

The Effects of Sertraline on Fathead Minnow (*Pimephales promelas*) Growth and Steroidogenesis

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Abstract The purpose of this study was to evaluate the steroidogenic effects of sertraline, a popular selective serotonin reuptake inhibitor, on larval fathead minnow (FHM; *Pimephales promelas*) and adult FHM. Larvae were exposed to 0.1, 1, and 10 µg/L sertraline for 28 days and analyzed for differential mRNA expression of 11β-hydroxysteroid dehydrogenase (11β-HSD), 20β-hydroxysteroid dehydrogenase (20β-HSD), aromatase (CYP19a), nuclear thyroid receptor alpha (TRα), and normalized to RP-L8. Adult FHM were exposed to 3 or 10 µg/L sertraline for 7 days and analyzed for differential expression of the same genes with the addition of thyroid receptor beta (TRβ). Larval FHM exposed to 0.1 µg/L had a significant upregulation of both 20β-HSD and TRα while adult FHM exposed to 10 µg/L had a significant upregulation of 11β-HSD expression in brain tissue. The significance of these findings with respect to survival, growth and reproduction are currently unknown, but represent areas for future research.

Keywords Pharmaceuticals · Fathead minnow · Endocrine disruption

Adverse side-effects from chronic exposure to low concentrations of the most frequently prescribed drugs is an important area of research. Recent studies suggest selective serotonin reuptake inhibitor (SSRI) such as fluoxetine

and sertraline may be present in wastewater treatment plant effluents at much higher concentrations than previously thought and may be therapeutically active in aquatic organisms (Brooks et al. 2003; Painter et al. 2009; Schultz et al. 2010; Schultz and Furlong 2008; Weinberger and Klaper 2014). More specifically, SSRIs have been found in aquatic systems at concentrations as high as 70 ng/L (Batt et al. 2008). Additionally, the effect some SSRIs might have on aquatic life is not well studied or understood (Brooks et al. 2003). While most pharmaceuticals may not pose an immediate threat to the health and safety of humans, chronic exposure to low concentrations of various pharmaceuticals may impact our ability to maintain normal biological processes, i.e., endocrine function (Taylor and Senac 2014), which forms the basis of the present work.

Steroid hormones are involved in many biological functions including: development, growth, reproduction, and many other chemical responses (Garcia-Reyero et al. 2014). Therefore, SSRIs or other pharmaceuticals in the environment may pose a threat to the reproduction and development of sensitive aquatic life. In addition, the possible endocrine disrupting attributes of popular SSRIs is an important toxicological topic and may prove to be invaluable in correlating serotonin with the differential expression of estrogen, testosterone, aldosterone, and/or cortisol catalytic enzymes. The effect of serotonin on steroidogenesis has been widely studied since the 1970s and is now becoming recognized as a key player in regions of the mammalian system. i.e., peripheral cell differentiation and mammary gland development (Dubé and Amireault 2007; Matsuda et al. 2004). In addition to adverse steroidogenic side-effects, thyroid function may also be altered by SSRIs. A recent study shows patients without depression and with normal thyroid function may have a decrease in triiodothyronine (T₃) and thyroxine (T₄) after SSRI treatment (de

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Morais and Traple 2010). This is important due to T_3 playing a significant role in fetal development, as well as influencing serotonin concentrations in the brain (Brochet et al. 1987).

In this study, we tested the endocrine disrupting potential of sertraline at environmental relevant concentrations via differential expression of 11β -hydroxysteroid dehydrogenase (11β -HSD), 20β -hydroxysteroid dehydrogenase (20β -HSD), aromatase (CYP19a), and nuclear thyroid receptor alpha/beta ($TR\alpha/\beta$) in adult and larval fathead minnow (FHM). 11β -HSD is the primary catalytic enzyme for the synthesis of corticosterone and cortisol from deoxycorticosterone and 11-deoxycortisol, respectively. 20β -HSD is the primary catalytic enzyme for the synthesis of $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one, which is a maturation inducing hormone in teleost fish, from 17α -OH-progesterone (Tokumoto et al. 2006). CYP19a drives the conversion of androgens to estrogens in gonadal tissue, more specifically estrone (E1) and estradiol (E2) from androstenedione and testosterone, respectively. Nuclear thyroid receptors, $TR\alpha/$ $TR\beta$, which recognize T_3 in the brain and regulate thyroid hormone function, were also screened. Differential neuroendocrine activity in aquatic organisms can play an imperative role in our knowledge of chronic exposure to environmental concentrations of antidepressants and give an insight to possible genetic ramifications (Valenti et al. 2012). Therefore, our primary objectives are to elucidate endocrine dysregulation following both an acute and long-term exposure towards the SSRI sertraline in fish depending on their life-stage (larval vs. adult).

Materials and Methods

For the adult exposure, 5 month old adult female FHM were obtained from the Tarrant County Regional Water District. Six adult female FHM were housed in three dechlorinated tap water flow through systems kept at $25 \pm 1^\circ\text{C}$ with 16:8 light/dark cycles and fed a small pinch of tetramine flakes twice daily. They were exposed to 3 and 10 $\mu\text{g/L}$ sertraline, as well as a dimethyl-formaldehyde (DMF) solvent control in three 5 L tanks with a flow rate of 13.9 mL/min (four turn-overs/day) for 7 days. For the larval exposure, FHM eggs were purchased from Aquatic Biosystems, Inc. (Fort Collins, CO). FHM larvae were fed 28 ± 8 mg (~ 1.5 mg/fish) *Artemia* and housed in a contained walk-in unit at $25^\circ\text{C} \pm 0.5$ with 16:8 light/dark cycles. The newly spawned FHM eggs (<48 h) were placed in glass beakers fitted with stainless-steel wire mesh false bottoms that allowed for proper water circulation and inhibition of egg congregation. FHM larvae were exposed in glass beakers (600 mL) which were separated into four groups: methanol solvent control, 0.1, 1, and 10 $\mu\text{g/L}$ sertraline, all in quadruplicate,

for 28 days. Beakers were filled with 500 mL water with 300 mL freshly prepared water replaced daily. In both adult and larval studies, reconstituted hard water was prepared according to EPA protocols for in vivo acute testing (EPA 2002). Prepared water fell within acceptable ranges for pH (7.92–8.6), hardness (168–180 mg/L), alkalinity (110 mg/L), dissolved O_2 (8.2–9.1 mg/L), and conductivity (0.0563–0.0604 S/m).

Water samples were collected from exposed beakers at day 0, 14, and 28 then analyzed via LC-MS/MS to insure ideal exposure concentrations of sertraline. Samples, except 0.1 $\mu\text{g/L}$ sertraline, were spiked with internal standard sertraline- D_3 (Cerilliant) and directly analyzed on the LC-MS/MS. Due to LC-MS/MS sensitivity ranges, 0.1 $\mu\text{g/L}$ samples had to be extracted via liquid extraction method and reconstituted at 1 $\mu\text{g/L}$ before analyzing. The liquid extraction process began with 10 mL of 0.1 $\mu\text{g/L}$ sample water spiked with 10x internal standard. Next, 5 mL of 1:1 hexane:ethyl-acetate was mixed with 10 mL of 0.1 $\mu\text{g/L}$ water sample and extracted ($\times 2$). The 10 mL of hexane:ethyl-acetate extract was then evaporated using nitrogen. The evaporated hexane:ethyl-acetate vial was then reconstituted in 1.5 mL of methanol and evaporated again using nitrogen until dry. Once dry, the 1.5 mL methanol vial was reconstituted in 100 μL methanol to a concentration of 1 $\mu\text{g/L}$ and then analyzed on the LC-MS/MS. Time-weighted sertraline concentrations for solvent control, 0.1, 1, and 10 $\mu\text{g/L}$ were 0.00, 0.09, 0.71, and 8.9 $\mu\text{g/L}$, respectively.

Following exposure periods, excised adult FHM brain and ovary tissue, as well as, pooled whole larval FHM were stored in RNAlater. RNA isolation occurred via the TRI-Reagent method according to the manufacturer's protocol. Isolated RNA was re-suspended in either tris-EDTA buffer (Sigma) or molecular grade RNase/DNase free water. After RNA isolation was concluded, all samples were treated for DNase contamination and analyzed for RNA integrity and purity/concentration. RNA purity/quantification was measured in duplicate and averaged using a BioTek Take3 micro-volume plate by calculating the ratio of absorbance values of RNA (260 nm) to the absorbance values of protein (280 nm). RNA samples were then diluted in molecular grade RNase/DNase free water or tris-EDTA buffer to 100 ng/ μL stocks and stored at -80°C . RT-qPCR assays were assessed using SYBR-green detection chemistry on a Rotor-Gene 6000 cycler (Corbett-Qiagen) (Heid et al. 1996). 11β -HSD, 20β -HSD, $TR\alpha/\beta$, CYP19a, and RP-L8 primers used in the RT-qPCR assays were previously published and cycler parameter optimization for each primer was achieved in house (Table 1) (Filby et al. 2007; Villeneuve et al. 2006). RT-qPCR analysis was evaluated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). We analyzed all RT-qPCR data using one-way analysis of variance

Table 1 Primers used for RT-qPCR analysis are provided below along with their NCBI accession numbers and forward/reverse sequences

Primer name	NCBI accession	Sequence 5'–3'	R ²	Efficiency (%)
11 β -HSD	DT228665	FW-GCATCGGCGAGC RV-CTCCTCGCCGTG	0.996	111
20 β -HSD	DT259130	FW-TGTCATGCTCTTC RV-CTTGCTAACAAAGC	0.981	117
CYP19a	AF288755	FW-TGCTGACACATGCA RV-CAGCTCTCCGTGGC	0.96	101
TR α	DQ074645	FW-ATGACCCAGAGA RV-CATCAGACACCA	0.984	115
TR β	AY533142	FW-GCCAACCAGTCAGG RV-AGCAACAGAAATGAGG	0.99	113
RP-L8	AY919670	FW-CATACCACAAGTACAAGGCCAAGA RV-ACCGAAGGGATGCTCAACAG	0.99	111

along with Tukey's post hoc test, but found no statistical significance. Therefore, RT-qPCR data was analyzed compared to control using the student's *t*-test ($p \leq 0.05$) and error identified as standard error of the mean (SEM).

Results and Discussion

Adult FHM ovary expression of steroidogenic enzymes did not show significant differential expression, but do imply a down-regulation trend in 11 β -HSD at 3 μ g/L sertraline (Fig. 1a). However, sertraline exposure caused a statistically significant up-regulation of 11 β -HSD ($p=0.03$) and 20 β -HSD ($p=0.0002$) at 10 μ g/L sertraline, in brain tissue (Fig. 1 b). CYP19a and thyroid receptors showed no change in either the ovary or brain tissue of adult FHM. Early life-stage results for 11 β -HSD and 20 β -HSD were

similar with an increase in transcript at 0.1, 1, and 10 μ g/L sertraline (Fig. 2). 20 β -HSD was the only significantly up-regulated gene with $p=0.01$ at 0.1 μ g/L sertraline (Fig. 2). 11 β -HSD and CYP19a were both differentially expressed, but lacked statistical significance. TR α transcripts were significantly increased in the 0.1 μ g/L exposure groups with $p=0.02$ (Fig. 2). CYP19a and TR α transcript amounts were inversely proportional to sertraline concentrations, but due to variance between samples they lacked significance at higher concentrations of sertraline.

Differential neuroendocrine activity in aquatic organisms can play an imperative role in our knowledge of chronic exposure to environmental concentrations of SSRIs and offer insight into possible genetic ramifications (Valenti et al. 2012). Due to sertraline targeting SERT transporters in neural tissue (Valenti et al. 2012), most of the differential gene expression effects realized in

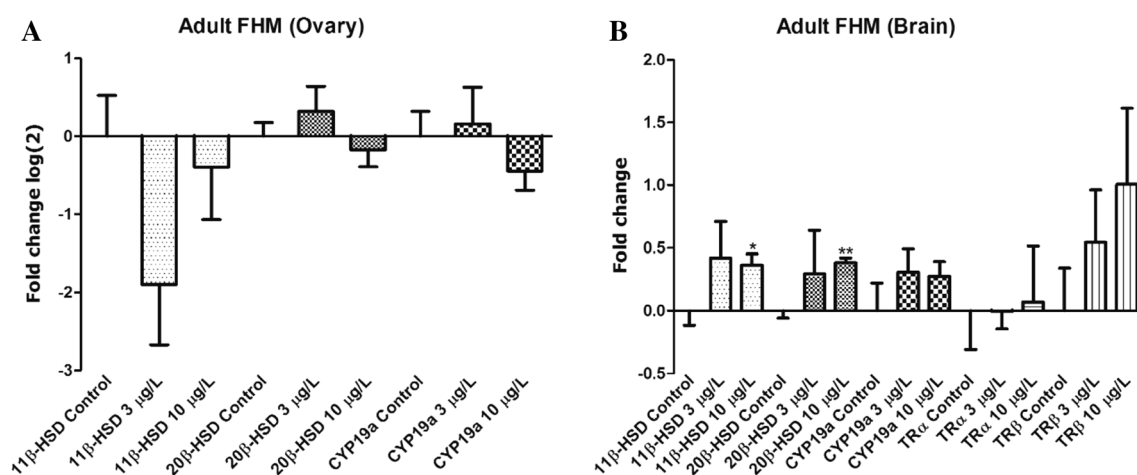


Fig. 1 **A** In adult ovary no significant changes in transcript abundance were observed in 11 β -HSD, 20 β -HSD, or CYP19a after exposure to either 3 or 10 μ g/L. **B** 11 β -HSD and 20 β -HSD are both up-regulated at 10 μ g/L ($p=0.03$ and 0.0002 , respectively). CYP19a

transcripts (10 μ g/L $p=0.054$). Ovary TR not displayed due to optimization deficiency. Ovary TR not displayed due to optimization deficiency. Results are relative to control. (*t*-test, * $p \leq 0.05$, ** $p \leq 0.01$, $n=5-6$) (\pm SEM)

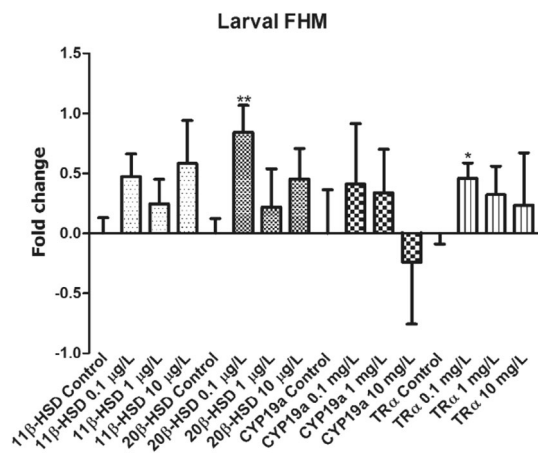


Fig. 2 Early life stage exposure of fathead minnow larvae for 28 days at 0.1, 1, and 10 µg/L sertraline. 20β-HSD and TRα were significantly up-regulated at 0.1 µg/L ($p=0.01$ and $p=0.02$, respectively), but had no effect at higher concentrations of sertraline. Results are relative to control. (t -test, $*p\leq 0.05$, $**p\leq 0.01$) ($n=5$ pooled samples, 2–5 fish per pool) (\pm SEM)

adult FHM occurred in the brain. At 10 µg/L sertraline, 11β-HSD and 20β-HSD transcripts in adult FHM brain tissue were increased compared to solvent control. Previously, prenatal SSRI exposures on male rodents have a decreased serotonergic receptor expression, serotonin concentrations, and even locomotion (Grzeskowiak et al. 2012). In addition, studies using serotonin transporter (5-HT) knockout rodents have shown a similar decrease in receptor expression and serotonin synthesis compared to control, implying a direction correlation between serotonin signaling and propagation of down-stream signaling (Grzeskowiak et al. 2012).

11β-HSD is the primary catalytic enzyme responsible for the conversion of 11-deoxycortisol to cortisol and/or the synthesis of other corticoid proteins, therefore increased cortisol concentrations should be influenced by an up-regulation of 11β-HSD. While glucocorticoids are elevated during stress and inflammation in both humans and fish, chronically increased plasma corticoid concentrations can lead to muscle wasting (Simmons et al. 1984). The results produced by this study suggest sertraline exposure may induce maturation in FHM as seen by the increase of 20β-HSD transcripts in the early-life stage study and adult FHM study. Previous studies show opposing effects with a decrease in 20β-HSD transcripts after prolonged levonorgestrel exposure, which has downstream effects on both cortisol and estradiol concentrations in teleost fish, therefore inhibiting maturation (Overturf et al. 2014). It is important to note the counteracting effects of sertraline, a popular prescription anti-depressant, to levonorgestrel, a popular over-the-counter birth control, on teleost fish endocrine function.

It has previously been reported that sertraline at environmentally relevant concentrations has a feminization effect on fish via reducing follicle stimulated hormone and testosterone while increasing endogenous estradiol concentrations (Mennigen et al. 2010). In the present study, differential expression statistical significance of CYP19a was not observed in larval or adult FHM; however, CYP19a transcripts were increased slightly compared to control groups ($p=0.054$) in adult FHM. Feminization effects have been documented in Japanese medaka via elevated estradiol and aromatase concentrations, therefore feminization may also occur in FHM with increased aromatase expression, but more research needs to be conducted on FHM development, maturation, and observed sex ratios following sertraline exposure (Kuhl and Brouwer 2005). Additionally, goldfish exposed to environmentally relevant concentrations of fluoxetine, another SSRI, led to increased estradiol concentrations and adverse side-effects relating to reproductive stability (Mennigen et al. 2010). Significant changes in steroidogenic gene expression did not occur in adult FHM ovary tissue most likely due to the short exposure period and the time it may take for differential gene expression to occur in down-stream organ tissues, e.g., hypothalamus–pituitary–gonadal axis.

In conclusion, results from this study suggest sertraline alters the regulation of steroidogenic enzymes in larval FHM at close to environmentally relevant concentrations, ≥ 0.1 µg/L sertraline. While widespread differential expression is not observed, it should be noted that one differentially expressed steroidogenic catalytic enzyme can lead to endocrine stress equating to detrimental effects on development and reproduction. An important aspect of this study that requires further attention is the consistency of 20β-HSD to be differentially regulated in both adult FHM and larval FHM. $17\alpha,20\beta$ -DP's ability to activate progesterone receptors is an important concept, therefore correlating membrane progesterone receptor (mPR) expression with 20β-HSD expression concentrations in the presence of SSRIs such as sertraline may incorporate an important piece of the SSRI/steroidogenesis puzzle. Additionally, differentially expressed maturation inducing hormones may provide further implications on development and/or subsequent generations. While no significance was observed in the end weight analysis of larval FHM, further research is needed due to characteristics of trend in this study. In situ hybridization, fecundity, and population analysis are necessary for the complete pharmacological toxicity of sertraline on FHM development and steroidogenesis, especially in the role of sertraline toxicity occurring in the wild. Lastly, we have shown sertraline, at concentrations close to those detected in the field, can potentially induce effects in population fitness, specifically, developmental and reproductive health.

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