

Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows

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ABSTRACT

Antidepressant pharmaceuticals have been reported in wastewater effluent at the nanogram to low microgram-per-liter range, and include bupropion (BUP), fluoxetine (FLX), sertraline (SER), and venlafaxine (VEN). To assess the effects of antidepressants on reproductive anatomy, physiology, and behavior, adult male fathead minnows (*Pimephales promelas*) were exposed for 21 days either to a single concentration of the antidepressants FLX, SER, VEN, or BUP, or to an antidepressant mixture. The data demonstrated that exposure to VEN (305 ng/L and 1104 ng/L) and SER (5.2 ng/L) resulted in mortality. Anatomical alterations were noted within the testes of fish exposed to SER and FLX, both modulators of the neurotransmitter serotonin. Additionally, FLX at 28 ng/L induced vitellogenin in male fish—a common endpoint for estrogenic endocrine disruption. Significant alterations in male secondary sex characteristics were noted with single exposures. Effects of single compound exposures neither carried over, nor became additive in the antidepressant mixtures, and reproductive behavior was not affected. Analysis of brain tissues from the exposed fish suggested increased uptake of FLX, SER and BUP and minimal uptake of VEN when compared to exposure water concentrations. Furthermore, the only metabolite detected consistently in the brain tissues was norfluoxetine. Similar trends of uptake by brain tissue were observed when fish were exposed to antidepressant mixtures. The present study demonstrates that anatomy and physiology, but not reproductive behavior, can be disrupted by exposure to environmental concentrations of some antidepressants. The observation that antidepressant uptake into fish tissues is selective may have consequences on assessing the mode-of-action and effects of these compounds in future studies.

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1. Introduction

Pharmaceutically active compounds (PhACs) enter wastewater treatment plants via human excretion and disposal of unwanted medications to sewage (Ruhoy and Daughton, 2008). With limited removal from these plants, PhACs and select degradates can remain biochemically active as they enter the aquatic ecosystem through wastewater effluent (Hiemke and Härtter, 2000; Dietrich et al., 2002), although concentrations in wastewater or wastewater-impacted surface water are typically less than 1 µg/L (Kolpin et al., 2002; Glassmeyer et al., 2005). Other sources of freshwater contamination have been cited and include leaching from landfills (Schwarzbauer et al., 2002) and pharmaceutical manufacturing (Larsson, 2007). Generally, these pharmaceuticals do not display

high acute ecotoxicity because lethal effects in aquatic life typically occur at concentrations greater than 1 mg/L (Cunningham et al., 2006). However, organisms may receive continuous exposure in “pseudopersistent” scenarios where chemical half-lives are exceeded by effluent introduction rates (Daughton, 2002), therefore justifying the need to characterize chronic, sublethal effects of these drugs on aquatic life (Flaherty and Dodson, 2005; Connors et al., 2009; Zeilinger et al., 2009).

Antidepressant pharmaceuticals and select degradates have been reported in wastewater effluent and effluent-receiving surface waters at the nanogram to low microgram-per-liter range (Schultz and Furlong, 2008; Lajeunesse et al., 2008; Metcalfe et al., 2010; Schultz et al., 2010), and include the selective serotonin reuptake inhibitors (SSRIs) fluoxetine (FLX) and sertraline (SER), the serotonin-norepinephrine reuptake inhibitor venlafaxine (VEN), and the norepinephrine-dopamine reuptake inhibitor (NDRI) bupropion (BUP) (Fig. 1). As the cellular receptors of these antidepressant pharmaceuticals are evolutionarily conserved (Fent

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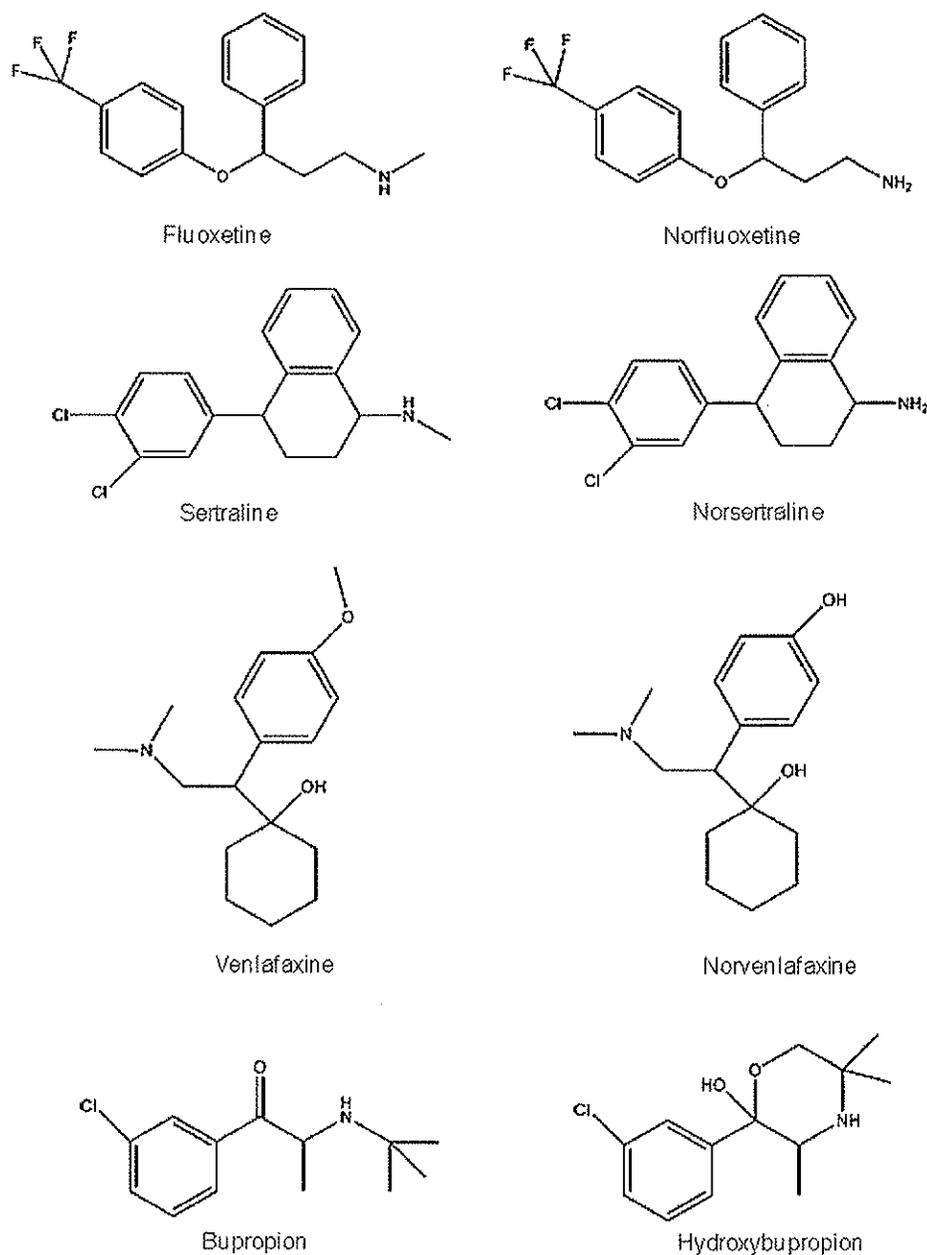


Fig. 1. Chemical structures of antidepressants (Column 1) and their respective metabolites (Column 2).

et al., 2006), aquatic life may experience exposure effects similar to the intended pharmacological response or its side effects such as disruption of neurohormonal homeostasis, alterations to the immune system and disruption of neurotransmission (van der Ven et al., 2005). Recently, analytical techniques have enabled detection of PhAC residues in tissues of freshwater fish living in effluent-impacted streams (Brooks et al., 2005; Ramirez et al., 2009; Schultz et al., 2010). While antidepressants were detected in fish brain tissue, a qualitatively different antidepressant profile was observed in concurrently collected streamwater samples (Schultz et al., 2010).

In vertebrates, these PhACs possess the ability to alter vital endogenous neurotransmitter levels. In aquatic organisms, serotonin influences critical stages of reproductive development (Gagné and Blaise, 2003) and also stimulates luteinizing hormone (LH) production in rats (Pinilla et al., 1997). In males, LH affects testosterone production in testicular Leydig cells, and testos-

terone promotes spermatogenesis (Hendrick et al., 2000). In female zebrafish, FLX decreased 17β -estradiol plasma concentrations and resulted in reduced egg production (Lister et al., 2009). Several studies have also described behavioral alterations in fish exposed to antidepressants, including abnormal positioning in the water column (Gaworecki and Klaine, 2008; Lister et al., 2009) a decreased ability to capture prey when the predatory fish was exposed to FLX (Gaworecki and Klaine, 2008) and a delayed escape response when the prey fish was exposed to FLX or VEN (Painter et al., 2009).

The present study aimed to evaluate the selective nature of antidepressant uptake in tissues and to assess effects of single compound and mixture antidepressant exposure on the anatomy and reproductive physiology of adult male fathead minnows. Additionally, since these drugs are often found in mixtures within the aquatic environment and since adverse behavioral alterations were noted in previous studies, mixture effects were further

Table 1

Measured concentrations (ng/L) of antidepressant pharmaceuticals in the single exposure treatments and their mixtures. Water samples were taken from the aquarium inflow on days 1, 3, 5, 10 and 20. The degradates, NFLX, NSER, NVEN, and HBUP, were not detected in any of the water samples.

| Compound | Low | | High | |
|---------------|-----|--------------------|------|--------------------|
| | n | Mean ± stand. err. | n | Mean ± stand. err. |
| Fluoxetine | 5 | 2.5 ± 0.99 | 5 | 28 ± 4.2 |
| Sertraline | 5 | 1.6 ± 0.46 | 4 | 5.2 ± 2.2 |
| Venlafaxine | 5 | 305 ± 32 | 5 | 1104 ± 89 |
| Bupropion | 5 | 7.4 ± 3.4 | 5 | 57 ± 23 |
| Mixture (MIX) | | | | |
| FLX | 5 | 2.2 ± 1.2 | 5 | 28 ± 2.5 |
| SER | 5 | 1.3 ± 0.78 | 5 | 22 ± 1.5 |
| VEN | 5 | 117 ± 47 | 5 | 798 ± 95 |
| BUP | 5 | 50 ± 19 | 5 | 466 ± 69 |

evaluated using a behavioral reproductive challenge. Thus, the study was designed to test two hypotheses. (i) Antidepressant pharmaceuticals affect reproductive anatomy (testis morphology), physiology (vitellogenin production) and behavior (reproductive assay) through the hypothalamus–pituitary–gonadal (HPG) axis. (ii) The uptake of antidepressant pharmaceuticals by the organism is selective. Together, this integrated assessment of uptake rates and biological consequences of antidepressant pharmaceuticals at environmentally relevant concentrations represents the first comprehensive laboratory analyses of these contaminants of emerging concern.

2. Materials and methods

2.1. Fish source and PhAC exposure

Three experiments were conducted at the St. Cloud State University Aquatic Toxicology Laboratory (St. Cloud, MN, USA). The first experiment exposed fish to either a blank water control, or one of two concentrations of BUP, SER or VEN. The second experiment included a blank and an ethanol carrier control and two concentrations of FLX. The third experiment consisted of a blank and a carrier control and low and high MIX treatments (Table 1). Adult male fathead minnows (six months old) were purchased from Environmental Consulting and Testing (Superior, WI, USA) and maintained at 22.9 °C ± 0.2 (mean ± standard error), pH 8.4 ± 0.02, under a constant 16:8 h light:dark photoperiod and fed frozen brine shrimp (*Artemia nauplii*) twice daily *ad libitum* (Denny, 1987). We focused on male fish only to avoid confounding effects associated with mixed sex exposures (dominance hierarchies, etc.). Fathead minnows were chosen as they are a well-established laboratory model for contaminants of emerging concern. In addition, they are an ecologically important and near-ubiquitous forage fish in North America, thus adding environmental relevance to the study, which is investigating environmentally realistic concentrations of antidepressants as reported from North America. Fish for the continuous 21-day flow-through (18-fold volume exchange/24 h) exposure were randomly placed in 16 L Pyrex® aquaria (n = 10 fish/aquarium, four aquaria/treatment; n = 40/treatment). Fish were exposed to either a blank well water control, a carrier control (ethanol), a single antidepressant FLX, SER, VEN, or BUP (at either of two concentrations), or to an antidepressant mixture (MIX, Table 1). Exposure concentrations were selected to represent antidepressant concentrations reported in surface waters and spanned at least a three-fold difference between low and high doses to account for temporal and spatial concentration variability observed in the aquatic environment (Lajuenesse et al., 2008; Schultz and Furlong, 2008).

Concentrated stock solutions were prepared in ethanol at the U.S. Geological Survey laboratories (Denver, CO, USA) and used to

draw daily spike aliquots. Masses of neat standards of bupropion hydrochloride (Toronto Research Chemicals, Toronto, ON), fluoxetine hydrochloride (U.S. Pharmacopeia, Rockville, MD) sertraline hydrochloride (U.S. Pharmacopeia), and venlafaxine hydrochloride (Bosche Scientific, New Brunswick, NJ) of between 98% and 99.9% minimum purity, were individually dissolved in unadulterated anhydrous ethanol to yield concentrated single-component stock solutions. From these stock solutions, daily spike aliquots were prepared with deionized well water in amber vials to deliver the desired concentrations (Table 1) with daily spikes stored at 4 °C for the entire experiment. One aliquot of the pertinent chemical was dissolved daily in 10 L of conditioned deionized water and gently agitated to ensure proper mixing. The 10 L solution was then delivered to a stainless steel mixing chamber using peristaltic pumps. Through use of a mixing chamber, all exposure aquaria within a treatment group were ensured similar exposure concentrations. Total ethanol solvent dosing to all the aquaria was less than 0.0001% (v/v), and ethanol controls were added to experiments. Water samples were collected from the outflow of each mixing chamber to determine actual exposure concentrations. Selecting the aquarium inflow as sampling point assured that our analysis quantified the chemical concentrations that would initially insult the fish, rather than aquarium concentrations that reflect biological breakdown and uptake mechanism that would confound our results. This sampling approach is also consistent with previous studies (Schoenfuss et al., 2008; Hyndman et al., 2010; Shappell et al., 2010). The extraction method and antidepressant determination are fully described elsewhere (Schultz and Furlong, 2008). Briefly, Waters Oasis® HLB cartridges (Milford, MA) were used for the solid-phase extractions. The extracts were analyzed by liquid chromatography tandem mass spectrometry with electrospray ionization (LC/ESI/MS/MS, Agilent 6410, Santa Clara, CA). Recoveries of parent antidepressants from matrix spiking experiments for the individual antidepressants ranged from 72% to 118% at low spiking concentrations (0.5 ng/L) and 70% to 118% at high spiking concentrations (100 ng/L). Method detection limits for the individual antidepressant compounds ranged from 0.19 to 0.45 ng/L. The limits of quantitation (LOQ) were defined as the concentrations that yielded signal-to-noise values ≥ 10 within the sample matrix.

Following exposure, 10 fish were randomly selected, sacrificed, body weight and total body length determined, blood samples drawn, and liver and testes removed for histological analysis. An additional 15–20 fish per treatment were sacrificed at the same time for brain tissue analysis. For the remaining 10 fish exposed to the antidepressant mixture, an additional four-day behavioral reproductive challenge followed the exposure (Schoenfuss et al., 2008; Hyndman et al., 2010). Research was conducted in accordance with St. Cloud State University Institutional Animal Care and Use permits.

2.2. Analysis

2.2.1. Secondary sex characteristics

A blind scoring system (Smith, 1978) based on a 0–3 scale (0 = no expression; 3 = pronounced secondary sex characteristic) was used to characterize the expression of nuptial tubercles, dorsal pad, and color expression. Scoring of each trait was done independently (0–3), before scores of all three traits were added to obtain a total expression value for each fish (0–9) following procedures described in Hyndman et al. (2010).

2.2.2. Body indices

Liver and testes were removed and weighed to the nearest 0.1 mg using an analytical scale. The gonadosomatic index (GSI) and hepatosomatic index (HSI) of each fish was determined by dividing the weight of the organ by the weight of the fish and then multi-

plying by 100. The condition factor (CF) was calculated as: [weight of fish (g)/total length (mm)³].

2.2.3. Histological examination

Tissues were prepared following modified procedures by Gabe (1976), then embedded in paraffin, sectioned to a thickness of 5 μ m, and hematoxylin and eosin stained (Carson, 1997). Testes slides were examined for interstitial cell (including Leydig cell) prominence (Fig. 5), and the abundance of spermatogonia and spermatozoa. Testes slides were also used to confirm sex and the sexual maturity stage of treated fish; females (that were misidentified as males due to their similarities to subordinate males) and immature males were excluded from all further analyses. Liver slides were examined for proliferation of vacuolization of hepatocytes. Each endpoint was ranked using a 0–4 scale (0 = low; 4 = high abundance/proliferation) following a modified US EPA procedure (Wolf, 2008).

2.2.4. Vitellogenin analysis

Blood was collected from the caudal vasculature of fish using a heparinized capillary tube and centrifuged (5900 \times g for 5 min) to isolate plasma. The plasma was stored in a -80°C freezer until analysis as described by Hyndman et al. (2010). Briefly, plasma vitellogenin levels were measured via a competitive antibody-capture enzyme-linked immunosorbent assay (ELISA). Microtiter wells were coated with fathead minnow VTG at an approximate concentration of 4 $\mu\text{g}/\text{mL}$ in coating buffer (0.35 M sodium bicarbonate, 0.15 M sodium carbonate, pH 9.6). A pre-incubation binding step was carried out where plasma samples or standards were diluted and mixed 1:1 (1:20,000 final dilution) with a polyclonal anti-fathead minnow VTG antibody (provided by Gerald LeBlanc, North Carolina State University) and incubated at 37°C for 2 h. Prior to the completion of the pre-incubation, plates were washed three times with wash buffer in an automated plate washer and 200 μL of each sample or standard was added. After 1 h incubation at room temperature, plates were washed and 200 μL of horseradish peroxidase labeled anti-rabbit IgG secondary antibody was added and incubated for 1 h at room temperature. After washing plates, 200 μL of tetramethylbenzidine substrate (Sigma, St. Louis, USA) was added and incubated in the dark for 15 min. Absorbance was read at 620 nm on a Multiskan EX microplate reader (Thermo Fisher Scientific, Waltham, MA). Standard curves were constructed and sample values calculated using the accompanying Multiskan software. The standards were prepared as a seven step, two-fold serial dilution with a range of 0.075 $\mu\text{g}/\text{mL}$ to 4.8 $\mu\text{g}/\text{mL}$. The resultant standard curves were robust, with *r*-squared values routinely higher than 0.99. Occasionally, the lowest standard (0.075 $\mu\text{g}/\text{mL}$) was removed from the curve to keep the *r*-squared above 0.99. Each plate contained an internal standard control of VTG at three dilutions within the standard curve range. The upper and lower limits of detection for vitellogenin were 5 mg/mL and 4 $\mu\text{g}/\text{mL}$, respectively. Any number that fell below the detection limit was given a value half that of the detection limit (2 $\mu\text{g}/\text{mL}$) (Hyndman et al., 2010). Vitellogenin data was log transformed prior to additional statistical analysis.

2.2.5. Behavioral reproductive challenge

Immediately following the 21-day exposure, male secondary sex characteristics were graded for a subset of ten males in the low and high MIX treatments. Each exposed male was then matched by rank with a control male, to remove social status as a confounding variable. Males were randomly assigned an upper or a lower caudal fin clip to visually identify each fish. Random fin clips assured that the observers remained unaware of the treatment status of each male, thus preventing the introduction of unintentional scoring biases. The two males were placed simultaneously into a tank

that contained one nest site. Fathead minnows practice paternal nest care (Unger, 1983) and fish almost instantaneously begin to compete for this structure (Schoenfuss et al., 2008; Hyndman et al., 2010). Twice daily, the fish that occupied and defended the nest site was noted using the caudal fin clip for identification. Observations were tallied, and a fish that occupied the nest site within a tank for >65% of the time, was credited with being that tank's reproductive challenge winner. Tanks in which a single fish did not occupy the nest site for >65% of the observations were excluded from further analysis (9 of 53 tanks total). We chose to exclude these tanks from further analysis as prolonged nest occupation by one male is necessary to ensure that fertilized eggs are protected from predators and conspecifics (Unger, 1983).

2.2.6. Brain tissue analysis

A subset of fish ($n = 15\text{--}20/\text{treatment}$) were processed for analysis of antidepressant chemical concentrations in the brain tissue. These fish were not included in other analyses. Brains were removed through an incision initiated at the foramen magnum and continued midsagittally to the frontal bone. Three to four brains from fish exposed to the same treatment were combined to assure sufficient tissue for analysis (approximately 0.05–0.08 g) (Schultz et al., 2010). Excised brains were flash-frozen in liquid nitrogen and stored at -80°C until analysis. Formic acid and acetonitrile were added to the thawed brain tissue and homogenized with an ultrasonic tissue disruptor. The brain sample was centrifuged; the supernatant removed and evaporated to dryness with nitrogen gas, and the final extract was reconstituted with 0.1% formic acid. Brain extracts were analyzed by the LC/ESI/MS/MS as described elsewhere (Schultz et al., 2010). The individual antidepressant recoveries ranged from 77% to 97%. The LOQ was 0.015 ng/g for all antidepressants.

2.2.7. Metabolite chemical analysis

Norfluoxetine (NFLX, >97%, Sigma–Aldrich, St. Louis, MO), norsertaline (NSER, >98%, Toronto Research Chemicals, Toronto, Ontario), hydroxy bupropion (HBUP, >98%, Toronto Research Chemicals), and norvenlafaxine (NVEN, Toronto Research Chemicals >98%, Sigma–Aldrich) are metabolites of FLX, SER, BUP, and VEN, respectively and were measured in the water and brain extracts (Fig. 1) as in Schultz and Furlong (2008) and Schultz et al. (2010), adapted to include HBUP and NVEN analysis. The mass spectrometry identification and quantitation parameters are described in Table 2.

2.2.8. Statistical analysis

The assumption of normality was tested with the Lilliefors test for normality prior to any additional analysis (Prism 4.01TM, GraphPad Software, Oxnard, CA, USA). Treatments that violated the assumption of normality were analyzed using the non-parametric Kruskal–Wallis test followed by Dunn's post-test. Normally distributed data were analyzed using one-way ANOVA followed by Tukey's Test. All statistical tests were conducted by experiment, even if experiment contained more than one test chemical (for example, experiment one). Nest holding ability in the reproductive challenge and fish survival was assessed using a two-tailed Fisher's Exact Test. For statistical analysis, all treatments were compared to the well water. A probability of $p < 0.05$ was set as the level of significance for all comparisons.

3. Results

3.1. Aqueous antidepressant concentrations

Antidepressants were not detected in the water of the control or the ethanol control treatments. While antidepressant concen-

Table 2

Mass spectrometer parameters and ion transitions for identification and quantitation for isotopically labeled and native antidepressants and their respective metabolites.

| Compound | Precursor ion (m/z) | Fragmentor voltage (V) | Primary product ion (m/z) | Collision energy primary ion (eV) | Secondary product ion (m/z) | Collision energy secondary ion (eV) |
|--|---------------------|------------------------|---------------------------|-----------------------------------|-----------------------------|-------------------------------------|
| Fluoxetine | 310 | 85 | 44 | 10 | 148 | 15 |
| Norfluoxetine | 296 | 90 | 30 | 10 | 134 | 5 |
| Sertraline | 306 | 85 | 275 | 10 | 159 | 15 |
| Norsertraline | 292 | 85 | 275 | 10 | 159 | 20 |
| Venlafaxine | 278 | 85 | 260 | 10 | 121 | 10 |
| Norvenlafaxine | 264 | 85 | 58 | 15 | 246 | 5 |
| Bupropion | 240 | 85 | 184 | 10 | 166 | 15 |
| Hydroxybupropion | 256 | 85 | 238 | 10 | 215 | 10 |
| Fluoxetine-d ₅ ^a | 315 | 85 | 44 | 10 | 148 | 15 |
| Sertraline-d ₃ ^b | 309 | 85 | 275 | 10 | 159 | 15 |

^a Surrogate.^b Internal standard.

trations generally correlated well between the single compound exposures and the composite mixtures, sertraline concentrations in the MIX-H treatment were four-fold greater than in the SER-H treatment and bupropion concentrations in MIX-H were eight-fold greater than in BUP-H. The degradates, NFLX, NSER, NVEN, and HBUP, were not detected in any of the water samples.

3.2. Fish survival body indices and vitellogenin induction

Survival in all control and ethanol control treatments exceeded 85% (Fig. 2). However, significant mortality occurred in the VEN-L ($p < 0.0001$), VEN-H ($p = 0.034$), and SER-H ($p = 0.005$) treatments. The GSI (Ex1: $p = 0.30$), (Ex2: $p = 0.10$), (Ex3: $p = 0.27$), HSI (Ex1: $p = 0.28$), (Ex2: $p = 0.054$), (Ex3: $p = 0.44$), and condition factor (Ex1: $p = 0.67$), (Ex2: $p = 0.61$), (Ex3: $p = 0.10$) did not differ significantly among groups (data not shown). A statistically significant induction of vitellogenin was present in FLX-H exposed fish ($p = 0.023$, Fig. 3).

3.3. Histological findings

Histological examination of the testes presented no statistically significant difference in spermatogonia (Ex1: $p = 0.36$), (Ex2: $p = 0.15$), (Ex3: $p = 0.15$) or spermatozoa abundance (Ex1: $p = 0.67$), (Ex2: $p = 0.63$), (Ex3: $p = 0.77$) (data not shown). However, a statistically significant difference was observed in testicular interstitial cell prominence in the SER-L ($p = 0.004$) and FLX-H ($p = 0.003$) treatment groups (Figs. 4 and 5). No statistically significant changes in abundance of hepatic adipocytes (Ex1: $p = 0.22$), (Ex2: $p = 0.35$), (Ex3: $p = 0.42$) or vacuolization of hepatocytes (Ex1: $p = 0.57$), (Ex2: $p = 0.22$), (Ex3: $p = 0.35$) were observed (data not shown).

3.4. Male secondary sex characteristics and nest holding ability

In the single compound exposure experiments, a statistically significant difference in male secondary sex characteristics (Ex1: $p = 0.016$) (Ex2: $p = 0.048$) was observed (Fig. 6, although the differences could not be resolved using Dunn's post-test). There was no statistically significant difference between the nest holding ability of control and ethanol control ($p = 0.39$), MIX-L ($p = 1.00$), or MIX-H ($p = 1.00$) treated fish (data not shown).

3.5. Brain tissue analysis

Analysis of brain tissues revealed differential uptake patterns for the antidepressant pharmaceuticals (Table 3). Compounds were only detected in brains of fish exposed to the respective compound(s) ruling out other sources of PhACs in the exposure system. In both low (L) and high (H) treatments, FLX, SER, and BUP were found in brains of exposed fish at higher concentrations than

suggested by water concentrations of the respective compounds (Tables 1 and 3, Fig. 7). In contrast, VEN-L exposed fish did not have detectable concentrations of VEN in their brains, while VEN-H exposed male fathead minnows only yielded occasional detections of VEN in three out of five brain composite samples despite the higher measured water concentrations of this compound compared to the other tested antidepressants. The only metabolite consistently detected in brains during the exposure studies was NFLX, which was found in all brain composites of FLX exposed fish (FLX-L, FLX-H, MIX-L, MIX-H). NSER was only detected in two brain composites of MIX-H, which had higher SER concentrations than the single SER exposures. This selective uptake of antidepressants into fish neural tissue supports the results of previous field research in two effluent-impacted streams (Schultz et al., 2010).

4. Discussion

The present study demonstrates that 21-day exposures to antidepressants at environmentally realistic concentrations reduced survival (VEN and SER), induced vitellogenin (FLX), and altered testis morphology (SER, FLX). The observed effects were often subtle and did not alter the reproductive behavior of adult male fish. Furthermore, the uptake of these compounds by the organism appears to be selective with several compounds being measured in the brain at much higher or lower concentrations than in the respective exposure water.

Fathead minnows exposed to environmentally relevant concentrations of SER and VEN suffered significant mortality up to 60% (VEN-L – 305 ng/L). The specific mortality-based mode of action is unknown; however, a medical study (Whyte et al., 2003) reported higher toxicity of VEN in humans when compared to other antidepressant pharmaceuticals. Furthermore, this data demonstrates the hazard posed by chronic exposure to antidepressant concentrations at an order of magnitude below published acute toxicity tests with much shorter exposure periods (e.g., SER LC₅₀ value for *Oncorhynchus mykiss* 96 h test: 380 µg/L; Minagh et al., 2008).

Antidepressants have been detected at high concentrations in liver tissues of fish (e.g., SER 545 ng/L in Ramirez et al., 2009); however, histological examination of adipocyte proliferation and vacuolization of hepatocytes in exposed fathead minnows did not find significant anatomical alterations. Histological examination of fish testes showed antidepressant exposure did not affect the process of spermatogenesis (spermatogonia and spermatozoa abundance were similar among treatments and controls) in adults. A statistically significant decrease in interstitial cell prominence was observed in the testes of fish exposed to SER-L, while significant interstitial cell hypertrophy was apparent in the FLX-H (28 ng/L) treatment. In mammalian studies (Hedger et al., 1995), serotonin administration resulted in alterations to intratesticular testosterone concentrations and changes to interstitial (Leydig) cell

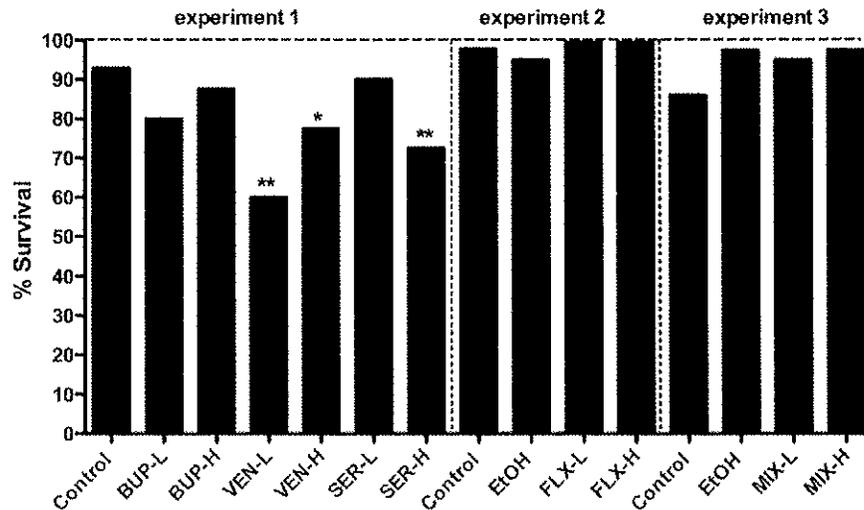


Fig. 2. Effects on the survival of adult male fathead minnows ($n=40$ at start of experiment) exposed to the antidepressant pharmaceutical bupropion (BUP), venlafaxine (VEN), sertraline (SER) and fluoxetine (FLX) at a low (L) or high (H) concentrations or in mixtures (MIX). Each experiment included a control and experiments two and three also an ethanol carrier control (EtOH). * indicates significance at $p < 0.05$ (Fisher's exact test against control), ** $p < 0.01$. Dashed vertical lines indicate separate experiments.

steroidogenesis in male rats. Furthermore, the effects of altering serotonin concentrations in male rats appear to be non-monotonic with different SSRIs producing dissimilar effects (Horn and Fink, 1985). In teleost fish, LH stimulates testosterone production in Leydig cells (Norris, 2007), and this LH-induced androgen elevation parallels functional alterations in Leydig cell morphology (Schulz et al., 1997). The changes to interstitial cell prominence observed in the current study suggest the potential for alteration of steroidogenesis in exposed fish and the opposing effects of two seemingly similar antidepressant pharmaceuticals (SER and FLX) are congruent with findings from the mammalian literature (Horn and Fink, 1985; Hedger et al., 1995). In fish, SSRI exposure is able to modulate brain serotonin activity (Gaworecki and Klaine, 2008). Serotonin can indirectly affect interstitial and Leydig cell prominence via the HPG axis through effects on gonadotropin-releasing hormone activity (Schulz and Goos, 1999), or by stimulating the release of LH from the pituitary (Trudeau, 1997). Somoza and Peter (1991) further demonstrated that by adjusting serotonin concen-

trations, differing effects on LH (through a $5HT_2$ -like receptor) can be observed. Although SER and FLX have similar modes of action as psychotherapeutic drugs in humans, it is possible that subtle changes in chemical structure, and subsequent pharmacokinetics, may provide this slight difference in serotonin concentration, which results in distinct effects on LH. In teleosts, LH stimulates testosterone production in Leydig cells (Norris, 2007), and this LH-induced androgen elevation parallels functional alterations in Leydig cell morphology (Schulz et al., 1997).

Antidepressants have been detected in many environmental matrices, however, only one study has examined the environmental concentrations of antidepressants in water as compared to the levels in fish tissue and sediment within the same stream system (Schulz et al., 2010), documenting qualitatively different profiles of antidepressants in the water, sediment, and brain tissue. This laboratory study confirms observations made in a field study where selective uptake of FLX, NFLX, SER, and NSER in the brain tissue was observed along with a selective exclusion of VEN (Fig. 7, Table 3).

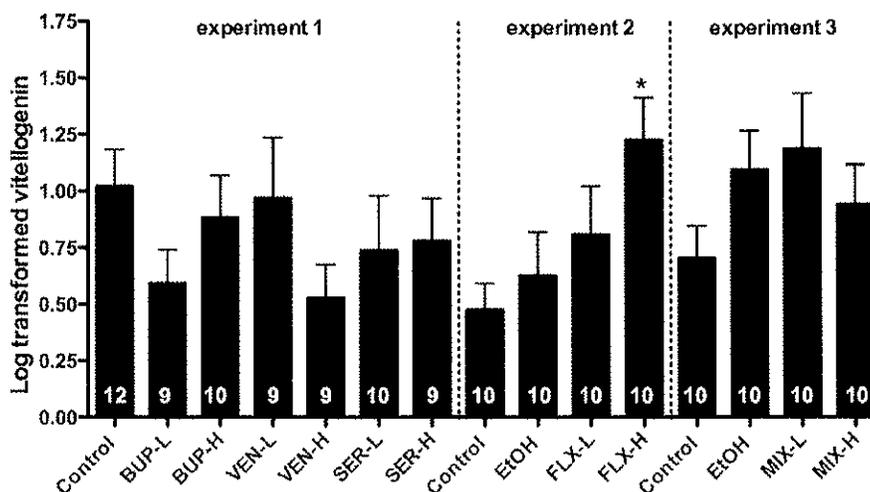


Fig. 3. Effects on plasma vitellogenin induction on adult male fathead minnow exposed to the antidepressant pharmaceutical bupropion (BUP), venlafaxine (VEN), sertraline (SER) and fluoxetine (FLX) at a low (L) or high (H) concentrations or in mixtures (MIX). Each experiment included a control and experiments two and three also an ethanol carrier control (EtOH). Data are log-transformed and presented as means \pm standard errors. An * indicates significance at $p < 0.05$ (Kruskal–Wallis test with Dunn's post-test). Vertical dashed lines indicate separate experiments. Sample size (n) indicated at base of each column. Differences in sample size are due to mortality, insufficient plasma for analysis, uneven numbers of fish needed for brain analysis or behavioral challenge.

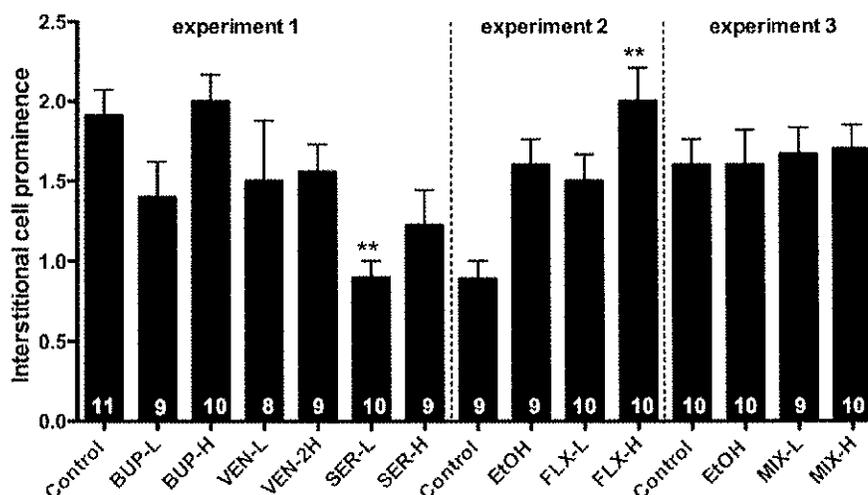


Fig. 4. Effects on interstitial cell prominence in adult male fathead minnow exposed to the antidepressant pharmaceutical bupropion (BUP), venlafaxine (VEN), sertraline (SER) and fluoxetine (FLX) at a low (L) or high (H) concentrations or in mixtures (MIX). Each experiment included a control and experiments two and three also an ethanol carrier control (ETOH). Data are presented as mean \pm standard error. An * indicates significance at $p < 0.05$ (Kruskal–Wallis test with Dunn's post-test) and a ** indicated significant at $p < 0.01$. Dashed vertical lines indicate separate experiments. Sample size (n) indicated at base of each column. Differences in sample size are due to mortality, insufficient tissue for analysis, uneven numbers of fish needed for brain analysis or behavioral challenge.

The lower abundance of VEN in the brain tissue may be explained in part by hydrophobicity. VEN has a lower $\log K_{ow}$ (-0.37) (Wyeth Pharmaceuticals, 2004) than FLX (1.22) and SER (1.37) (Kwon and Armbrust, 2008). It was beyond the scope of this study to assess the fate of the antidepressants in all tissues of fathead minnows and it is possible that VEN is accumulating in other target organs (e.g., liver, muscle) of the fish as well. However, considering the neuroactive nature of the compounds tested as well as the existing data on selective uptake in a field study (Schultz et al., 2010), we focused our tissue analysis on the brain.

Metabolites can be as pharmacologically active as the parent compound, as is the case with some antidepressants, and are thought to be largely responsible for the pharmacologic activity of the parent drug (Hiemke and Härtter, 2000). NFLX has shown to retain the biological activity of SSRIs and possesses a longer half-life than FLX; thus, suggesting it may exhibit a higher potential for bioaccumulation (Hiemke and Härtter, 2000; Paterson and Metcalfe, 2008). HBUP, the active metabolite of BUP, was until recently a drug candidate for treatment of depression under the trade name of Radafaxine® (Glaxo Smith Kline, 2006). The active ingredient in Pristiq®, a current prescription antidepres-

sant, is NVEN (also commonly known as desvenlafaxine) (Perry and Cassagnol, 2009). In this study, the detection of NFLX in the fish brain tissue (Table 3) suggests that the metabolite is likely pharmacologically active and is likely inhibiting the presynaptic reuptake of serotonin (Hiemke and Härtter, 2000). The NFLX concentrations were higher than parent FLX concentrations in three out of the four FLX exposures (FLX-H, MIX-L, and MIX-H). Previous studies have shown higher concentrations of the metabolites, NFLX and NSER, as compared to the parent antidepressant compounds, FLX and SER, in brain neural tissue of fish residing in effluent-impacted streams (Brooks et al., 2005; Schultz et al., 2010). Similar observations were made in laboratory studies that examined levels of FLX and NFLX in Japanese medaka (*Oryzias latipes*) that were only exposed to FLX (Nakamura et al., 2008; Paterson and Metcalfe, 2008). NSER was also detected in brain tissue; however studies have not found it to contribute to therapeutic effect (Hiemke and Härtter, 2000). NVEN and HBUP were not observed in the fish brain tissue.

We hypothesize that impaired testosterone production in antidepressant-exposed fish may be the cause of the diminished sex characteristics observed in fish exposed to single antidepressants (Fig. 6) with decrease in scored secondary sex characteristics in

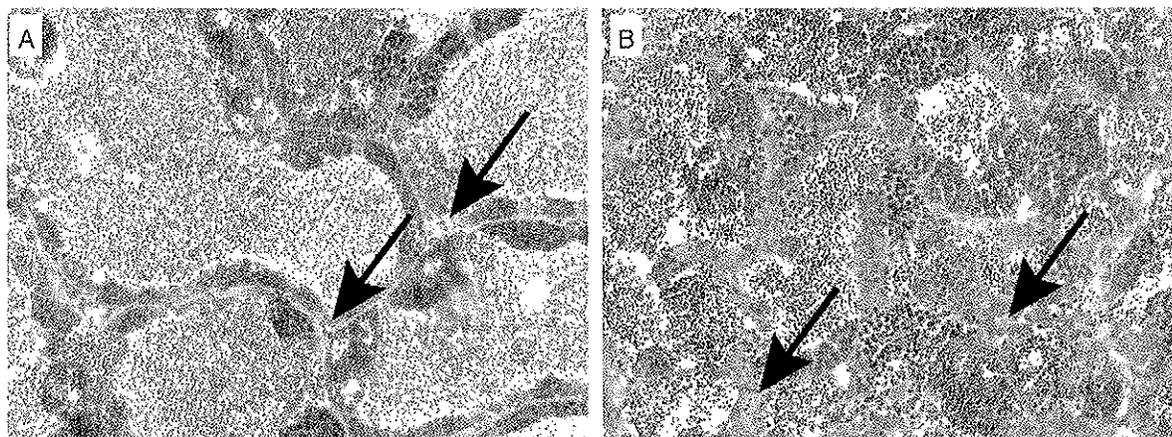


Fig. 5. Representative micrograph images illustrating the appearance of the testis interstitial space with little (A) and much (B) prominence (400 \times magnification). Arrows indicate interstitial space. Images were optimized for illustration using Adobe Photoshop (CS5).

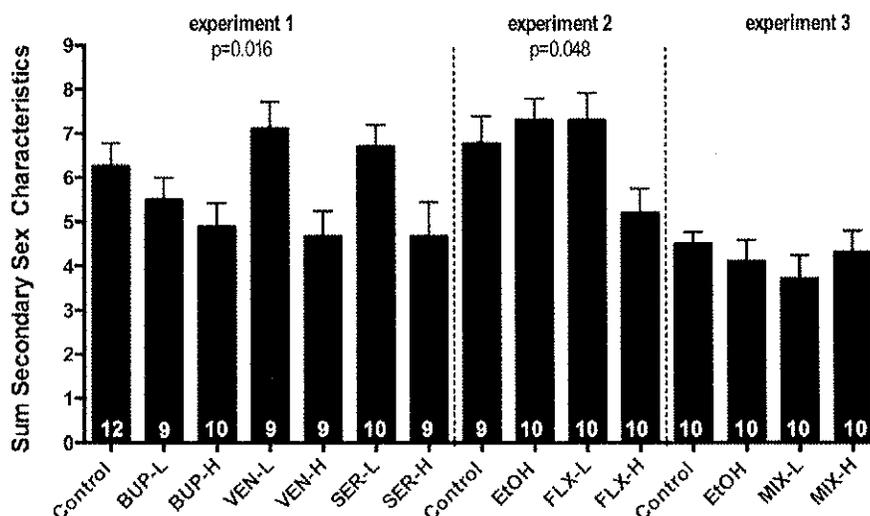


Fig. 6. Sum of secondary sex characteristics of adult male fathead minnows exposed to the antidepressant pharmaceutical bupropion (BUP), venlafaxine (VEN), sertraline (SER) and fluoxetine (FLX) at a low (L) or high (H) concentrations or in mixtures (MIX). Each experiment included a control and experiments two and three also an ethanol carrier control (EtOH). For each fish, the expression of nuptial tubercles, dorsal pad, and color expression were characterized. Scoring of each trait was conducted independently (0–3), and then the scores of all three traits were added together to obtain a sum for each fish (0–9). Data are presented as mean \pm standard error. *p* Value listed indicates significance using Kruskal–Wallis test for each experiment (not resolved by the Dunn's post-test). Dashed vertical lines indicate separate experiments (see text for additional details). Sample size (*n*) indicated at base of each column. Differences in sample size are due to mortality, uneven numbers of fish needed for brain analysis or behavioral challenge. Treatment concentrations follow abbreviation of each individual compound (ng/L). Treatment water concentrations are listed in Table 2.

FLX-H-exposed fish (mean control sum of characteristics: 6.8 ± 0.6 ; FLX-H sum: 5.2 ± 0.6). In fathead minnows, testosterone induces development of secondary sex characteristics (Norris, 2007) and decreased intratesticular testosterone levels have been correlated with Leydig cell hypertrophy (Sharpe et al., 1984). FLX-H exposure also induced the egg yolk protein vitellogenin, which in male fish is often associated with an exogenous compound binding to hepatic estrogen receptors (Palmer and Palmer, 1995).

Apparent disruption in normal testicular morphology and reproductive physiology is present in adult male fish exposed to single antidepressant compounds, yet these effects neither carry over, nor become additive within the antidepressant mixtures. Fur-

thermore, nest-holding ability of male fathead minnows exposed to two doses of antidepressant mixtures was not compromised. However, other adverse behavioral responses of aquatic organisms exposed to environmentally relevant levels of antidepressants have been previously described. Decreased activity was seen in the Crustacea (*Gammarus pulex*) exposed to 10–100 ng/L of FLX (De Lange et al., 2006). In hybrid striped bass exposed to FLX at 2300 ng/L, a decreased ability to capture prey was observed (Gaworecki and Klaine, 2008). Tadpoles (*Xenopus laevis*) exposed to FLX and SER at 0.1–10 μ g/L concentrations experienced impaired growth and development during metamorphosis (Connors et al., 2009). Additionally, larval fathead minnows exposed to FLX, VEN, BUP, and a

Table 3

Average concentrations (ng/g) \pm standard deviation of antidepressant pharmaceuticals (fluoxetine, sertraline, venlafaxine, and bupropion), their respective metabolites (norfluoxetine (NFLX), nortsertraline (NSER), norvenlafaxine (NVEN), and hydroxybupropion (HBUP)), and controls (ethanol (EtOH)) in fish neural brain tissue. The range of concentrations measured for each experiment is in parentheses.

| | n ^a | FLX | NFLX | SER | NSER | VEN | NVEN | BUP | HBUP |
|-----------------------|----------------|------------------------------------|------------------------------------|------------------------------------|---------------------------------|----------------|------|------------------------------------|------|
| Control | 13 | ND | ND | ND | ND | ND | ND | ND | ND |
| EtOH control | 3 | ND | ND | ND | ND | ND | ND | ND | ND |
| FLX low | 5 | 0.108 \pm 0.063 (<LOQ–0.161) | 0.055 \pm 0.021 (<LOQ–0.084) | ND | ND | ND | ND | ND | ND |
| FLX high | 5 | 0.173 \pm 0.067 (0.089–0.270) | 0.308 \pm 0.150 (0.123–0.511) | ND | ND | ND | ND | ND | ND |
| SER low | 7 | ND | ND | 0.023 \pm 0.025 (<LOQ–0.066) | ND | ND | ND | ND | ND |
| SER high ^b | 5 | ND | ND | 0.060 \pm 0.017 (0.034–0.082) | ND | ND | ND | ND | ND |
| VEN low ^b | 7 | ND | ND | ND | ND | ND | ND | ND | ND |
| VEN high ^b | 5 | ND | ND | ND | ND | 0 (ND–<LOQ) | ND | ND | ND |
| BUP low | 6 | ND | ND | ND | ND | ND | ND | 0.025 \pm 0.021 (<LOQ–0.054) | ND |
| BUP high | 8 | ND | ND | ND | ND | ND | ND | 0.062 \pm 0.073 (<LOQ–0.178) | ND |
| Mixture low | 5 | 0.025 \pm 0.056 (ND–0.127) | 0.204 \pm 0.153 (ND–0.424) | 0.047 \pm 0.105 (ND–0.235) | ND | ND | ND | 0.012 \pm 0.024 (ND–0.061) | ND |
| Mixture high | 5 | 0.218 \pm 0.222 (ND–0.575) | 1.632 \pm 0.838 (0.554–2.583) | 0.305 \pm 0.343 (ND–0.702) | 0.199 \pm 0.301 (ND–0.680) | 0 (ND–<LOQ) | ND | 0.261 \pm 0.150 (0.139–0.436) | ND |

ND = no detect, LOQ = limit of quantitation, S/N = signal to noise.

<LOQ is where the analyte was detected; however, the S/N \leq 10. Typically, the <LOQ was 0.009 ng/g. A zero was substituted for ND or <LOQ when determining averages.

^a *n* refers to each pool of three to four brains that were combined for sufficient tissue mass required for analysis.

^b Exposures resulted in significant mortality (Fig. 1).

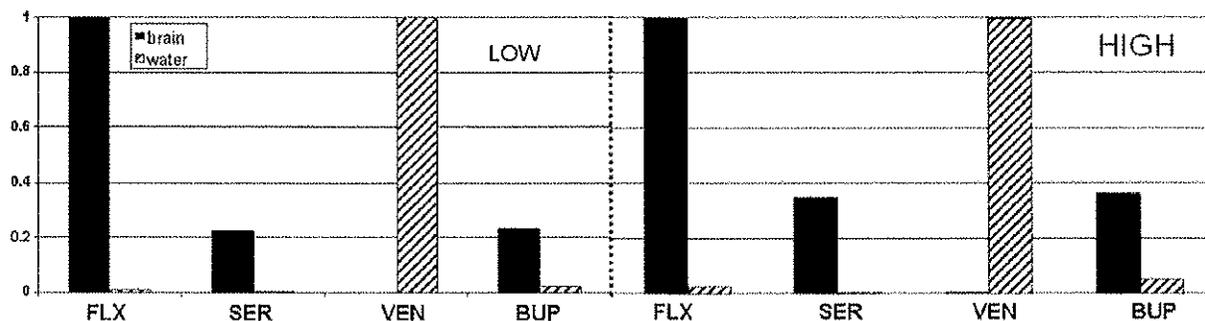


Fig. 7. Qualitative concentration profiles of antidepressant pharmaceuticals (fluoxetine [FLX], sertraline [SER], venlafaxine [VEN], and bupropion [BUP]) observed in the water and fish neural tissue from the individual low and high exposure experiments. Concentrations were normalized to the highest single antidepressant concentration observed in each sample type. In the aqueous samples, venlafaxine was the most abundant antidepressant observed, so its concentration was assigned the value of 1, and the other aqueous concentrations measured were scaled accordingly. Fluoxetine was the abundant antidepressant measured in the brain tissue, so its concentration was assigned the value of 1 and the other tissue concentrations were scaled accordingly. The concentrations of observed metabolites are not included in this graph. The actual antidepressant concentrations measured in the water and brain tissue are reported in Tables 1 and 3, respectively.

MIX, at comparable concentrations to those applied in this study, exhibited diminished predator avoidance escape behaviors compared to controls (Painter et al., 2009). These findings imply that (i) reproductive behavioral endpoints are not as sensitive as locomotor behavioral responses, and/or (ii) early life stages are more sensitive than adults.

The absence of dose-dependent relationships and differing effects for related antidepressant pharmaceuticals is comparable to previous findings regarding other antidepressants. For example Horn and Fink (1985) found dissimilar effects on LH levels in rats exposed to two SSRIs (alaproclate and zimelidine). Lister et al. (2009) discussed findings by Gaworecki and Klaine (2008) and suggested that the expression of physiological endpoints may be affected by dose and length of exposure. This hypothesis is supported by the observed differences in the expression of endpoints by control fish in the three experiments, which indicate that even consecutive monthly batches of fish from the same culture and housed in the same lab exhibit considerable differences in morphological and physiological parameters. This biological variability may also factor in the observed lack of carry-over effects from single compounds to mixtures. While this study was not designed to examine the interactions of pharmaceuticals in mixtures, these results suggest the need for additional investigations.

The present study demonstrates that survival of freshwater fish exposed to the antidepressants SER (5.2 ng/L) and VEN (305 ng/L and 1104 ng/L) at environmentally relevant concentrations (Schultz et al., 2010) is compromised within 21 days. This finding underscores the impact of “pseudopersistent” exposure scenarios often associated with the near continuous release of these compounds through treated wastewater effluent, resulting in fish encountering antidepressant compounds at concentrations substantially below lethality levels for extended periods. Furthermore, it has been observed that the SSRIs, SER and FLX, have the ability to alter testicular morphology in fish exposed at low nanogram-per-liter concentrations. Recent findings in the literature (Painter et al., 2009; Schultz et al., 2010) clearly indicate the potential for adverse environmental effects of SSRIs that warrant expanded studies to include assessment of uptake routes and of long-term and generational exposure effects.

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