examined through 24 h. Plasma E2 concentrations were significantly reduced as early as 4 h after initiation of exposure to either 5 or 50 µg fadrozole/L

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and remained depressed throughout 24 h in the 50 µg/L exposure. *Ex vivo* T concentrations remained unchanged throughout the time-course. Expression of transcripts involved in steroidogenesis increased within the first 24 h suggesting rapid induction of a mechanism to compensate for fadrozole inhibition of aromatase. Microarray results also showed fadrozole exposure caused concentration- and time-dependent changes in gene expression profiles in many HPG-axis pathways as early as 4 hrs. This study provides insights into the very rapid effects of aromatase inhibition on steroidogenic processes in fish.

Keywords

Endocrine Disruption; Estrogen; Microarray; Compensation; Fish

1. Introduction

Estrogens are essential for a number of processes related to normal reproduction, growth, and development in vertebrates. In teleost fish, for example, estrogens synthesized primarily in the ovary are secreted into the blood stream and induce production of vitellogenin, a yolk precursor protein needed for normal oocyte development. Cytochrome P450 (CYP) aromatase (*cyp19a1a*) is a key steroidogenic enzyme responsible for 17β-estradiol (E2) biosynthesis in ovarian granulosa cells. Cyp19a1a catalyzes three consecutive hydroxylation reactions, converting C19 androgens (e.g., testosterone) to C18 estrogens such as E2 (Miller 1988). Expression and activity of aromatase is the control point for maintaining a homeostatic balance between androgens and estrogens which in turn is critical to regulating reproductive development and function in females.

A variety of endocrine-disrupting chemicals (EDCs) have the potential to impair reproduction and development in wildlife and humans through effects on the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis uses complex networks and feedback loops to regulate and maintain homeostatic conditions needed for reproduction. Although EDCs can interfere with the endocrine system at multiple levels, many are known to inhibit enzymes involved in steroidogenesis, including aromatase (Cheshenko et al., 2008; Sanderson 2006). Chemicals can inhibit aromatase activity through at least two mechanisms. Those with steroid-like structures can inhibit aromatase by binding to its active site, whereas non-steroidal aromatase inhibitors interfere with the electron transfer via the CYP heme group (Petkov et al., 2009). Multiple environmentally-relevant chemicals, including some organochlorines, fungicides, pesticides, and drugs, have been shown to inhibit aromatase *in vitro* (Andersen et al., 2002; Drenth et al., 1998; Heneweer et al., 2004; Letcher et al., 1999; Ohno et al., 2004; Sanderson et al., 2002; Vinggaard et al., 2000). Many of these chemicals are found in the aquatic environment, suggesting the potential for adverse effects due to aromatase inhibition in fish.

In early investigations of the impacts of aromatase inhibition on HPG function and reproduction in fish, 21 d studies were performed with fathead minnows (*Pimephales promelas*) exposed to the model aromatase inhibitor, fadrozole (FAD) (Ankley et al., 2002). Female fathead minnows showed a significant concentration-dependent decrease in plasma

E2, vitellogenin, and egg production. That same basic pattern of response was observed in multiple other studies with fish exposed to aromatase inhibitors, leading to description of an adverse outcome pathway (AOP) linking aromatase inhibition to reproductive dysfunction in fish (Villeneuve 2016). In addition to key biological events captured in the AOP description, time-course studies with fish exposed to steroid biosynthesis inhibitors, including FAD, also focused on identifying indirect (compensatory) effects along the HPG axis in addition to more direct effects of the enzyme inhibition (Ankley and Villeneuve, 2015). For example, Villeneuve et al. (2009a) showed an increase in ovarian expression of mRNA coding for proteins involved in steroid production within 24 to 48 h in fathead minnow females exposed to FAD, consistent with compensation for decreased circulating E2. Other time-course experiments with prochloraz, a fungicide known to inhibit aromatase and other steroidogenic CYPs, showed the beginning of a compensatory response in as little as 24 hours after initial exposure (Ankley et al., 2009; Skolness et al., 2011). These data suggest that an examination of the direct and compensatory responses within a finer time period (i.e., within the first 24 h) after exposure is needed to more fully understand and model the dynamic nature of the consequences of aromatase inhibition in fish.

The objective of the present study was to characterize the early molecular and biochemical responses of female fathead minnows exposed to FAD. Two independent time course experiments were performed, one sampling adult female fathead minnows at relatively fine intervals over the course of 6 h of exposure to two concentrations of FAD, and another that sampled fish after 6, 12, or 24 h of exposure to a single concentration of FAD. Endpoints included plasma E2 concentrations, *ex vivo* steroid (E2, testosterone [T]) production, ovarian expression of transcripts involved in HPG function by quantitative polymerase chain reaction (QPCR), and global gene expression analysis using oligonucleotide microarrays. This study provides further insights into the rapid direct effects of aromatase inhibition on steroidogenic processes in fish, as well as their ability to compensate for observed inhibition. This type of information contributes to both fundamental understanding of fish HPG axis function and development of computational models that can quantitatively simulate the dynamics of that axis in order to predict effects of aromatase inhibitors on reproductive processes in fish (e.g., Wittwehr et al. 2016; Conolly et al. 2016).

2. Materials and Methods

2.1 Fadrozole exposure

Two independent experiments were conducted with female fathead minnows. In the first, fish were exposed to 5 or 50 µg FAD/L for 0.5, 1, 2, 4 or 6 h. In the second, fish were exposed to just one concentration, 50 µg FAD/L, for 6, 12, or 24 h. Although independent, these experiments were conducted in a similar manner and examined the same suite of endpoints. The methods described apply to both studies unless otherwise noted.

Solvent-free stock solutions of FAD (a gift from Novartis, Inc., Summit, NJ) were prepared in filtered and UV-sterilized Lake Superior water. Stock solutions were diluted in Lake Superior water to achieve the desired nominal concentrations of 0 (control), 5 or 50 µg FAD/L. Target test concentrations were chosen based on effects observed in a previous 21 d fathead minnow reproduction assay (Ankley et al., 2002). Glass aquaria (20 L) were divided

into three sections using nylon mesh screens. Each tank contained 10 L of Lake Superior water (control) or FAD solution, delivered at a continuous flow-rate of approximately 45 mL/min. Delivery of FAD solution was initiated 48 h prior to the addition of fish to ensure that equilibrium test concentrations were achieved. Exposures commenced by placing two reproductively mature (5-6 month old) female fathead minnows into each of the three sections of the tanks. Four replicate tanks were used for each treatment at each time period. Fish addition times were staggered within each replicate and treatment to permit all samples from a given experiment to be sampled within 10 min of the target exposure duration for the shorter exposures (0.5, 1, and 2 h) and within 30 min for the longer exposures (4, 6, 12, and 24 h). Fish were maintained at $25 \pm 1^\circ\text{C}$ for the duration of the exposures and were not fed during the test. Animals used for this experiment were from an on-site culture facility at the US Environmental Protection Agency (USEPA) in Duluth, MN and all laboratory procedures were approved and by the Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

After 0.5, 1, 2, 4, 6, 12, or 24 h of exposure, two fish from each replicate tank were sampled, yielding a total of eight fish sampled per treatment at each time period. Fish were euthanized in buffered tricaine methanesulfonate (MS-222; Argent, Remond, WA), and the sampling time recorded. Whole body wet weight was measured for each fish. Blood was collected from the caudal vasculature using heparinized microhematocrit tubes, separated by centrifugation, and plasma stored at -80°C until extracted and analyzed for E2. Whole ovaries were removed from each fish and weights recorded for calculation of gonadosomatic index (GSI). Each ovary was then sectioned into separate pieces for various analyses (See Villeneuve et al., 2009a for details). A subsample was immersed in tissue culture media for an *ex vivo* steroid production assay, while a second subsample was snap-frozen in liquid nitrogen and stored at -80°C until extraction for gene expression analysis. All tools used for dissection were washed between each sample with RNaseZap (Ambion, Austin, TX) to prevent sample cross-contamination and RNA degradation by RNases.

2.2 Chemical Measurements

Water samples (1 mL) were collected from all test tanks at the start of the sampling period for the 0-6 h exposure and at the start and end of the 6, 12, 24 h test to verify FAD concentrations. Fadrozole measurements were determined by high pressure liquid chromatography with diode array detection (Ankley, et al., 2002; Villeneuve et al., 2009a). A number of quality assurance samples were also analyzed for each experiment. Mean recoveries of FAD in spiked Lake Superior water samples were 98% ($n=2$) and the agreement among duplicate samples was 99% ($n=4$). The method detection limit was 1 $\mu\text{g/L}$.

2.3 Biochemical Measurements

Ex vivo steroid production from ovary subsamples (mean \pm SD; 11.4 ± 7.17 mg for 0-6 h and 16.0 ± 6.21 mg for 6, 12, 24 h) were assessed using a method adapted from McMaster et al. (1995) and described in detail by Villeneuve et al. (2009a). Briefly, each subsample was immersed in 500 μL of Medium 199 (M2520; Sigma, St. Louis, MO) supplemented with 0.1mM isobutylmethylxanthine (Sigma I7018) and 1 μg 25-hydroxycholesterol/ml and

incubated for 12 h at 25° C. Tissue viability was not assessed, but previous studies have shown steroid production/release from the tissue to increase in a relatively linear fashion for at least 30 h (Breen et al. 2007). Following incubation, medium was collected and each tissue subsample was weighed. Medium samples were stored at -20° C until analyzed and blanks incubated in the same manner, but without tissue were used as blanks in the analysis. Steroids were extracted from *ex vivo* medium or plasma by liquid-liquid extraction with diethyl ether and quantified with a small volume radioimmunoassay (RIA) method (Jensen et al., 2001). Both E2 and T were quantified in the *ex vivo* culture medium. Plasma sample volumes generally were not sufficient to accommodate measurement of both E2 and T, so only plasma E2 was quantified.

2.4 Real-time PCR (QPCR) analyses

Expression of selected genes in ovary subsamples associated with each time period and treatment was analyzed using QPCR. Total RNA was extracted from each subsample using Tri Reagent (Sigma-Aldrich, St. Louis, MO). The total RNA was resuspended in RNase-free water (Integrated DNA Technologies, Coralville, IA), and quantity and purity were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA), respectively. The RNA samples used for QPCR had optical densities for the 260/280 ratios between 1.8 and 2.0. A total of 250 ng of RNA were reverse transcribed to cDNA in 20 µl reaction containing 250 ng of random primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Synthesized cDNA samples were diluted to 10 ng/µL.

Target genes known to be involved in HPG axis function, including several previously shown to be influenced by FAD exposure (Villeneuve et al., 2013; 2009a; 2007; 2006) were analyzed. Transcripts measured in the ovaries included those coding for three CYPs involved in steroidogenesis: cytochrome P450 side-chain cleavage (*cyp11a*), cytochrome P450 17 alpha hydroxylase/17,20 lyase (*cyp17*) and *cyp19a1a*. Two additional transcripts measured were steroidogenic acute regulatory protein (*star*) and follicle-stimulating hormone receptor (*fshr*). The QPCR measurements were also used to confirm microarray results as probes for all transcripts, except *fshr*, were present on the microarray employed in this study (see below). Primer sequences for the genes measured have been described previously (Villeneuve et al., 2009a; 2007).

The QPCR assays were conducted in a 96-well format using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Each 20-µL reaction contained 10 µL of 2x master mix, 200 nM forward and reverse primers, and 20 ng cDNA. QPCR assays were run using a 7500 Real-Time PCR system (Applied Biosystems). Cycling parameters were 95°C for 15 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 1 min. Primer specificity was determined by melt curve analysis. Both no-reverse-transcription and no-template controls were used to confirm a lack of genomic DNA contamination and primer dimerization. Results for the 0-6 h time course were normalized to 18s rRNA and fold-changes calculated using the Cq method without correction for amplification efficiency (Livak and Schmittgen, 2001). Sample sizes analyzed for the 0-6 h time course were n=6 for *star*, *cyp11a*, and *cyp19a1a* but only n=4 for *cyp17* and *fshr*. Results for the 6, 12, 24 h time

course were normalized to the geometric mean of three reference genes, including hypoxanthine phosphoribosyltransferase 1 (*hprt1*), TATA-box binding protein (*tbp*), and ribosomal protein 18 (*rpl8*) whose expression has been shown not to be influenced by the effects of estrogens in fish (Filby and Tyler, 2007). The stability of the reference genes for normalization was examined using BestKeeper© software (Pfaffl et al., 2004) and NormFinder software (Andersen et al., 2004). Sample sizes analyzed for the 6, 12, 24 h time course were n=7-8.

2.5 Microarray Analysis

For both experiments, microarray analysis was conducted on ovarian subsamples from four randomly selected individuals collected from each treatment and time period. Total RNA was isolated from each ovary subsample using RNeasy kits (Qiagen, Valencia, CA, USA). RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent) and quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Total RNA was stored at -80°C until used for microarray analysis. Microarray analysis was conducted using a custom fathead minnow 15k microarray (GEO Platform Accession GPL9248) purchased from Agilent Technologies (Palo Alto, CA). Microarrays were hybridized according to the manufacturer's protocols (Agilent) for One-Color Microarray-Based Gene Expression Analysis (version 5.7) using 1 μg of total RNA. Complementary DNA synthesis, cRNA labeling, amplification, and hybridizations were performed following the manufacturer's kits and protocols (Quick Amp labeling kit; Agilent). Scanning was conducted at 5 μm resolution using an Axon GenePix 4000B Microarray Scanner (Molecular Devices Inc., Concord, Ontario, Canada). Data were extracted from microarray images using Agilent Feature Extraction Software. Text versions of the Agilent raw data from each of these studies has been deposited at the Gene Expression Omnibus website (GEO: <http://www.ncbi.nlm.nih.gov/geo/:GSE70936>; Supplementary Table S28).

2.6 Statistical Analysis

Statistical analyses focused on cross-treatment differences within time-points rather than changes across time. Statistical analyses for morphological and biochemical measurements were conducted using Statistica 10 (Statsoft, Inc., Tulsa, OK) and GraphPad Prism v. 5.02 (GraphPad Software, San Diego, CA). Data were tested for normality and homogeneity of variance using a Kolmogorov-Smirnov test and Levene's test, respectively. When data conformed to parametric assumptions, unpaired t-tests (two treatments) or one-way analysis of variance (ANOVA; three treatments) were used to test for differences across chemical treatment groups. When applicable, Duncan's multiple range or Dunnett's post hoc tests were used to determine differences between chemical treatments. When data did not meet parametric assumptions, they were transformed (\log_{10}) or analyzed using Mann-Whitney or Kruskal-Wallis and Dunn's test. In the case of *ex vivo* steroid production data, ovary subsample weights were included as a factor in the analysis using General Linear Models ANOVA. Because weight was not a significant factor determining *ex vivo* E2 or T production, results were not normalized to tissue weight. Differences were considered significant at $p \leq 0.05$ unless otherwise noted.

For QPCR results, significant differences in fold-change between treatments and control at each time-point were calculated using a Pair-Wise Fixed Reallocation Randomization Test implemented in the Relative Expression Software Tool (REST©) software (Pfaffl et al., 2002). Two thousand random allocations were performed and differences were considered significant at $p = 0.05$.

All raw microarray datasets ($n = 60$ microarrays for the 0-6 h exposure and $n = 24$ microarrays for the 6, 12, 24 h exposure) were imported into GeneSpring GX 12 (Agilent). The data were subjected to quantile normalization. Two approaches were applied to identify significant differentially expressed genes (DEGs) due to the FAD treatment(s). The first approach examined each study separately to identify DEGs between the 5 or 50 μg FAD/L treatment and controls at each time-point. Significant DEGs from the 0-6 h exposure were identified by one-way ANOVA followed by pairwise Tukey's HSD test ($p < 0.05$). The DEGs from the 6, 12, 24 h exposure were identified by t-tests ($p < 0.05$).

The second approach examined temporal changes in gene expression during the 24 h exposure by identifying significant DEGs between the 50 μg FAD/L treatment at each time point and a single pooled control composed of the control expression data from all sampling time points. All normalized data for the 50 μg FAD/L concentration and controls from both experiments were combined and batch effects removed using an empirical Bayes method (COMBAT; Johnson et al., 2007) implemented in R (<http://www.R-project.org>; v 2.15.2). To distinguish differential expression due to chemical-specific impacts rather than potential random or systematic variation in the controls over time, normalized and batch-corrected expression data for control samples from each time point were pooled to provide a single control. Significant DEGs were identified by one-way ANOVA ($p < 0.05$) with no multiple testing correction. Functional analysis of the DEGs identified within and across the 24 h exposure to 50 μg FAD/L compared to the pooled control was performed using Ingenuity Pathway Analysis (IPA; Redwood City, CA). The fathead minnow probe identifications (IDs) were mapped to orthologous Entrez human gene IDs where feasible and these were input into IPA to identify statistically enriched canonical pathways ($p < 0.05$); the lists of genes were included in the pathway enrichment.

3. Results

3.1 Exposure Characterization

Mean measured concentrations (SD, n) of FAD in the 5 and 50 μg /L treatment tanks during the 0-6 h exposure were 6.4 (0.05, 4) and 54.6 (0.45, 4) μg /L, respectively. Mean concentrations of FAD in the 50 μg /L treatment tanks during the 6, 12, 24 h exposure were 51 (1.69, 8) μg /L. No FAD was detected in control water samples from either experiment nor in method blanks ($n = 4$ for each experiment).

3.2 Morphological and Biochemical Analysis

There was no FAD-induced mortality nor obvious effects on behavior during the testing periods. Neither female mass (not shown) nor GSI (Supplementary Figure 1) varied significantly as a function of treatment or time.

Exposure to FAD had a significant effect on plasma E2 concentrations (Figure 1A). Both the 5 and 50 µg FAD/L treatments significantly reduced plasma E2 concentrations within 4 h of exposure initiation. Plasma E2 levels remained significantly reduced through 24 h for the 50 µg/L treatment.

The *ex vivo* steroid production assay measures the rate of steroid release into tissue culture medium following in vivo exposure to a test chemical, in this case FAD. *Ex vivo* ovarian E2 production was significantly reduced by the 5 µg FAD/L treatment within 6 h of exposure (Figure 1B). *Ex vivo* ovarian E2 production was significantly reduced by the 50 µg FAD/L treatment within 2 h and remained decreased through 24 h. *Ex vivo* ovarian T production did not vary significantly in any treatment group (Figure 1C).

3.3 Targeted Gene Expression

Ovarian expression of targeted gene transcripts measured by QPCR displayed significant time- and concentration-dependent effects in FAD-exposed fish (Figure 2). Expression of *star* was significantly up-regulated at 0.5 h in the 5 µg FAD/L treatment (Figure 2A). In contrast, *star* expression was significantly downregulated after 1 and 6 h of exposure to 50 µg FAD/L. *Cyp11a* was significantly upregulated at 1 h by both the 5 and 50 µg FAD/L treatments and significantly downregulated at 6 h by the 50 µg FAD/L treatment, but only in one of the two experiments (Figure 2B). There was no significant effect of either FAD treatment on *cyp17* expression (Figure 2C). *Cyp19a1a* was significantly upregulated at 1 h by both the 5 and 50 µg FAD/L treatments (Figure 2D). However, it was significantly downregulated at 2 h in both the 5 and 50 µg FAD/L treatments and remained downregulated at 6 h (both experiments) in the fish exposed to 50 µg FAD/L. Ovarian expression of *fshr* was significantly downregulated after 0.5 h and significantly up-regulated at 1 h in the 50 µg FAD/L treatment, while both the 5 and 50 µg FAD/L treatments significantly down-regulated *fshr* expression at 2h (Figure 2E).

3.4 Global Ovarian Transcriptome Response and Functional Analysis

The effects of FAD on transcripts measured by microarray analysis were relatively consistent with observations in terms of magnitude and direction of expression changes from the QPCR. However, based on microarray analysis *cyp19a1a* was not detected as significantly down-regulated by FAD treatment at any time point before 24 h, potentially due to the different number of samples analyzed (microarray n=4, QPCR n=6-8; Supplementary Figure 2). The increasing trajectory from 6 h to 24 h was similar for both methods, but significant up-regulation in the 50 µg FAD/L treatment at 24 h was only detected by the microarray analysis.

The number and identity of genes significantly up- or down-regulated in the ovaries varied considerably with time and FAD concentration (Table 1; Supplementary Tables S1-5). Fish exposed to 5 µg FAD/L had more significant DEGs within the first hour of exposure than the number of significant DEGs at the other three time points combined. The majority of the significant DEGs (59% at 0.5 h; 74% at 1 h) were up-regulated. Fish exposed for 4 h had the fewest significant DEGs and the greatest percentage of down-regulated genes (60%).

Overall, fish exposed to 5 µg FAD/L had more significantly up-regulated genes (60%) than significantly down-regulated genes over the first 6 h.

There were also significant effects on gene expression in females exposed to 50 µg FAD/L (Supplementary Tables S6-13). Fish exposed to 50 µg FAD/L for 1 h and 12 h had the most significant DEGs with the majority (73% at 1 h; 59% at 12h) being up-regulated. Relatively few significant DEGs were identified for fish exposed for 0.5 h, but this sampling time had the greatest percentage of genes down-regulated (51%).

There were several significant DEGs in common between the 5 and 50 µg FAD/L treatment at each time point during the 0-6 h exposure (Table 1; Supplementary Tables S15-19). The 1 h time point had the greatest number of common DEGs between the two FAD treatments, with the majority of those (84%) up-regulated. Fish exposed for 4 h had the fewest common significant DEGs between FAD treatments, which again corresponded with the greatest percentage of down-regulated genes among the time points (58%).

To better understand the possible biological impacts of exposure to 50 µg FAD/L over the first 24 h, we identified significant canonical pathways based on the DEGs identified both across and within time-points using IPA (Supplementary Table S20). Overall, there were 137 significantly enriched canonical pathways associated with the DEGs identified across the 24 h exposure period (Supplementary Table S20). Enriched pathways related to HPG-axis signaling and steroid hormone-related pathways were mostly down regulated including androgen receptor signaling and several pathways regulated by E2 involved in cell proliferation and signaling. Ten out of the 16 enriched pathways that were mostly up-regulated were related to oxidative stress responses (cardiac hypertrophy, mitochondrial dysfunction) or inflammation (oncostatin M signaling, G beta gamma signaling, IL-1 signaling, chemokine signaling, ephrin B signaling, IL-6 signaling, IL-17A signaling in gastric cells, ERK5 signaling).

Examination of enriched pathways within time-points showed the greatest number of enriched pathways associated with the 2 h exposure, while the fewest enriched pathways were detected after 24 h of exposure (Supplementary Tables S21-S27). There were no enriched pathways in common among all individual time-points. However, there were 23 enriched pathways that were common to at least two consecutive time points (Table 2). Among those, genes associated with the Huntington's disease signaling pathway were impacted at the first four time points, while hereditary breast cancer signaling and PI3/AKT signaling were impacted at the first three (Table 2). Telomerase signaling was the only other pathway that was significantly enriched at four different time points (at least two being consecutive), and inhibition of matrix metalloproteinases was the only one for which all associated genes were up-regulated across all time points in enriched pathways (Table 2). The greatest overlap between time points was for the 1 h and 2 h, with 12 enriched pathways impacted at both time points (Table 2). Interestingly, enriched pathways related to HPG-axis and steroid hormone signaling were only observed during the first four sampling time points (0.5, 1, 2, and 4 h). In general, there were no enriched HPG-axis pathways reported after 6 h.

4. Discussion

Fadrozole is a pharmaceutical that was developed as a treatment for estrogen-sensitive breast cancer by functioning as a potent nonsteroidal competitive aromatase inhibitor (Browne et al., 1991). Because of its specific mode of action for aromatase inhibition, FAD has been used as a model chemical to assess effects of aromatase inhibition on the HPG-axis in fish (Ankley et al., 2002; Bhandari et al., 2004; Dapeng et al., 2009; Villeneuve et al., 2013; 2009a; 2009b; 2006; Zhang et al., 2008). Some of these studies have employed time-course designs focused on defining direct effects and compensatory responses to aromatase inhibition in fish. For example, Villeneuve et al. (2009a; 2013) showed significant direct effects of FAD on E2 and vitellogenin concentrations in female fathead minnows and compensatory changes in gene expression within 24–48 h of initial exposure; however, prior studies had not been conducted at earlier sampling times. In the current study we used a time-course design to intensively sample time-points prior to 24 h to determine how rapidly effects are observed, and how soon after exposure compensatory responses may be triggered.

It is recognized that toxicokinetic considerations play an important role in determining the rates at which effects are observed following exposure to a chemical. While the present study did not specifically characterize the dynamics of uptake, available evidence suggests FAD concentrations rapidly reach steady state concentrations *in vivo*. Fadrozole has a log Kow around 2.2, suggesting significant bioconcentration is unlikely. In a previous study, Villeneuve et al. (2013) showed that females exposed to 30 µg FAD/L for 24 h accumulated plasma concentrations that were roughly equivalent to water concentrations (27±7 ng FAD/ml plasma; mean ±SD, n=3). Additionally, in female fathead minnows exposed for 8 d and then held in a continuous flow of control water for 24 h, plasma FAD concentrations declined from 22±4.5 ng/ml to 2.1±0.3 ng/ml (approximately 90%; Villeneuve et al. 2013). In a short-term time-course study with letrozole, another aromatase inhibitor with a log Kow around 2.6, mean (±SD) plasma concentrations after 2 h of exposure (32.5±9.6 ng/ml, n=12) were ≈75% of those measured after 24 h of continuous exposure (43±15 ng/ml; unpublished data). Thus we postulate that steady state plasma concentrations of FAD were likely reached within the first few hours of exposure.

Consistent with an AOP describing the effects of aromatase inhibition on reproductive performance of female fish (<https://aopwiki.org/wiki/index.php/Aop:25>; Villeneuve et al. 2016), significant direct effects of FAD on *ex vivo* and *in vivo* E2 status in female fathead minnows were observed in the present study. The E2 production by ovary explants collected from females exposed to 50 µg FAD/L *in vivo* for as little as 2 h was significantly reduced. However, significant reduction in E2 production by ovary explants from females exposed to 5 µg FAD/L was not observed until the females had been exposed for 6 h. Based on the mechanistic understanding of the biology (i.e., that most circulating E2 is synthesized in the ovary) and the AOP, it would be expected that the rate of E2 production by ovary tissue would be impacted before plasma E2 concentrations would decline significantly. The results for the 50 µg FAD/L treatment were concordant with that expectation, as significant impacts on *ex vivo* E2 production and plasma E2 concentrations were detected after 2 and 4 h of exposure, respectively (Figure 1). At 5 µg FAD/L, the effects were more modest, as one would expect at a lower dose, but a significant impact on plasma E2 was detectable at 4h,

which was 2 h before a significant impact on *ex vivo* E2 production was measured (Figure 1). In evaluating whether this represents a deviation from the causal relationship between E2 production in the ovary and circulating E2 concentrations, it is important to consider differences in the measurement methods used to evaluate the two endpoints. In the case of *ex vivo* E2 production, inhibition over the 12 h incubation period relies on FAD that had accumulated in the tissue during *in vivo* exposure. It is reasonable to assume that some FAD may dissociate from the tissue into the medium during incubation. Assuming the exposure concentration in the tissue is increasing over the first few hours *in vivo* and some dissociation from the tissue occurs during incubation, at the 5 µg/L, it may have required at least 6 h to accumulate sufficient FAD in the tissue to yield a significant effect on E2 production *ex vivo*, even though under *in vivo* conditions, with intact circulation, E2 production was being impacted earlier in the time-course. Likewise, the *ex vivo* assay tends to be more variable, conferring less statistical power than the plasma E2 measurements. With these methodological considerations in mind, the effects on E2 status overall were consistent the proposed AOP and support observations that the direct effects of aromatase inhibition were apparent within 4 h of exposure initiation.

The effects on female *ex vivo* E2 production and plasma E2 concentrations observed in this study are the most rapid reported impacts associated with chemical inhibition of aromatase in any fish species studied to date. Previous observations with fathead minnows exposed to another aromatase inhibitor, prochloraz, did not show a significant reduction in plasma E2 concentrations until after 12 h of exposure (Skolness et al., 2011). This difference in timing of inhibition is likely due to the greater potency of FAD for inhibiting aromatase as well as toxicokinetic differences as compared to prochloraz (log Kow 4.1). Although the present study showed rapid direct effects of FAD, the impacts on *ex vivo* E2 production and plasma E2 concentrations at 24 h in this study are consistent with observations made at 24 h for female fathead minnows exposed to FAD in more extended time course studies (Villeneuve et al., 2013; 2009a). As such, the current study further supports the generalized motif of biological response to aromatase inhibition in fish that has been observed in previous studies and documented using the AOP framework (Villeneuve et al. 2016). These results demonstrate that the biochemical impacts of aromatase inhibition can occur far more rapidly than has generally been studied in experiments with EDCs.

In addition to observing the effects of the chemical on E2 production, a key event in the associated AOP, we were interested in examining whether the early responses to FAD exposure would provide insights into the rapidity and mechanisms through which compensatory responses are triggered in response to aromatase inhibition. Therefore, we examined ovarian expression of a number of genes previously shown to be indicators of compensatory responses due to aromatase inhibition (Skolness et al., 2011; Villeneuve et al., 2009a; 2006). *Star* and *cyp11a* both code for proteins that are rate-limiting for steroidogenesis, with *star* mediating the transport of cholesterol, the precursor of steroid synthesis, to the inner mitochondria membrane and *cyp11a* catalyzing the first cleavage of cholesterol to steroids (Miller, 1998; Stocco, 2001). *Fshr* is expressed in the granulosa cells in the ovary and important for follicle stimulating hormone (FSH)-mediated regulation of steroidogenesis in fish (Dickey and Swanson, 1998; Gharib and Wierman, 1990). *Cyp19a1a* codes for the aromatase isoform predominantly expressed in the gonads (Tchoudakova and

Callard, 1998), and *cyp17* codes for an enzyme with dual hydroxylase and lyase activity that is essential for androgen and estrogen synthesis (Miller 1988). While expression of each of these genes (with the exception of *cyp17*) was significantly impacted, the profile of response was somewhat different than observed in longer term experiments. For example, over the course of an 8 d waterborne exposure to FAD, Villeneuve et al. (2009a) observed relatively persistent compensatory upregulation of *cyp19a1a*, *fshr*, *cyp11a*, and *star*. In the present study, *cyp19a1a*, *fshr*, and *cyp11a* were all upregulated at 1 h of exposure, but then were down-regulated relative to controls at 2 and/or 6 h (Figure 2). Previous studies involving exposure for several days showed that *cyp19a1a* and *fshr* expression was upregulated relative to controls, which continued for as long as exposure is maintained (Villeneuve et al. 2009a, 2013). Here, expression of *star* appeared to oscillate between control levels and significant down-regulation. Because the temporal resolution of prior studies (e.g., Villeneuve et al. 2009a, 2013) was on a scale of days rather than hours, we cannot rule out the possibility that similar oscillatory dynamics may have been occurring on an hourly basis between the daily sampling points characterized in the previous studies. Nonetheless, the contrast between the dynamic variation in expression of these genes over the first 24 h compared to more persistent changes over longer time scales suggest that the signaling mechanism(s) regulating expression of these genes in the early hours of exposure may differ from those operating after a sustained period of systemic depression of E2 concentrations. Notably, Stocco et al. (2008; citing Fitzpatrick and Richards, 1991) reported that 24-48 h is required for FSH to induce aromatase expression. Consequently, an endocrine feedback response mediated through increased FSH signaling may not be feasible over the hourly time-scale examined in the present study. The gene expression dynamics observed may instead be driven by autocrine/paracrine effects (for additional discussion see supporting information).

Nonetheless, within 4 h of exposure, it is clear that circulating estrogen concentrations were being impacted. Thus, on or around 4 h it would be reasonable to infer that an endocrine feedback response mediated through the HPG axis may be initiated. In the fathead minnow there is not an antibody available to specifically measure circulating FSH concentrations. Thus it remains uncertain if, and exactly when, a negative feedback response to declining E2 concentrations may have been triggered. However, the major shift in expression profile moving from 2 to 4 to 6 h (Table 2) may reflect transition from a predominantly autocrine/paracrine response to a more systemic endocrine response around that time. Qualitatively, many of the enriched pathways identified at 12 and 24 h were less obviously linked to FAD's mode of action. Such a transition may shift the system from early dynamic oscillation in expression of *cyp19a1a*, *fshr*, and *star* toward the more seemingly stable upregulation observed in longer terms studies (e.g., Villeneuve et al. 2009), but again, without conducting sampling at hour-scale resolution after longer term exposures one cannot be sure.

From a practical standpoint, these results have implications relative to the use of gene expression data as biomarkers of exposure to aromatase inhibitors. A variety of longer time-course studies with aromatase inhibitors have found increased ovarian *cyp19a1a* and *fshr* expression to be fairly reliable hallmarks of aromatase inhibition or reduced E2 concentrations (Villeneuve et al. 2006, 2009a, 2013; Ankley et al. 2009). While on one hand, the present data indicate similar responses may be observed in as little as 1 h following

initiation of exposure, they also indicate that expression may be highly dynamic over the initial period of exposure. A longer exposure duration may be needed to establish a more stable profile that would be more reliable as a biomarker response. In contrast, from the standpoint of attempting to elucidate chemical mode of action based on transcriptional response profiles, the earliest portion of the exposure provided a signal that could most readily be linked to the inhibition of aromatase by FAD. As one moves further from the rapid, direct effects of the chemical, toward secondary and tertiary responses reflective of the system responding to that perturbation it may become increasingly difficult to elucidate a chemical's mode of action based on transcript profiles. In that respect, dynamic changes in expression profiles can be seen to parallel the AOP framework being used to describe such generalizable patterns of response to stressors (Villeneuve et al. 2014; Ankley et al. 2010). Early key events in an AOP are temporally more proximal to the perturbation and specifically linked with a chemical's mode of action, while later key events are more proximally tied and reflective of the adverse biological consequence of those perturbations. This suggests that if the goal of testing is to define a chemical's mode of action, it may be beneficial to examine gene expression quite early in the time course of exposure, while if the goal is to identify a profile that is diagnostic of apical impact, longer exposures should be employed.

5. Conclusion

The HPG axis is a dynamic system capable of quickly responding to stressors to maintain homeostasis through various feedback loops. This study demonstrates that statistically significant biochemical and molecular responses to FAD exposure occur within the first 24 h of exposure. The effects on E2 production and circulating concentrations are rather consistent over this early period, while gene expression changes appear more dynamic. We postulate that initial variation of *cyp19a1a* and *fshr* during the first 6 h of exposure are reflective of altered autocrine and paracrine signaling within the granulosa cells and ovary, respectively. Moving beyond 6 h, the evidence seems to suggest a transition toward an endocrine, HPG-axis, regulated feedback response that results in a significant shift in ovarian expression profiles and a more stable and sustained compensatory upregulation of *cyp19a1a* and *fshr* as observed in previous studies. This study is the first to demonstrate just how rapid the HPG-axis in fish can respond to aromatase inhibition and that compensatory responses can be observed within hours of exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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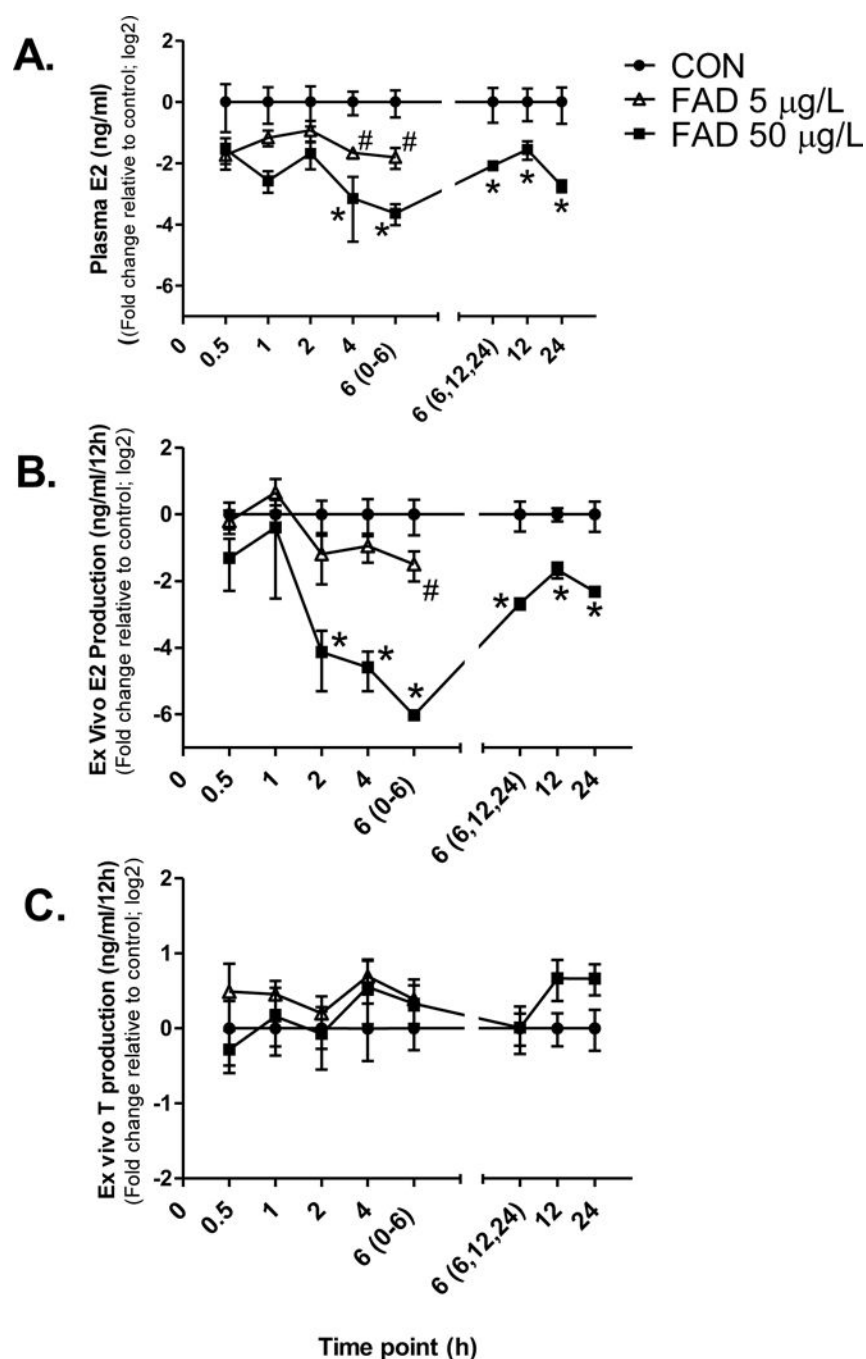
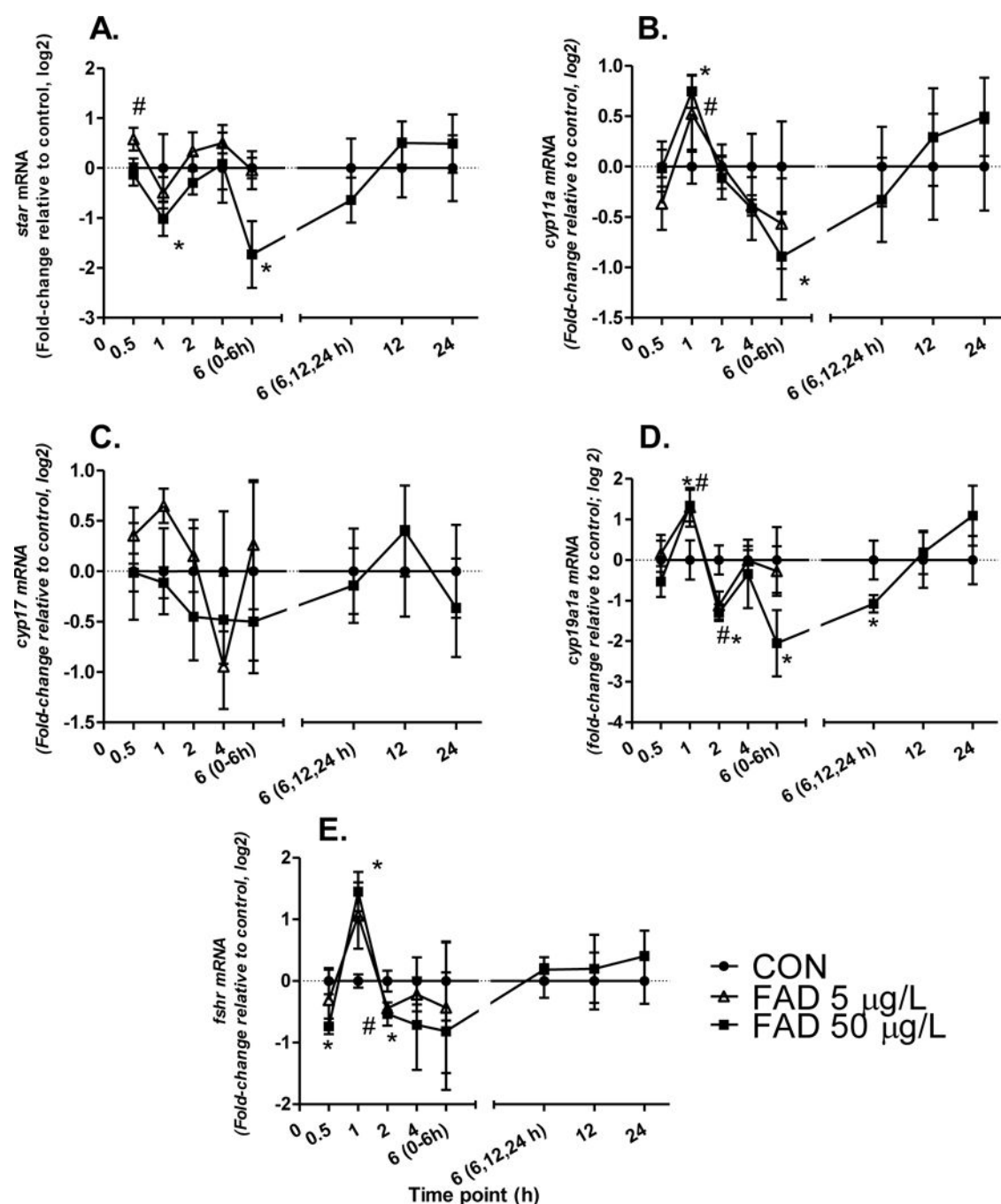


Figure 1.

Effects of fadrozole (FAD) on (A) Plasma 17 β -estradiol (E2) concentration, (B) *ex vivo* E2 production, and (C) *ex vivo* testosterone (T) production of female fathead minnows. Data points represent the mean \pm standard error (n=5-8) of the fold-change relative to controls from the same time point, expressed as log2 transformed units. *indicates significant difference from control at 50 μ g FAD/L, # indicates significant difference from control at 5 μ g FAD/L.

**Figure 2.**

Effects of fadrozole (FAD) on fathead minnow ovarian expression of transcripts for: (A) steroidogenic acute regulatory protein (*star*), (B) cytochrome P450 side-chain cleavage (*cyp11a*), (C), cytochrome P40 hydroxylase/lyase (*cyp17*), (D) cytochrome P450 aromatase (*cyp19a1a*), and (E) follicle stimulating hormone receptor (*fshr*). Data points represent the mean \pm standard error of the fold-change relative to controls from the same time point, expressed as log2 transformed units. Sample sizes analyzed for the 0-6 h experiment were n=6 for *star*, *cyp11a*, and *cyp19a1a* and n=4 for *cyp17* and *fshr*. Sample sizes analyzed in the

6, 12, 24 h experiment were n=7-8. *indicates significant difference from control at 50 µg/L (FAD-50), # indicates significant difference from control at 5 µg/L (FAD-5).

Table 1

Gene expression changes as determined by the 15k feature fathead minnow microarray in the ovaries of female fathead minnows exposed to 5 or 50 µg fadrozole/L over the course of 24 h. Differentially expressed genes based on t-tests or ANOVA with Tukey post hoc test ($p < 0.05$). Numbers in parentheses indicate genes downregulated and upregulated, respectively.

Time Point (h)	Total DEGs	Control vs Fadrozole (5 µg/L) DEGs	Control vs Fadrozole (50 µg/L) DEGs	Common DEGs between Fadrozole Treatments
0.5	978	533 (216, 317) Suppl. Table S1	362 (185, 177) Suppl. Table S6	141 (69, 72) Suppl. Table S15
1	1969	1204 (315, 889) Suppl. Table S2	1037 (280, 757) Suppl. Table S7	564 (91, 473) Suppl. Table S16
2	1272	285 (141, 154) Suppl. Table S3	880 (422, 458) Suppl. Table S8	149 (69, 72) Suppl. Table S17
4	471	110 (66, 44) Suppl. Table S4	236 (118, 118) Suppl. Table S9	29 (17, 12) Suppl. Table S18
6(0-6)	578	154 (62, 92) Suppl. Table S5	311 (100, 211) Suppl. Table S10	29 (17, 12) Suppl. Table S19
6 (6,12,24)	–	N/A	415 (201, 214) Suppl. Table S11	N/A
12	–	N/A	1123 (458, 665) Suppl. Table S12	N/A
24	–	N/A	453 (217, 236) Suppl. Table S13	N/A

“N/A” indicates the fadrozole concentration was not tested at this time

Table 2

Pathways identified as significantly enriched for two or more consecutive time points as a result of exposure to 50 µg fadrozole/L. Numbers in each cell indicate the proportion of differentially expressed genes (DEGs) associated with the pathway that were up-regulated. Red shading indicates predominant up-regulation (0.66); green shading indicates predominant down-regulation (0.33); yellow shading indicates fairly even split between up- and down-regulated DEGs (0.34–0.64).

Pathway	0.5 h	1 h	2 h	4 h	6 h	12 h	24 h
Huntington's Disease Signaling	0.55	0.83	0.33	0.6			
Estrogen Receptor Signaling	0.71	0.75					
Hereditary Breast Cancer Signaling	0.6	0.33	0.57				
PI3K/AKT Signaling	0.6	0.66	0.5				
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency		1	0.25				
NGF Signaling		0.75	0.4				
Sertoli Cell-Sertoli Cell Junction Signaling		0.4	0.57			1	
Androgen Signaling		0.75	0.6				
Tight Junction Signaling		0.5	0.71				
UVA-Induced MAPK Signaling		0.66	0.71				
Breast Cancer Regulation by Stathmin1		1	0.5				
UVB-Induced MAPK Signaling		0.5	0.75			0.6	
Cardiac α -adrenergic Signaling		1	0.5		0.66		
Estrogen-Dependent Breast Cancer Signaling	0.66		0.57	0.33			
Telomerase Signaling	0.6		0.5	0.33		0.66	
Protein Ubiquitination Pathway			0.45	0.6		0.6	
GNRH Signaling			0.57	0.33			
p70S6K Signaling	0.2		0.5	0.66			
cAMP-mediated signaling				0.2	0.33		
Inhibition of Matrix Metalloproteases			1		1	1	
Cardiac Hypertrophy Signaling			0.375		0.5	0.6	
Dopamine-DARPP32 Feedback in cAMP Signaling					0.66	0.6	
NRF2-mediated Oxidative Stress Response						0.25	0.75