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Human Therapeutic Plasma Levels of the Selective Serotonin Reuptake Inhibitor (SSRI) Sertraline Decrease Serotonin Reuptake Transporter Binding and Shelter-Seeking Behavior in Adult Male Fathead Minnows

Theodore W. Valenti Jr.[†,‡,#,*], **Georgianna G. Gould**[§], **Jason P. Berninger**[‖,‡], **Kristin A. Connors**[‖,‡], **N. Bradley Keele**[‖,⊥], **Krista N. Prosser**[‡], and **Bryan W. Brooks**[†,‖,‡]

[†]The Institute of Ecological, Earth, and Environmental Sciences, Baylor University, Waco, Texas 76798, United States

[‡]Department of Environmental Science, Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas 76798, United States

[§]University of Texas Health Science Center at San Antonio, Department of Physiology, MC 7756, 7703 Floyd Curl Drive, San Antonio, Texas 78229, United States

[‖]Institute of Biomedical Studies, Baylor University, Waco, Texas 76798, United States

[⊥]Department of Psychology and Neuroscience, Baylor University, Waco, Texas 76798, United States

Abstract

Selective serotonin reuptake inhibitors (SSRIs) represent a class of pharmaceuticals previously reported in aquatic ecosystems. SSRIs are designed to treat depression and other disorders in humans, but are recognized to elicit a variety of effects on aquatic organisms, ranging from neuroendocrine disruption to behavioral perturbations. However, an understanding of the relationships among mechanistic responses associated with SSRI targets and ecologically important behavioral responses of fish remains elusive. Herein, linking Adverse Outcomes Pathways (AOP) models with internal dosimetry represent potential approaches for developing an understanding of pharmaceutical risks to aquatic life. We selected sertraline as a model SSRI for a 28-d study with adult male fathead minnows. Binding activity of the serotonin reuptake transporter (SERT), previously demonstrated in mammals and fish models to respond to sertraline exposure, was selected as an endpoint associated with therapeutic activity. Shelter-seeking behavior was monitored using digital tracking software to diagnose behavioral abnormalities. Fish plasma levels of sertraline exceeding human therapeutic doses were accurately modeled from external exposure

[*]**Corresponding Author**: valenti.ted@epa.gov. .

[#]**Present Address**: U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecological Division, Duluth, MN 55804.

ASSOCIATED CONTENT

Supporting Information

The patterns of swim velocity for individual adult male *Pimephales promelas* in control and sertraline exposed treatments. This information is available free of charge via the Internet at http://pubs.acs.org

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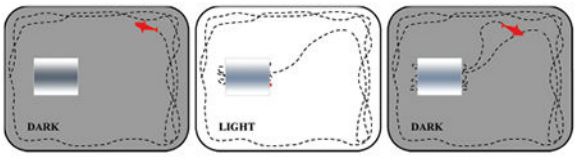
concentrations when pH influences on ionization and log D were considered. We observed statistically significant decreases in binding at the therapeutic target (SERT) and shelter-seeking behavior when fish plasma levels exceeded human therapeutic thresholds. Such observations highlights the strengths of coupling physiologically based pharmacokinetic modeling and AOP approaches and suggest that internal dosimetry should be monitored to advance an understanding of the ecological consequences of SSRI exposure to aquatic vertebrates.



1. INTRODUCTION

Detections of human pharmaceuticals and personal care products in ecosystems and the tissues of aquatic organisms have raised environmental concerns.[1] Selective serotonin reuptake inhibitors (SSRI) are a class of pharmaceuticals often detected in municipal wastewater[2–4] and have been shown to bioaccumulate in aquatic organisms inhabiting surface waters below discharges.[3–9] Widely prescribed to treat depression, anxiety, and other disorders in humans, SSRIs elicit therapeutic effects through increasing serotonergic neurotransmission by blocking reuptake of serotonin by presynaptic serotonin reuptake transporters (SERT) at synapses.[10]

Serotonergic drug targets are well described in humans and rodents[11,12] and appear to be largely conserved among vertebrates.[13] For example, Gunnarsson et al.[14] reported ~65% ortholog homology between humans and fish for the serotonin receptor and the SERT. Two genes coding for SERT in zebrafish (*Danio rerio*) were identified by blast search and cloned; these are $SERT_a$ and $SERT_b$ with 66−69% and 75% sequence homology with human SERT.[15] Gould et al.[16] used [^{3}H] labeled citalopram to compare the pharmacological profiles of SERT binding sites in whole brain homogenates from fathead minnows (*Pimephales promelas*), zebrafish, and laboratory rats *(Rattus norvegicus)*, and found similar K_D and B_{max} values for high-affinity binding sites among the model organisms. The study also demonstrated decreased SERT binding site density in zebrafish following a 21-d dietary exposure to the SSRI sertraline.[16] This observation is a critical consideration because a decrease in SERT binding in rodents following chronic administration accompanies therapeutic activity.[17]

SSRIs trigger a variety of toxicological effects on a range of model aquatic organisms. When standardized endpoints presently used by regulatory agencies are considered (e.g., cladoceran survival, reproduction), individual SSRIs appear to present relatively low ecological risk.[2,18] However, sublethal fish responses to SSRIs, such as disruption of behavior,[18–25] neuroendocrine function,[26–30] and other metabolic pathways,[31,32] appear more sensitive and occur at levels far lower than detected by standardized endpoints. Therefore, sublethal fish responses are particularly important for assessing the environmental safety of SSRIs[2,21,25] and pharmaceuticals.[33,34]

Researchers have long recognized the potential applicability of animal behavior as indicators of sublethal stress during laboratory bioassays with fish. Most early studies focused solely on directly quantifiable response measures, such as avoidance, coughs, or body tremors.[35] More recently, there has been a transition to identify changes in behaviors that have greater ecologically relevance, such as foraging,[36,37] feeding rates,[21,25,38] predator –prey interactions,[39] reproduction,[40] and social hierarchies.[41] Further, neuropsychopharmacology, neuropathology, and psychopathology studies commonly employ fish models to understand anxiety, stress, and fear.[42,43] SSRIs may elicit physiologically and ecologically important adverse outcomes at subtoxic thresholds of exposure. Therefore, when previous reports of SSRI bioaccumulation in wild fish are considered together with the evolutionary conservation of SSRI targets among vertebrates and available pharmacology and toxicology data, the application of behavior responses is particularly germane for ecological risk assessments (ERA).

In addition to monitoring surface water concentrations of pharmaceuticals to define environmental exposures, it is essential that relationships between internal dosimetry and mechanistic and physiological perturbations are explored more fully so that the wealth of pre-existing mammalian pharmacological safety data may be leveraged to predict thresholds of response in fish.[33,44,45] Huggett et al.[45] detailed an approach for predicting the partitioning of therapeutics from water to plasma of fish based on physicochemical properties using a model for superhydrophobic chemicals developed by Fitzsimmons et al.[46] The researchers further suggested that predicted fish plasma drug concentrations could then be compared to human therapeutic thresholds (e.g., C_{max}) to generate an Effect Ratio (ER; ER = human C_{max}/predicted fish plasma concentration). The conceptual foundation of this approach is based on receptor homology and other pathway similarities between fish and mammals and, therefore, may be an effective means to identify relative risks among pharmaceuticals.[47,48] However, there is little data relating water concentrations of human therapeutics to internal doses in fish, and even less information linking internal dosimetry to specific mechanistic and physiological alterations. A study by Cuklev et al.[49] specifically addressed the relationship between blood plasma levels of the pharmaceutical diclofenac in fish and effects on the molecular level. Interestingly, the Cuklev et al.[49] study provided evidence supporting assumptions made by Huggett et al.[45] as effects were discernible when plasma levels in the fish approached human therapeutic doses.

Recently, Ankley et al.[50] proposed the use of Adverse Outcome Pathways (AOP) for use in ERA. AOPs are conceptual frameworks that incorporate existing knowledge about linkages among molecular initiating events (anchor 1) and cascading responses that result in adverse outcomes at higher levels of biological organization used for ERA (anchor 2: individual and population levels).[50] Our experiment specifically tested the utility of employing an AOP framework for a model pharmaceutical by examining responses at the therapeutic target level (SERT binding; anchor 1) and a novel, ecologically relevant response at the individual level (shelter-seeking behavior; anchor 2). The objectives of our study were thus 3-fold: (1) to determine if water exposure can be used to predict internal doses of an ionizable weak base SSRI in a fish model when pH is considered; (2) to determine if human therapeutic plasma doses result in decreased SERT binding in fish brain tissue; and (3) to determine if changes in SERT function are associated with quantifiable changes in fish behavior. For this

experiment, the SSRI sertraline and adult fathead minnows were selected because previous research identified pH influence on the onset and magnitude of toxicity, including behavioral effects, following sertraline exposure in juvenile fathead minnows.[21]

2. MATERIALS AND METHODS

2.1. Test Organisms.

Fathead minnows were bred and reared according to an approved Baylor University animal care protocol. Individuals were housed in a flow-through system supplied with aged, dechlorinated tap water at a constant temperature of 25 ± 1 °C under a 16:8 light/dark photoperiod. For the first 30 d following hatch individuals were fed twice daily with newly hatched *Artemia* spp. and thereafter were fed a mixture of *Artemia* spp. and certified test-grade flake food. Individuals were aged to 120 d before experiments were initiated. Only adult male fish with pronounced reproductive features were selected for this study.

2.2. Experimental Design.

The experiment was completed in 20-L experimental units (aquariums) filled with 18 L of treatment level solutions. Each exposure aquarium housed 5 adult male *P. promelas* and had three breeding tiles as shelters, which consisted of 10 cm sections of 3-in PVC cut in half. Dechlorinated tap water was used as the control treatment and the dilution water for each of the 3 sertraline treatment levels. A stock of sertraline hydrochloride (Sigma Aldrich, St. Louis, MO, USA) was prepared and used to create the sertraline concentrations. Treatment levels included the control and three concentrations (3, 10, 30 μg sertraline/L) predicted to result in internal plasma concentrations exceeding the human C_{max} of sertraline (142 ng/mL) at pH 8.5 (described further in Section 2.5 below). Each treatment level included 3 replicate experimental units; thus, there were a total of fifteen individual adults per treatment level. Studies for each set of replicates were staggered by one day to accommodate the substantial time associated with completing the behavioral trials.

The study was 28 d in duration and 75% v/v water renewals were performed daily. During the exposure period, organisms were fed certified test-grade flake food daily. All aquaria were housed in a single walk-in incubator set at 25 ± 1 °C with a 16:8 light/dark photoperiod. Temperatures in the aquaria were monitored daily. Water quality parameters, including dissolved oxygen, conductivity, pH, chlorine, and ammonia were measured 3 times a week.

2.3. Behavioral Observations.

To examine *P. promelas* behavior, we employed the NOLDUS Ethovision XT (http://www.noldus.com/animal-behavior-research/products/ethovision-xt), a software package that allows automated tracking and analysis of animal movement and activity. The system processes video images, has been widely applied to study behavior in mammalian models, and is often employed during drug development. A random number table was used to determine the order in which fish were removed from each treatment level and to determine their position in observation arenas.

A SONY Handycam (HDR-SR11) equipped with a visible light filter (850 nm) was positioned over the center of four observation arenas. Each observation arena consisted of a white plastic container (20 L, 35 cm ×40 cm) that housed one breeding tile for shelter and was filled with 15 L of dechlorinated tap water. The camera and NOLDUS video settings were calibrated to optimize tracking. Two high-powered 3.0-W infrared lights (LEDTRONICS, Inc. R30-123-851-120 AS Manufacturer) were placed below the arenas and positioned to allow for indirect illumination with infrared light. The inside of the support structure was lined with foil and the infrared lights were positioned to allow for consistent, indirect illumination. The infrared lights remained on throughout the duration of each observation period. Two light fixtures fitted with 60-W bulbs were positioned above the arenas, which were either turned on or off to represent light and dark conditions.

One fish was transferred from the exposure system to the observation arena and each NOLDUS trial was initiated within 10 s of the last fish being introduced. After the first 300 s of the trial, the overhead visible lights were remotely switched off, and then turned on again after another 300 s. The process of alternating light conditions was repeated a total of 3 times per observation period. The NOLDUS software was used to analyze the total distance traveled, swim velocity, and overall movement for each fish. The time that the fish spent in the shelter was calculated manually by reviewing digital video files to ensure that the fish was actually inside of the shelter and not merely swimming above it.

Following each completed NOLDUS trial, fish were removed, weighed, length measured, and then euthanized by cervical dislocation. Brain tissues were stored at −80 °C for future analysis, and heparinized hematocrit tubes (StatSpin, Westwood, MA) were used to collect blood. The blood from all fish ($n = 5$) in a replicate experimental unit ($n = 3$) was pooled to ensure an adequate volume for analytical measurements. Plasma of the pooled samples was separated at 10 000 rpm for 3 min using a refrigerated microcentrifuge. Plasma was then immediately diluted 1:10 with 100 mM phosphate buffered saline (PBS) with preservatives at pH 7.0, filtered through a 0.22-μm filter (Immunalysis Corporation, Ponoma, CA), and stored at 4 °C.

2.4. Analytical Quantification of Sertraline.

Due to small volumes of plasma in adult fathead minnows, sertraline direct ELISA kits (Immunalysis Corporation, Pomona, CA) were used to quantify plasma sertraline concentrations in control and exposed fish. To ensure that absorbance readings were within the standard curve range developed for the ELISA, 25, 50, and 100 μL of fish plasma were examined in triplicate by ELISA. After incubation, absorbance was measured at 450 and 630 nm using a Bio-Tek ELx 800 microplate reader (Winooski, VT, USA) within 30 min of loading the last sample. Sertraline direct ELISA was also used to confirm nominal concentrations of sertraline in exposure water, which performed consistently with LC-MSMS analyses previously reported from our group.[20]

2.5. Comparing Measured versus Predicted Fish Plasma Concentrations.

Measured plasma concentrations of sertraline were compared to predictions from a model developed by Huggett et al.[45] derived from a plasma bioconcentration model by

Fitzsimmons et al.[46] with minor modifications to account for pH influences. Sertraline is a weak base with a pK_a of 9.47 previously demonstrated to be more toxicity to aquatic life at higher pH.[21] Because prior research has shown that site-specific pH affects the ionization, bioavailability, and toxicity of SSRIs,[21,51] our group recently proposed accounting for these effects by using log D in model for predicting fish plasma concentrations rather than K_{OW}.[1,52] Therefore, log D (3.77 at pH 8.5; ACD/Laboratories, Toronto, ON, Canada) was substituted for the log K_{OW} value in eq 1. The treatment levels of 3, 10, and 30 μg/L were used as the aqueous concentrations in eq 2.[45] The human therapeutic plasma concentration (C_{max}) was defined as either 142 ng/mL[53] or 190 ng/mL (sertraline product registry), each of which were used to calculate ER values (eq 3).[45]

$$\mathrm{Log}P_{\mathrm{Blood:Water}} = 0.73 \times \mathrm{Log}D_{\mathrm{oct:water}} - 0.88 \quad (1)$$

$$\mathrm{Fish}_{\mathrm{plasma}} = [\mathrm{Aqueous}] \times P_{\mathrm{Blood:water}} \quad (2)$$

$$\mathrm{ER} = C_{\mathrm{max}} / \mathrm{Fish}_{\mathrm{plasma}} \quad (3)$$

2.6. Saturation Radioligand Binding to Serotonin Transporters in Whole Brain Homogenates.

SERT saturation binding to [^{3}H] citalopram in membrane homogenate preparations from fathead minnow whole brains was performed as previously described by Gould et al.[16] Whole brains from three fish from each replicate were randomly selected and pooled. The remaining two brains were preserved for future studies. Therefore, three homogenates were prepared for each treatment level. Tissue was dispersed at 30 000 rpm for 20 s in 25 mL of 50 mM Tris, 120 mM NaCl, and 5 mM KCl, pH 7.4 at 26 °C buffer using a tissue homogenizer (Polytron 3100, Kinematica, Bohemia, NY). The homogenate was spun for 10 min at 30 600 rpm in a 4 °C centrifuge (Avanti A-J, Beckman-Coulter, Brea, CA). The supernatant was discarded and the pellet was resuspended in 5 mL of buffer on ice using a hand-held tissue homogenizer. Another 20 mL of buffer was added and the homogenate was centrifuged again for 10 min at 30 600 rpm. The final pellet was resuspended to obtain a protein concentration near 1 mg/mL, as determined with Bradford reagent (Sigma, St. Louis, MO) and measured on a spectrophotometer (DU-640, Beckman, Corona CA). The homogenate was incubated at 26 °C for 1 h in buffer at two concentrations of [^{3}H] citalopram, 7 nM and 48 nM, (PerkinElmer, Boston, MA) to examine effects at both K_D and B_{max} concentrations. These concentrations were chosen based on a K_D of 7 nM for [^{3}H] citalopram found in golden shiner minnows in Gould et al.[16] At the 48 nM concentration, calculated occupancy at the [^{3}H] citalopram binding sites was 87%. In Gould et al.,[16] a K_D of 18 nM was also reported for fathead minnow brain homogenate saturation binding to [^{3}H] citalopram when a single site NLR curve fit was used, but it is possible that a second binding

site might have contributed to the higher K_D found in this species. Therefore, the lower K_D estimate determined from the golden shiner was used to capture only the activity of high-affinity SERT-like binding sites.

Nonspecific binding was defined by 50 μM sertraline (Pfizer, Groton, CT). Triplicate tubes were incubated in the presence and absence of sertraline and incubation was terminated by addition of 4 mL of 4 °C pH 7.4 buffer. Labeled homogenates were captured by filtration under vacuum with a tissue harvester (Brandel, Gaithersburg, MD) onto Whatman GF/B filter paper strips (Brandel) presoaked in 5% polyethyleneimine (Sigma). Filters were washed twice more with 4 mL of buffer. [^{3}H] citalopram trapped in membrane tissue on the filters was determined using a liquid scintillation counter (LS 6500, Beckman) with 40% efficiency.

2.7. Statistical Analysis.

A comparison between sertraline water column concentration and sertraline fish plasma concentration was determined using linear regression. SERT binding in adult male *Pimephales promelas* brain tissue was analyzed by ANOVA with fish plasma concentration as the main experimental factor. Fish behavior (e.g., time in shelter and velocity) was analyzed by 2-factor ANOVA with sertraline concentration (4 levels: 0, 3, 10, 30 μg/L) and lighting condition (2 levels: light, dark) as the factors. For statistical comparisons of fish velocities, mean velocities for each fish were calculated in 15 s intervals. Dunnett's post hoc comparisons were then completed between exposed groups and the control with $\alpha = 0.05$. These statistical approaches were used to define no-observable-adverse effect concentration (NOAEC) and lowest-observable-adverse effect concentrations (LOAEC). All statistical analysis was performed with the computer software Statistica 10 (StatSoft, Tulsa, OK). Prior to analysis, data were checked for normality using the Shapiro–Wilk Test and for homogeneity using the Lovene test for homogeneity of variance.

3. RESULTS

3.1. Analytical Quantification of Sertraline.

Nominal treatment concentrations for water exposures were very close to those measured and a significant linear dose-dependent relationship between concentrations of sertraline in water and plasma concentration in exposed fish was observed ($p = 0.001$; Figure 1). Sertraline was not detected in the plasma of control fish but was quantifiable for the other treatment levels (Figure 1). These measured sertraline plasma concentrations were very similar to those predicted by the fish plasma concentration model based on water column exposure concentrations based on eq 2 when log D at exposure pH was considered (Figure 1). Furthermore, the ER values based on the ratio of measured fish plasma levels and human therapeutic plasma doses calculated from eq 3 were all <1, which suggested that biological responses related to therapeutic activity would be observed in exposed fish (Table 1).

3.2. SERT Binding.

Functional binding changes of the therapeutic target (SERT) were observed in brain tissue of fish with elevated plasma concentrations of sertraline following the 28-d study. Sertraline

treatments significantly reduced the B_{max} (F[3,8] = 6.9, $p = 0.01$) and K_D (F[3,8] = 4.169, $p = 0.04$) during [^{3}H] citalopram binding assays. Dunnett's post hoc analysis indicated that mean SERT binding site densities in fish exposed to all three levels of sertraline were significantly lower than controls for both B_{max} and K_D ($p < 0.05$; Figure 2).

3.3. Behavioral Observations.

Sertraline concentration and lighting, as well as their interactions, significantly affected the time adult male fathead minnows spent in the shelter. Dunnett's post hoc analysis indicated that control fish spent significantly greater time in the shelter during light periods compared to the other treatment levels ($p < 0.05$; Figure 3). Interestingly, during the initial light period when fish were first introduced to the chamber in which behavior was assessed, nearly all organisms sought shelter. For example, control fish spent 258 ± 22 (mean ± SD) s in the shelter, whereas sertraline exposed fish spent between 173 and 203 s (approximately 67–78% of the time that control fish spent in the shelter during the same interval; Figure 3). During the next two light intervals, control fish again spent a substantial amount of time in the shelter (e.g., 217 ± 31 and 239 ± 36 s, respectively), whereas fish exposed to sertraline spent between 18 and 42% less time in the shelters (Figure 3). There was very little difference in the amount of time fish spent in the shelter during the dark periods as individuals for each treatment level only spent between 4 and 33 s per interval (Figure 3).

Mean patterns of velocity (cm/sec) for exposed fish also differed from those of control fish during light and dark conditions (Figure 4). NOLDUS data for one trial was not collected because of a recording malfunction; however, data pertaining to the time spent in the shelter was collected by watching the video. Sertraline concentration and lighting, as well as their interactions, had significant effects on the velocity of adult male fathead minnow. The Dunnett's post hoc analysis indicated that control fish spent swam significantly slower than fish in the sertraline treatment levels ($p < 0.05$; Figure 4). (See Figures S1–S4 for velocities of individual fish).

4. DISCUSSION

Behavior can be defined as the visual response of an organism to a culmination of biotic and abiotic stimuli. Such responses to these cues are dependent on physiological (internal) signals and environmental or social (external) factors.[54] Contaminant-induced alterations in behaviors may therefore reveal connections among the biochemical, individual, and population levels of biological organization.[39] For these reasons, behavioral endpoints may be more robust and comprehensive for ERA than either physiological or biochemical parameters.[55] However, because variability of various fish behavioral responses inherently has limited their use in risk assessment, future interlaboratory variability studies coupled with semifield experiments (e.g., with –cosms) are necessary to examine the utility of employing fish shelter-seeking, feeding, and other behaviors as robust measures of effect in risk assessment.

In the present study, exposure to sertraline caused statistically significant changes in shelter-seeking behavior in adult male fathead minnows at water concentrations as low as 3 μg/L, which is lower than a previously reported feeding behavior threshold for juvenile fathead

minnows (e.g., 15 μg/L).[21] Whereas the effect on shelter-seeking behavior was observed at sertraline concentrations higher than those previously reported in surface waters, total SSRI concentrations below wastewater effluent discharges have been reported as high as 3.2 μg/L.[4] Furthermore, fish in aquatic ecosystems may be exposed to SSRIs through both dietary and inhalational routes of exposure. The physicochemical properties of some SSRIs may facilitate their partitioning to aquatic organisms and sediment depending on ambient pH conditions; for example, higher concentrations of SSRIs are found in sediment than in the water column.[3] Therefore, these additional routes of exposure may lead to higher internal doses than observed here, emphasizing the importance of considering internal dosimetry when performing ERAs for SSRIs.

The present study provides a novel demonstration of a cascading series of effects in adult male fathead minnows following 28-d water exposure to sertraline that led to internal plasma doses exceeding human therapeutic threshold concentrations (Figure 1). As previously noted, plasma levels of sertraline in fish exposed to all of the treatment levels tested in the present study exceeded therapeutic plasma doses for humans based on the model described by Huggett et al.[45] when appropriate modifications for exposure pH and log D were considered.[52] In fact, this study supports a growing literature highlighting the importance of accounting for pH during bioaccumulation and toxicity studies of ionizable therapeutics.[21,51,52,56–59] Furthermore, all sertraline treatments produced similar physiological and behavioral effects due likely to the maximum stimulation of the drug target and thus lower exposure concentrations may also trigger such effects. The importance of relating internal dose to biological responses is emphasized by Homberg et al.[60] who examined the effects of the SSRI citalopram on fish behavior; no effects on aggression in rainbow trout fry or on sexual behavior in male guppies were observed when internal levels were below human therapeutic doses. Therefore, it is important that future research efforts attempt to identify sertraline thresholds at which an external exposure concentration culminates in an internal dose that cause physiological and behavioral effects.

It is also important to note that the exposure concentrations used in our study were well below those previously reported to cause acute mortality[21] and; therefore, focused on mode-of-action related effects rather than those associated with gross intoxication resulting from narcosis. The calculated ER values based on predicted and measured fish plasma concentrations were quite similar and all were below 1, suggesting potential hazard associated with therapeutic activity (Table 1). These internal doses resulted in significant modification of binding to the therapeutic target of sertraline (SERT-like binding sites; Figure 2),[17] which was also observed in 21-d dietary study with zebrafish exposed to sertraline.[16] Whereas our assays demonstrated decreases in SERT-like binding sites, other populations of different transporters may have also been recruited as noted in a review by Daws.[61] Furthermore, changes in SERT-like binding sites in our study are more likely attributable to B_{max} differences as SERT binding sites may be downregulated with chronic treatment (see 17 for examples). Regardless, decreased SERT-like binding in all sertraline treatments culminated in a potentially ecologically important behavioral change in male fathead minnows (e.g., change in shelter-seeking behavior; Figure 3).

Interestingly, altered fish behavior following sertraline exposure was only apparent when light was present and was not observed in dark conditions. The reduced propensity for exposed fish to seek shelter during light periods suggests that sertraline elicits an anxiolytic effect in teleost fish. Previous researchers have observed that teleosts have natural preferences for dark environments.[62,63] Therefore, under the present study conditions, the shelter was the only dark area during light conditions in the observation arena; therefore, unexposed fish apparently retreated to the shelter to reduce anxiety, whereas fish exposed to sertraline appeared to display reduced anxiety and did not exhibit this behavior. Similar patterns of behavior have been observed in rodent models during elevated plus-maze tests designed to measure anxiety. Montgomery[64] first used the concept of an elevated plus-maze and observed that rats were less likely to exhibit exploratory behaviors in “open” arms compared to “closed” arms, which was attributed to an avoidance of open alleys due to the fact that they engender higher levels of anxiety. In a review of how the elevated plus X-Maze may be used to study anxiety and anxiety-modulating drugs, Handley and McBlane[65] noted that the antianxiety drug diazepam increased the ratio of open/total arm entries, while the pro-anxiety drug picrotoxin diminished this ratio for rats. Consequently, based on observations in the present study, we hypothesize that fish exposed to sertraline displayed reduced levels of anxiety; therefore, fish were more willing to explore the observation arena during both light and dark conditions.

Serotonin is a key neurotransmitter in pathways related to reproduction, stress, appetite regulation, and behavioral functions in fish.[26] Several laboratory and field studies have demonstrated that exposure to SSRIs leads to bioconcentration and bioaccumulation of these therapeutics in fish.[3–9] Following a 28-d study with the model SSRI sertraline and adult male fathead minnows, we observed significant decreases in the therapeutic target (SERT binding) and shelter-seeking behavior when adult male fish plasma levels exceeded human therapeutic concentrations. Such observations highlight the importance of defining internal dosimetry and employing AOP approaches to advance an understanding of the ecological consequences of pharmaceutical exposure and to reduce uncertainty during future ecological risk assessments involving aquatic vertebrates, particularly for biologically active compounds.

Previous experiments with other fish species have also suggested that SSRIs may induce anxiolytic effects. For example, Egan et al.[66] observed reductions in erratic movements and a greater propensity of zebrafish to spend time in the upper half of a swim tank following 14 d of exposure to the SSRI fluoxetine. Painter et al.[22] reported a suppression of predator avoidance (C-start) behavior in juvenile fathead minnows exposed to sertraline, other SSRIs, and antidepressants with different modes of action. Previous studies have reported decreased locomotor activity in fish exposed to SSRIs,[67,68] which may be the result of an anxiolytic effect. However, our experimental design employed a shelter or “safe haven” for fish, which was not provided in previous experiments performed during light conditions.

For behavior to be used effectively as a tool in ERA, it is essential that observed behavioral modifications be specifically related to survival or ecological fitness.[35] Based on the design of this study, we did not directly identify whether the decrease in shelter-seeking behavior by adult male *P. promelas* exposed to sertraline during light periods was specifically due to

anxiolytic effects or another reason (e.g., neuroendocrine change, altered visual perception); however, observation of such a behavioral effect by sertraline exposure likely has direct implications for both survival and fitness of fish. Individuals willing to spend more time away from shelters face greater predation risk and thus their overall survival rate may be reduced. In terms of reproduction, fathead minnows typically spawn and attach adhesive eggs to the underside of aquatic plants, stones, or woody debris.[69] Shelter-seeking and nest-guarding behavior in males are intrinsically linked for adult male fathead minnows as establishing and defending territories is important for attracting mates.[70] Martinovic et al.[40] previously observed that nest-guarding behavior of *P. promelas* exposed to environmental estrogens ultimately led to complete reproductive failure. Thus, as suggested previously,[18,21,23,25] the results of the present study identify the importance of considering important behavioral modifications of fish following SSRI exposure during ERAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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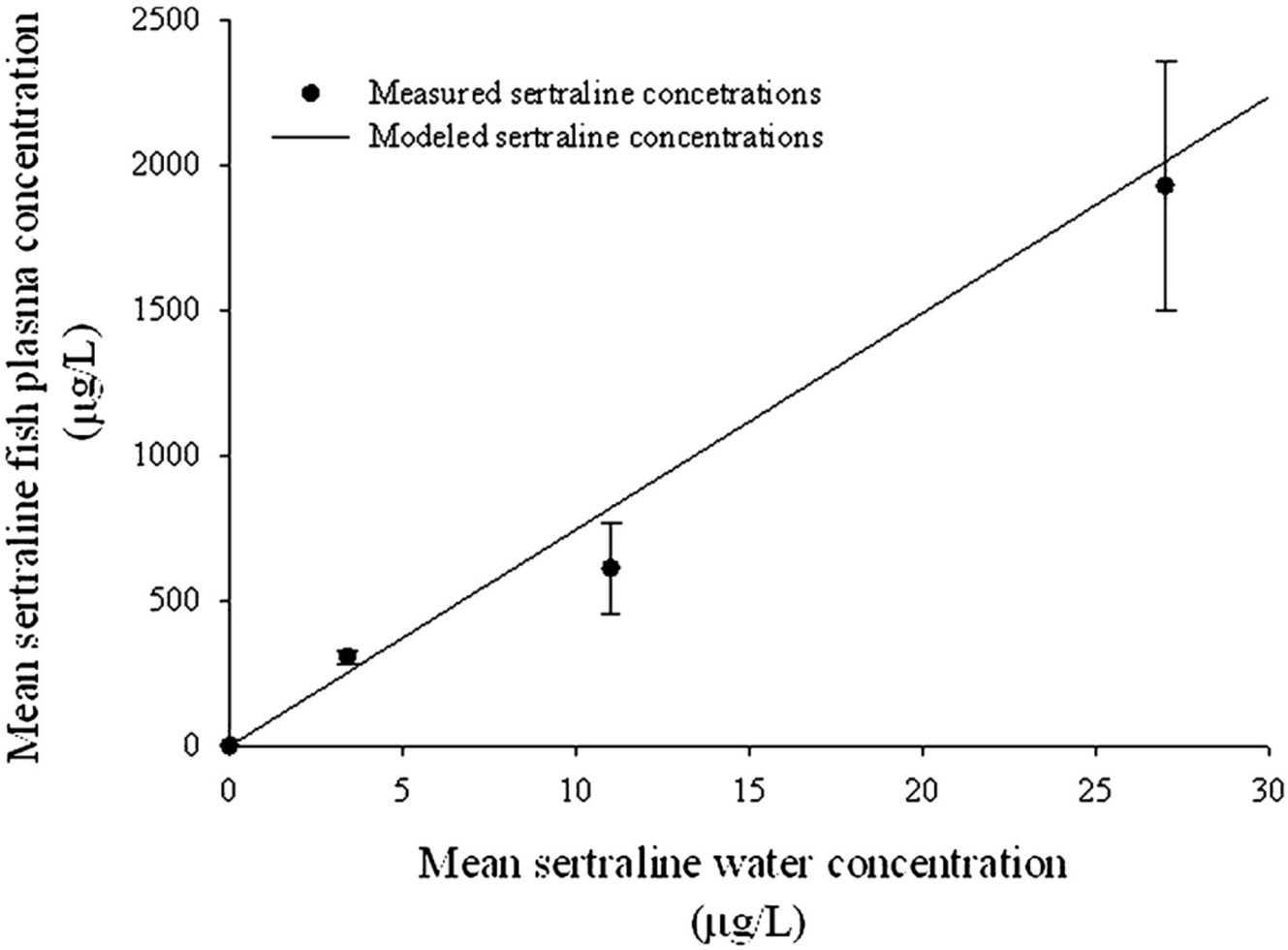

Figure 1.

Relationship between mean (n = 3; ± standard deviation), measured (dots), and modeled (solid line) plasma concentration of sertraline in adult male *Pimephales promelas* following a 28-d study, based on concentrations quantified in the water column. The modeled plasma concentration is a based on an equation described by Fitzsimmons et al.[46] in which log D was substituted for log K_{ow} (Berninger et al.[52]). $y = 70.1(x) - 15.3$; $r^2 = 0.98$; $p = 0.0001$.

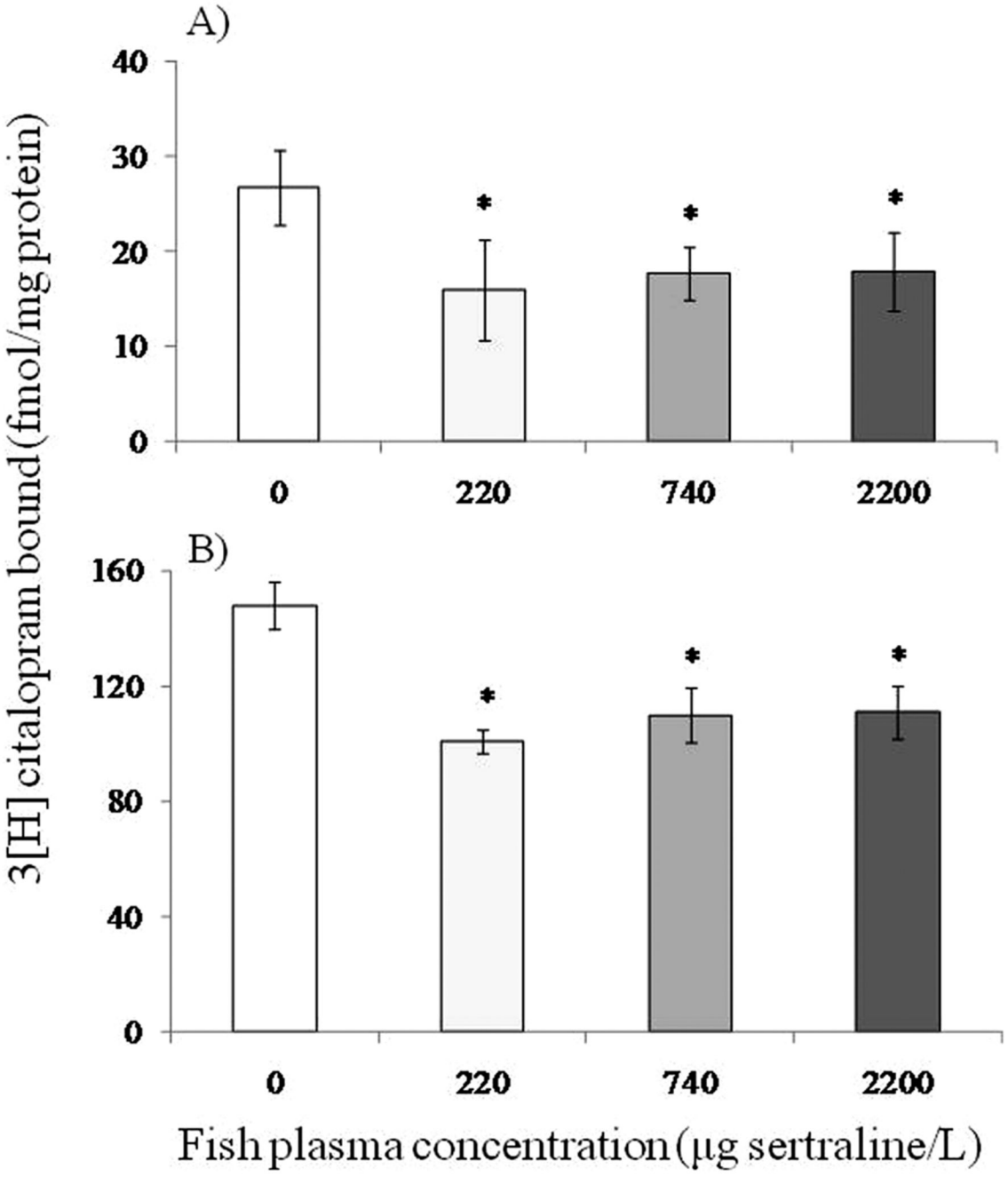

Figure 2.

Mean ($n = 3$; ± standard error) serotonin reuptake transport binding at B_{max} (A) and K_d (B) values for adult male *Pimephales promelas* brain tissue following a 28-d study with a control and three treatment levels of sertraline. *: $p < 0.05$.

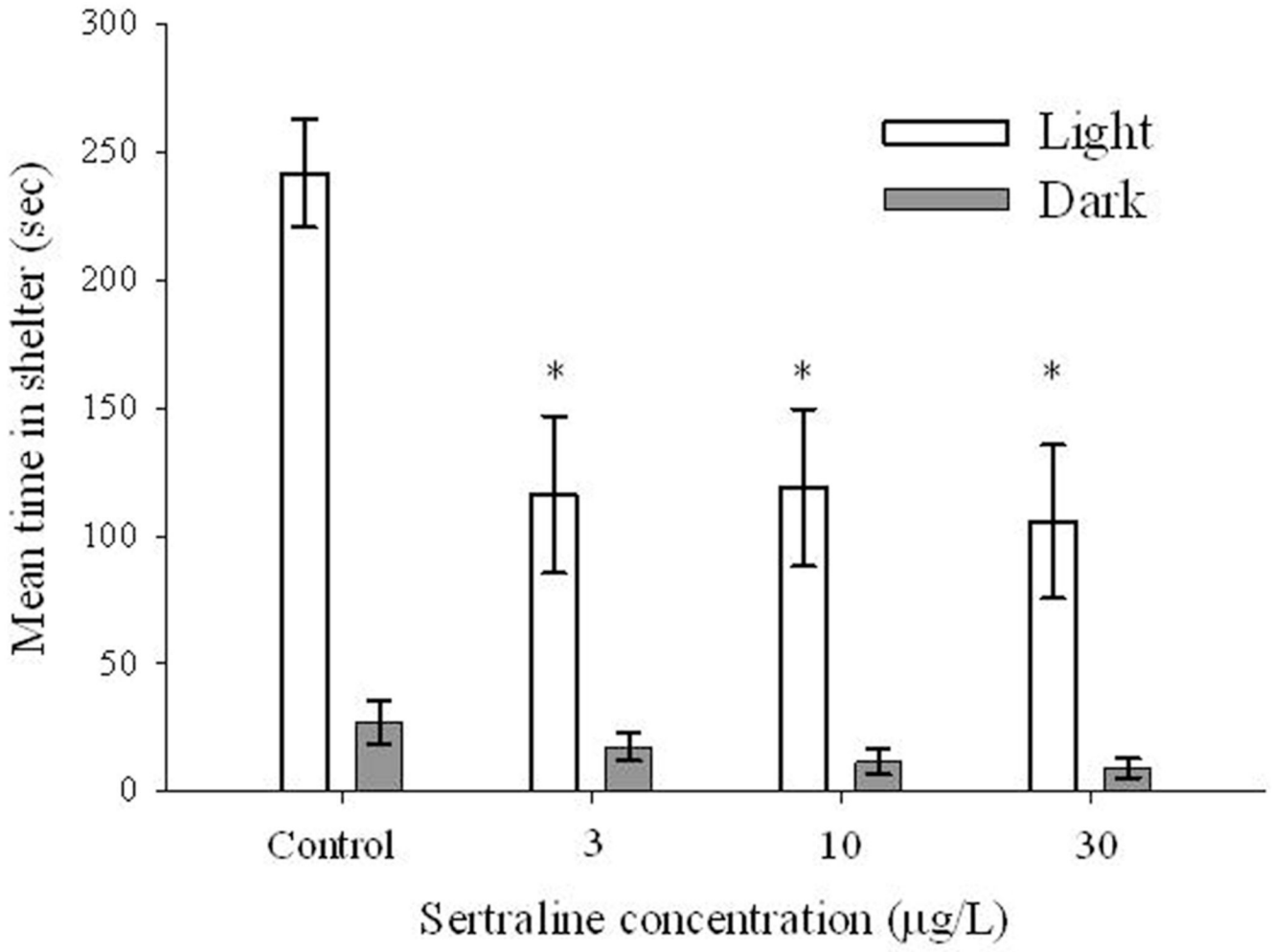

Figure 3.

Mean (n = 3; ± standard error) time (sec) adult male *Pimephales promelas* spent in shelters during light or dark conditions following a 28-d study with a control and three treatment levels of sertraline. *: $p < 0.05$.

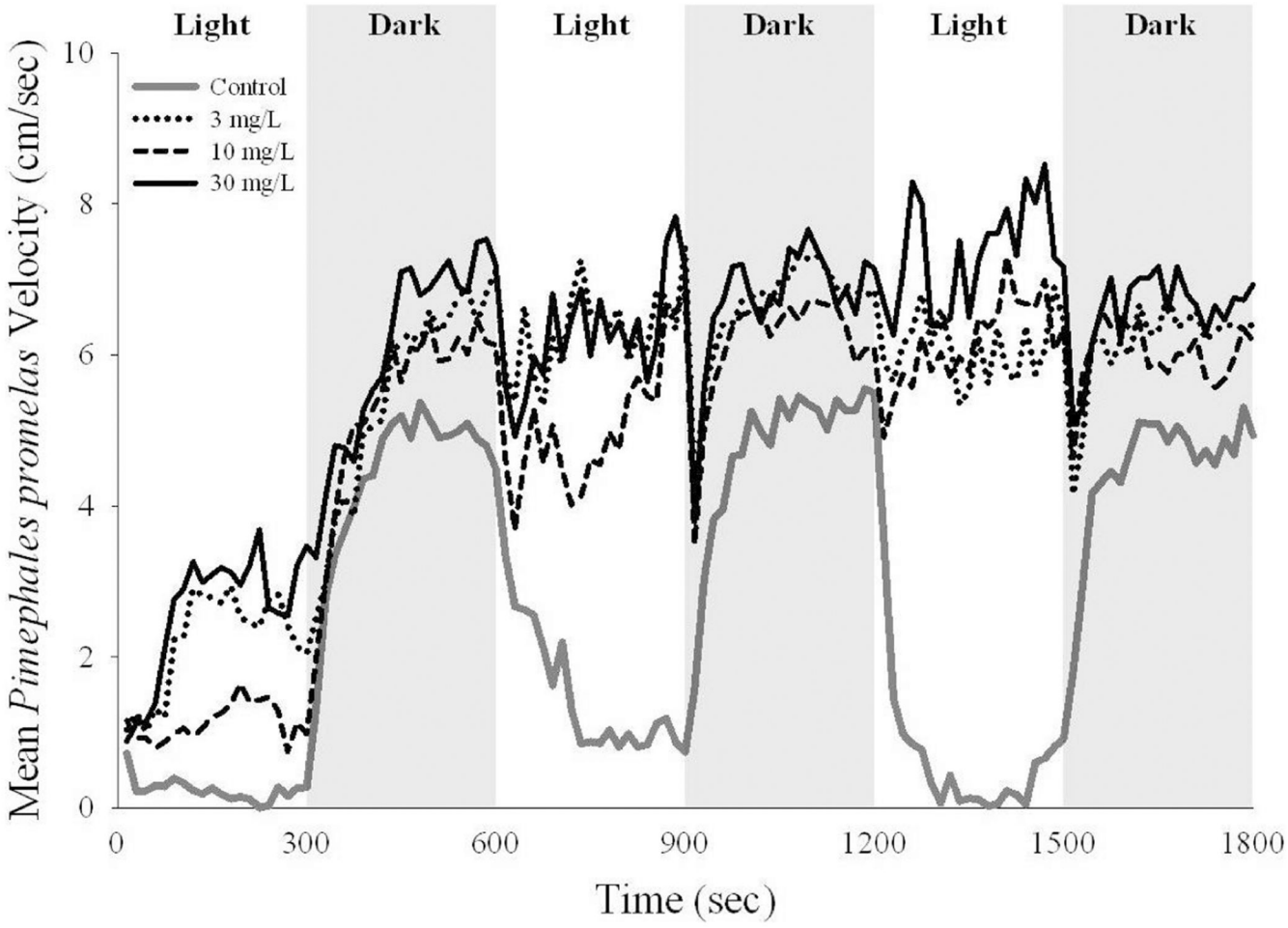

Figure 4.

Mean velocities of adult male *Pimephales promelas* during oscillating periods of light and dark conditions following a 28-d study with sertraline. Each line represents that mean for 13 individual fish to show the general patterns of swim velocities. The swim velocities were calculated using NOLDUS software.

Table 1.

Modeled and Measured Sertraline Concentrations in the Plasma of Adult Male *Pimephales promelas* Following 28-d Aqueous Exposures and Associated Effects Ratio Defined As [$Fish_{plasma}$]:C_{max}

[aqueous] (μg/L)	mean $fish_{plasma}$ [a] (μg/L)	effects ratio [b]
3 [c]	223 [e]	0.64 [f]–0.85 [g]
10 [c]	745 [e]	0.19 [f]–0.26 [g]
30 [c]	2235 [e]	0.06 [f]–0.09 [g]
2.8 [d]	305 [d]	0.47 [f]–0.62 [g]
9.4 [d]	610 [d]	0.23 [f]–0.31 [g]
28.1 [d]	1927 [d]	0.07 [f]–0.10 [g]

[a] [Aqueous] × $P_{blood:water}$.

[b] $Fish_{piasma}/C_{max}$.

[c] Nominal concentration.

[d] Measured concentration.

[e] Modeled concentration.

[f] C_{max} 142 μg/L (Thomson 2008).

[g] C_{max} 190 μg/L (Product registry).