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**ENDOCRINE AND REPRODUCTIVE EFFECTS OF THE PHARMACEUTICAL
FLUOXETINE ON NATIVE FRESHWATER MUSSELS: PROXIMITY TO
MEASURED ENVIRONMENTAL CONCENTRATIONS**

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ABSTRACT

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) drug commonly prescribed as an antidepressant, is increasingly being detected in surface waters impacted by treated municipal wastewater. Because of its serotonergic action, fluoxetine (and other SSRIs) has been used to manipulate reproductive processes in mollusks. We evaluated the effects of acute water-only fluoxetine exposure on reproductive activities, including release of gametes, parturition of glochidia (larvae), and mantle lure display behavior in native freshwater mussels (Family Unionidae). In *Elliptio complanata*, 3000 µg/L of fluoxetine significantly induced release of spermatozeugmata in male mussels during a 48 h exposure. Fluoxetine significantly induced parturition of nonviable glochidia from adult female *E. complanata* exposed to 300 (p = 0.0118) and 3000 µg/L (p < 0.0001) when compared to controls. Fluoxetine exposure also resulted in stimulation of mantle flap display behavior in adult female *Lampsilis fasciola* and *Lampsilis cardium*. Mussels exposed to 300 µg/L (p = 0.0075) and 3000 µg/L (p = 0.0001) of fluoxetine were more frequently observed in the most advanced stages of display (stages 5 and 6) compared to control mussels. Accordingly, mussels in the 300 µg/L (p = 0.0341) and 3000 µg/L (p = 0.0006) fluoxetine treatments were less frequently observed in less advanced display stages (stages 1 & 2) compared to control mussels. Although the lowest observed effects concentrations for fluoxetine in these tests (300 µg/L) are greater than concentrations measured in surface water, they demonstrate that fluoxetine exposure has the potential to disrupt several aspects of reproduction in freshwater mussels native to the United States, a faunal group recognized as one of the most imperiled in the world. Additional research is needed to elucidate the effects of chronic, low-level exposure to fluoxetine and other drugs with the same mechanism of action.

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SUMMARY AND CONCLUSIONS

The results from this research demonstrated that fluoxetine has the potential to disrupt reproduction of native mussel species by inducing release of spermatzeugmata from male mussels, inducing release of nonviable glochidia (larvae) from gravid female mussels, as well as altering the mantle flap display behavior of female mussels. The lowest observed effect concentration (LOEC) of fluoxetine in our study (300 µg/L) is greater than those measured to date in the environment (maximum 0.1 µg/L); however, our test durations were relatively short ($48 \leq 96$ h) for all experiments. The LOEC for release of nonviable glochidia by female *E. complanata* in the present study was 300 µg fluoxetine/L, but the next lowest test concentration was 30 µg/L, thus the true threshold for effects (in acute tests) lies somewhere between 30 and 300 µg/L.

To our knowledge this is the first report of reproductive effects with male unionid mussels exposed to an SSRI drug. Male *E. complanata* exposed to 3000 µg fluoxetine/L released spermatzeugmata (aggregates of hundreds to thousands of spermatozoa) but those exposed to the next lowest concentration, 300 µg/L, did not. The effects of fluoxetine on viability of spermatzeugmata or individual spermatozoa after release from the sperm sphere have not been elucidated.

Finally, consistent with other studies of mollusks in which fluoxetine stimulated reproductive processes, fluoxetine exposure resulted in a concentration-dependent stimulation of mantle lure display behaviors by female *Lampsilis fasciola* and *Lampsilis cardium*. It is currently unclear if such behavioral effects would preclude successful attraction of a suitable host fish for glochidia infestation. Additionally, it is not known if fluoxetine (or other SSRIs) would induce display behaviors in female mussels that are not gravid (i.e., out of season), or prematurely induce displays in those that contain immature or nonviable glochidia.

RECOMMENDATIONS

Currently little is known about the effects of chronic, low-level fluoxetine exposure (consistent with surface waters receiving treated wastewater effluent) on freshwater mussel reproduction. Therefore, additional research is needed on the potential long-term effects of fluoxetine on mussel reproduction and behavior, especially in surface waters receiving treated wastewater effluent. Moreover, because freshwater mussels are sediment-dwelling organisms, there remain uncertainties about the potential bioavailability and toxicity of sediment-bound fluoxetine and other SSRIs to mussels. Additional research is also required to elucidate the effects of fluoxetine on other ecologically-relevant behaviors of mussels such as burrowing and feeding. Because the SSRIs are most commonly associated with municipal wastewater effluents, they generally exist as mixtures rather than individual chemicals in surface waters. Thus, it would be prudent to evaluate the potential for surface waters to disrupt neuroendocrine pathways based on total SSRI concentration and activity rather than focus on individual drugs because the combined total SSRI activity in a stream may better estimate effects on critical biological endpoints.

1. INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) have been detected in surface waters, effluent from municipal wastewater treatment facilities, seawater, and groundwater (for a review, see Fent et al., 2006). Although these compounds are generally not as persistent as traditional priority pollutants (e.g., polychlorinated biphenyls and organochlorine pesticides), the continuous release of PPCPs into our rivers and streams presents similar exposure conditions as that of a persistent organic pollutant (Johnson et al., 2005). Previous studies indicate that many of these compounds enter the environment in their parent form (un-metabolized) or as a mixture of metabolites (Daughton and Ternes, 1999; Johnson et al., 2005; Kolpin et al., 2002; Metcalfe et al., 2003; Vieno et al., 2007). In addition, some PPCPs have been found to accumulate in fish in effluent-dominated streams (Brooks et al., 2005). Therefore, compounds that were manufactured with the intent of being bioactive are being discharged into surface and ground waters and may be responsible for acute and chronic effects in aquatic organisms including endocrine disruption (Colborn et al., 1993; Routledge et al., 1998).

Native freshwater mussels (family Unionidae) are long-lived (30-100 yr) filter- and deposit-feeding benthic organisms that live burrowed in sediments of streams and rivers. They are among the most rapidly declining faunal groups in North America; approximately 67% of the nearly 300 freshwater mussel species found in North America are considered vulnerable to extinction or already extinct (Bogan, 1993; Williams et al., 1993). The decline of mussel populations in North America has occurred steadily since the mid 1800s and has been attributed to pollution, construction of dams and impoundments, sedimentation, navigation, and habitat degradation (Bogan, 1993; Brim Box and Mossa, 1999; Neves, 1997; Vaughn and Taylor, 1999). Principally, the stressors associated with human development and urbanization have hastened these population declines over the past 20 to 50 years.

Unionids have a unique life history that includes an obligate parasitic larval stage (glochidia). Gravid female mussels release glochidia which must successfully attach to gills or fins of a suitable host fish. Mussels of the tribe Lampsilini use elaborate and highly specialized mantle tissue displays to mimic prey items and attract potential host fish (Haag et al., 1995; Haag and Warren, 1999). As the fish strikes the mantle flap “lure,” the gill epithelial tissue is ruptured and hundreds to thousands of glochidia are released into the buccal cavity of the fish where some attach to gills and undergo transformation into juvenile mussels before releasing from the fish and settling into the sediment to become adults. Because of this unique life history, unionid mussels may be among the groups of aquatic organisms most susceptible to PPCPs and other endocrine disrupting compounds (EDCs) that are now found in our surface waters.

One of the PPCPs detected frequently in surface waters is fluoxetine, the active ingredient in Prozac[®] and other anti-depressant drugs (Kolpin et al., 2002; Metcalfe et al., 2003; Ternes, 1998). Fluoxetine is a selective serotonin reuptake inhibitor (SSRIs) drug and exerts its action by reducing the clearance rate of serotonin, (5-hydroxytryptamine) a neurotransmitter, in the synapse following nerve signal transmission.

Serotonin is a key mediator for a wide variety of physiological functions in mollusks. In bivalve mollusks serotonin regulates a reproductive processes including oocyte maturation (Fong et al., 1994; Hirai et al., 1988) spawning (Hirai et al., 1988; Ram et al., 1993) and parturition (release of glochidia; Fong et al., 1996). Serotonin, fluoxetine and other SSRIs have been used to artificially induce spawning in freshwater bivalves for aquaculture purposes (Cunha and Machado, 2001; Gibbons and Castagna, 1984) and have been investigated as a potential chemical control mechanism (i.e., disruptor of reproduction) for exotic bivalve species like the zebra mussel *Dreissena polymorpha* (Fong et al., 1994; Fong et al., 1996; Ram et al., 1992). In light of the increasing number of surface waters with measurable fluoxetine (and other SSRI) concentrations and the imperiled status of freshwater mussel fauna, determination of the effects of environmental exposures of fluoxetine to native freshwater mussels is of utmost importance.

The goal of the present study was to resolve the effects of fluoxetine on reproduction in native freshwater mussels. Specific objectives were to determine 1) the concentration of fluoxetine required to induce parturition of glochidia from brooding female mussels, 2) if fluoxetine alters mantle flap display behavior used to attract a fish host, 3) and if fluoxetine exposure induces spawning from male mussels.

2. MATERIALS AND METHODS

2.1 Test organisms

Adult *Elliptio complanata* mussels were collected from the Eno River near Hillsborough, North Carolina and Little Creek near Wilson, North Carolina, in July 2004, June 2005, April 2006 and July 2006. These are relatively uncontaminated, rural, forested streams in the central Piedmont of North Carolina. The mussels were transported using methods of Cope et al. (2003) to the Aquatic Toxicology Laboratory at North Carolina State University. Adult female *Lampsilis cardium* (length 93 to 123 mm) displaying mantle flap lures were collected from Pool 8 of the Upper Mississippi River near La Crosse, Wisconsin, in September 2005 and shipped to the Aquatic Toxicology Laboratory at North Carolina State University via overnight courier following methods described by Cope et al. (2003). Adult female *Lampsilis fasciola* (length 45 to 82 mm) displaying mantle flap lures were collected from a rural, largely forested reach of the Little Tennessee River near Franklin, North Carolina, in May 2006 and transported to the Aquatic Toxicology Laboratory at North Carolina State University (Cope et al., 2003). Upon arrival at the laboratory, all mussels were acclimated to reconstituted soft water (ASTM, 1993) over 24 h and maintained at 18-20°C for at least 24 h prior to beginning any experiments to ensure that spawning or release of glochidia during experiments was not a result of handling or transport stress.

2.2. Test Chemicals

Fluoxetine hydrochloride (Sigma-Aldrich, St. Louis, Missouri, USA; 98% purity) and serotonin creatinine sulfate (Acros Organics, Geel, Belgium; 99% purity) were purchased from VWR International (West Chester, PA, USA). Working solutions of fluoxetine (8 mg/mL and 0.008 mg/mL) were prepared in deionized water to a final volume of 10 mL. The working solution of serotonin (20 mg/mL) was also prepared in deionized water. Test containers were pre-conditioned with appropriate test solutions for 24 h before experiments began.

2.3. Water Chemistry

Test solutions were prepared in reconstituted soft water for all experiments. Water chemistry (temperature, pH, dissolved oxygen, conductivity, alkalinity, and hardness) was measured according to standard methods (APHA, 1995) in one replicate of each treatment at least at the beginning and end of each test. A calibrated multiprobe (YSI Model 556 MPS, Yellow Springs Instruments, Yellow Springs, OH, USA) was used for analysis of pH, dissolved oxygen, conductivity and temperature. Alkalinity was determined by titration with 0.02 N H₂SO₄ to pH 4.5, and hardness by titration with 0.01 M ethylenediaminetetraacetic acid (EDTA).

2.4. Glochidia Parturition in *Elliptio complanata*

Glochidia release by brooding female *E. complanata* mussels (length from 55 to 77 mm) following exposure to fluoxetine was determined in three trials (July 2004, June 2005, and July 2006).

Female mussels that had not released glochidia during acclimation were placed in 3.75-L glass aquaria (one mussel per aquaria) containing 2 L of gently aerated reconstituted soft water. The mussels were exposed to one of five fluoxetine treatments (0, 0.3, 3.0, 30, 300, or 3000 µg/L), with 3, 5, or 6 replicates per treatment (depending on the trial) for 96 h. In addition, a serotonin treatment (40 mg/L) was included as a positive control. Mussels were examined at 24 h intervals for the duration of the exposure. Endpoints included time from initiation of exposure to parturition of >100 glochidia and viability of released glochidia, determined by response of a sub-sample (n = 50 -100) of the released glochidia to a saturated solution of NaCl (ASTM, 2006). Composite water samples (10 mL from each replicate in a given treatment) were collected from each treatment for analysis of fluoxetine at time 0 and at the time of first release of glochidia in a replicate.

2.5. Mantle Lure Display and Glochidia Parturition in *Lampsilis cardium* and *L. fasciola*

Effects of fluoxetine on mantle flap display behavior and glochidia parturition were evaluated in *L. cardium* in September 2005 and in *L. fasciola* in May 2006. Mussels were maintained in 3.75-L glass aquaria (1 mussel/aquaria) containing 2 L of gently aerated reconstituted soft water (ASTM, 1993) and 12 mm of artificial substrate (inert aquarium gravel) at 18-20°C for 24 h prior to beginning the experiment to ensure that the females were displaying their mantle flaps and that spawning or release of glochidia was not a result of handling or transport stress. Only mussels that displayed fish lures and had not released glochidia during acclimation were used for the experiment. The mussels were exposed to one of five fluoxetine treatments (0, 0.3, 3.0, 30, 300, or 3000 µg/L) with 6 replicates per treatment for 96 h in static renewal tests (total of 36 experimental units). All mussels were monitored for mantle flap display and the release of glochidia continuously for the first 6 h of the experiment and then observed at 2 hour intervals during 8 h blocks over the remaining exposure duration. Mantle flap display behaviors were categorized as: Stage 1- shell closed; Stage 2- shell gaped but no mantle tissue exposed; Stage 3- shell gaped and mantle tissue partially extended; Stage 4- shell gaped and mantle tissue fully extended with fish lure visible; Stage 5- shell gaped, fish lure fully extended, and marsupial gills extended beyond shell margin; Stage 6- shell gaped, fish lure fully extended, marsupial gills fully extended, and fish lure pulsating (number of beats/min was quantified). In addition, time to release of glochidia (>100) was recorded and viability was determined. A 100% renewal of exposure water was completed at 24-h intervals to maintain target fluoxetine concentrations. Composite water samples (200 mL from each of 6 replicates) were collected for fluoxetine analysis from each treatment at the time mussels were initially placed in fluoxetine treatments.

2.6. Spermatozeugmata Release by *Elliptio complanata*

Effects of fluoxetine on release of spermatozeugmata or “sperm spheres” (Ishibashi et al., 2000; Waller and Lasee, 1997) were evaluated in male *E. complanata* in April 2006. Because *E. complanata* is not a sexually dimorphic species, we initiated the experiment with 48 non-gravid adult mussels of unknown gender ranging in length from 52 to 74 mm. Mussels were randomly assigned to one of three treatments: control, 300 µg fluoxetine/L, or 3000 µg fluoxetine/L. An experimental unit consisted of a 3.75-L glass aquarium with 1 mussel and 2 L of gently aerated reconstituted soft water (ASTM, 1993).

Temperature was maintained at 20°C throughout the test by partially submerging aquaria in a water bath (Living Stream[®], Frigid Units, Toledo, Ohio, USA). Water samples (10 mL) were collected from each aquarium at the start of the experiment (before addition of fluoxetine) and examined at 40x magnification under a dissecting microscope for the presence of spermatozuogmata or glochidia. No mussels had released gametes or glochidia by the start of the experiment. A release of spermatozuogmata was defined as >10 spermatozuogmata per 10-mL sample of exposure water and glochidia parturition was documented when > 100 glochidia were expelled. Water samples were collected and examined at 2-hr intervals for the first 12 h of the experiment and from 24-36 h. A final 10-mL sample was examined at 48 h, after which mussels that had not released sperm or glochidia were exposed to serotonin (80 mg/L) for ≤ 8 h to stimulate release of spermatozuogmata or glochidia they may be carrying. Test solutions were renewed (100%) at 24 h.

2.7. Fluoxetine Extraction and Quantification

Exposure water was acidified with formic acid (pH 2.7), filtered (0.45 µm, Whatman, Brentford, Middlesex, UK), spiked with surrogate internal standard, ¹³C-phenacetin, and extracted using 47 mm C18 Empore[™] disks (Varian, Palo Alto, California, USA) that were conditioned according to manufacturer instructions. Following extraction, the disks were dried under vacuum, placed in 50-mL centrifuge tubes, and shaker table extracted with 20 mL of methanol. Methanol was decanted and the extraction was repeated twice more for a total of 60 mL methanol. Extracts were combined and nitrogen evaporated to 4 mL methanol. The extract was then filtered with a UniPrep[™] filter (Whatman, Brentford, Middlesex, UK), evaporated to approximately 1 mL, quantitatively transferred to a glass vial and evaporated to dryness. The sample was resuspended in 450 µl of methanol and 50 µl of 2-chlorolepidine was added as a recovery standard. All analyses were performed with an Agilent 1100 liquid chromatograph coupled to an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The parent and daughter ions used for each compound were as follows: ¹³C-phenacetin 181—110, 2-chlorolepidine 179—143, and fluoxetine 310—44 and 310—148. The transitions identified are consistent with previous work on fluoxetine (Brooks et al., 2005). The mass spectrometer was operated in electron spray ionization positive mode. Separation was performed using a Zorbax C18 column (Agilent ZORBAX Eclipse XDB; 250 mm x 5 mm; 5 µm). Solvent A was 10 mM ammonium formate in water (adjusted to pH 2.9 with formic acid). Solvent B was 10 mM ammonium formate and 5 % water in methanol (with an equivalent volume of formic acid). A gradient elution starting with 90 % A ramped linearly to 40 % A over 12 min was used for the separation of the compounds. The system was returned to the initial conditions after 17 min and equilibrated for 6 min. The flow rate was 0.5 mL/min. Samples were quantified using at least a four point calibration curve, which was not forced through the origin. The calibration solutions were not extracted.

2.8. Statistical Analyses

For statistical comparisons of glochidia release, the three trials of the experiment in which we evaluated glochidia release in *E. complanata* exposed to fluoxetine were treated as replicates in

time ($n = 3$) and the mean percentage of mussels releasing glochidia in each treatment was determined (total of 21 experimental units). JMP (SAS, Cary, North Carolina, USA) statistical software was used to test for homogeneity of variances (Bartlett's Test) and differences in glochidia release between treatment groups and controls was evaluated with analysis of variance (ANOVA) followed by Dunnett's Test ($\alpha = 0.05$). Display behavior (i.e., frequency of occurrence in each stage) was analyzed by multivariate analysis of variance (MANOVA) followed by Dunnett's Test for means comparison of treatments to controls for each stage ($\alpha = 0.05$, $n = 36$ experimental units). For simplicity, frequency of occurrence results were combined in to three categories: stages 1 and 2, stages 3 and 4, and stages 5 and 6. Preceding analysis, frequencies of occurrence in each of the three categories were transformed (arcsine) to achieve homogeneity of variance. JMP (SAS, Cary, North Carolina, USA) statistical software was used for all statistical analyses.

3. RESULTS

3.1 Water Chemistry

Dissolved oxygen concentrations were greater than 80% of saturation at all times during all experiments. Other water quality parameters were consistent among experiments as well: pH ranged from 7.7 to 8.1, alkalinity ranged from 30 to 34 mg CaCO₃/L and hardness was 42 to 48 mg CaCO₃/L. Mussel survival was 100% in all experiments.

3.2 Fluoxetine Analyses

Measured concentrations of fluoxetine ranged from 74.7 to 120.0% of target concentrations for the July 2004 trial and from 85.7 to 111.4% of target concentrations for the June 2005 trial. The most rigorous sampling for determination of fluoxetine in laboratory exposure water was during the July 2006 trial in which samples were collected at time 0, 12 h, 24 h and 48 h. Measured fluoxetine concentrations ranged from 78 to 169% of target concentrations throughout the experiment (Table 1). Surrogate (2-chlorolepidine) recovery ranged from 80 to 100 % for all *E. complanata* experiments.

Table 1. Measured fluoxetine concentrations in composite water samples (from 6 replicates) from laboratory *Elliptio complanata* exposures during June 2005; these values are similar to measured concentrations from the *E. complanata* experiment in July 2006.

Target fluoxetine concentration (µg/l)	Measured fluoxetine concentration (µg/l)					
	Time (hour)				Mean	SD ^a
	0	12	24	48		
Control	BDL ^b	BDL	BDL	BDL	--	--
0.3	0.5	0.26	0.29	0.20	0.3	0.1
3	3.8	2.4	1.2	2.5	2.5	1.1
30	36.8	15.8	15.4	23.2	22.8	10.0
300	331.3	243	190.9	283.5	262.2	59.7
3000	2340.5	2597.0	1629.7	NA ^c	2189.0	501.1

^a Standard deviation

^b Below detection limit

^c No samples collected

3.3 Glochidia Parturition by *Elliptio complanata*

Fluoxetine induced parturition of nonviable glochidia. A significantly greater percentage of

mussels in the 300 $\mu\text{g/L}$ ($p = 0.0118$) and 3000 $\mu\text{g/L}$ ($p < 0.0001$) fluoxetine treatments released nonviable glochidia compared to controls (Figure 1). Mussels in the 0.3 $\mu\text{g/L}$ fluoxetine treatment also released nonviable glochidia; however, this was not significantly different from controls ($p = 0.9998$). One control mussel in the June 2005 trial released nonviable glochidia but no control mussels released nonviable glochidia in the other two trials. Some mussels in each treatment released viable glochidia but there was not a significant difference ($p = 0.8176$) in the percentage of mussels in each treatment that released viable glochidia (Figure 1). Glochidia release by mussels exposed to serotonin, the positive control, was similar to the release by mussels exposed to the highest fluoxetine concentration (Figure 1). Mussels exposed to 300 and 3000 $\mu\text{g/L}$ of fluoxetine and serotonin had marked increase in foot volume, many to the point at which they were unable to close their shell.

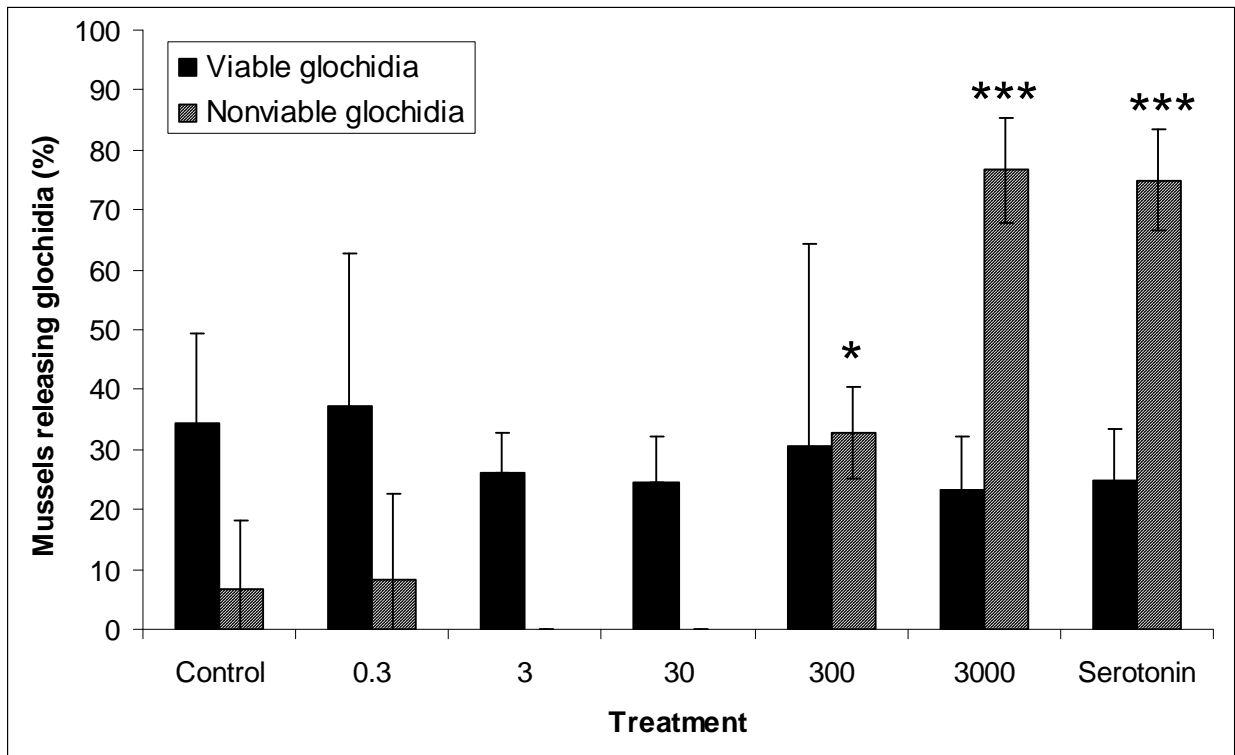


Figure 1. Mean percent of mussels ($N = 14$) per treatment that released glochidia during a 48-h exposure to fluoxetine (0 – 3000 $\mu\text{g/L}$) or serotonin (40 mg/L). Error bars indicate \pm one standard deviation. Asterisks indicate a significant difference compared to control (Dunnett’s Test): * $P \leq 0.05$, *** $P \leq 0.0001$.

3.4 Mantle Lure Display and Glochidia Parturition

Fluoxetine substantially altered mantle flap display behavior in *L. cardium* and *L. fasciola* in a similar pattern.

In *L. fasciola*, for example, mussels exposed to 300 $\mu\text{g/L}$ ($p = 0.0075$) and 3000 $\mu\text{g/L}$ ($p = 0.0001$) of fluoxetine were more frequently observed in the most advanced stages of display (stages 5 and 6) compared to control mussels. Concomitantly, mussels in the 300 $\mu\text{g/L}$ ($p = 0.0341$) and 3000 $\mu\text{g/L}$ ($p = 0.0006$) fluoxetine treatments were less frequently observed in less advanced display stages (stages 1 & 2) compared to control mussels (Figure 2). The trend toward more advanced stages of display behavior was more pronounced in mussels exposed to 3000 $\mu\text{g/L}$ of fluoxetine compared to the 300 $\mu\text{g/L}$ treatment group, indicating a concentration-response relationship. Additionally, variability of observation frequency in the various stages among replicates also decreased in the mussels exposed to 3000 $\mu\text{g/L}$ (Figure 2). The results of the experiment with *L. cardium* were consistent with those of *L. fasciola* in terms of a concentration-related response in mantle lure display behavior, except that only mussels in the 3000 $\mu\text{g/L}$ fluoxetine treatment were more frequently observed ($p = 0.0001$) in the most advanced stages of display (stages 5 and 6) compared to control mussels (data not shown).

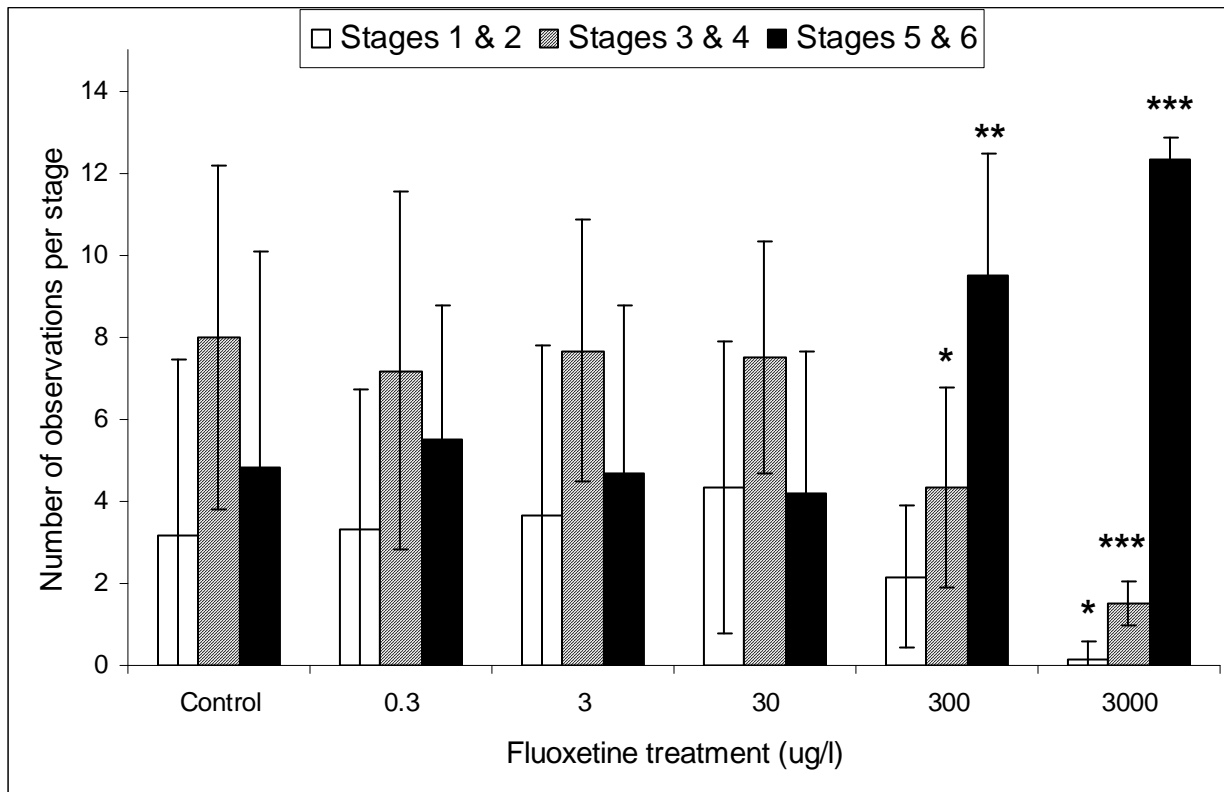


Figure 2. Summary of mantle flap display behavior data for *Lampsilis fasciola* exposed to fluoxetine (0 – 3000 $\mu\text{g/L}$) for 96 h. Mean number of observations ($N = 16$ total observations) per display stage ($N = 6$ replicates). Stages 1 and 2 were no visible mantle flap, stages 3 and 4 were partial displays, and stages 5 and 6 were full displays. Error bars indicate \pm one standard deviation. Asterisks indicate a significant difference compared to control (Dunnett’s Test): * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Parturition of glochidia occurred during the mantle lure display experiments with *L. cardium* and *L. fasciola*, as in experiments with *E. complanata*. However, in both of these tests, only mussels in the 3000 µg/L fluoxetine treatment released a significantly ($p < 0.0001$) greater percentage of glochidia compared to controls. All of the glochidia released from mussels during these two experiments were viable glochidia, unlike in the tests with *E. complanata*, in which viable and nonviable glochidia were released. In these tests, the number of glochidia released by mussels exposed to serotonin, the positive control, was again similar to those released by mussels exposed to the highest fluoxetine concentration. Also, as observed in the experiments with *E. complanata*, the foot volume of *L. fasciola* and *L. cardium* was substantially increased in mussels exposed to the two highest fluoxetine concentrations.

3.5 Spermatozeugmata Release

Fluoxetine induced release of spermatozeugmata in seven of 16 (43.8%) *E. complanata* of unknown gender in the 3000 µg/L treatment, whereas 1 of 16 (6.3%) mussels in each of the control and 300 µg/L fluoxetine treatments released spermatozeugmata (Figure 3). Release of spermatozeugmata by mussels in the 3000 µg/L fluoxetine treatment was significantly different ($p = 0.0056$) from other treatments. In addition, five of the 16 (31.3%) mussels in the 300 µg/L fluoxetine treatment and 4 of 16 (25.0%) in the 3000 µg/L fluoxetine treatment released glochidia, whereas 2 of 16 (12.5%) released glochidia in the control treatment (Figure 3). Of the 12 remaining mussels that had been exposed to fluoxetine but had not released spermatozeugmata or glochidia within 48 h, 2 released nonviable glochidia within 2 h of exposure to serotonin (80 mg/L). The 10 remaining mussels did not release glochidia or gametes within 24 h of exposure to serotonin.

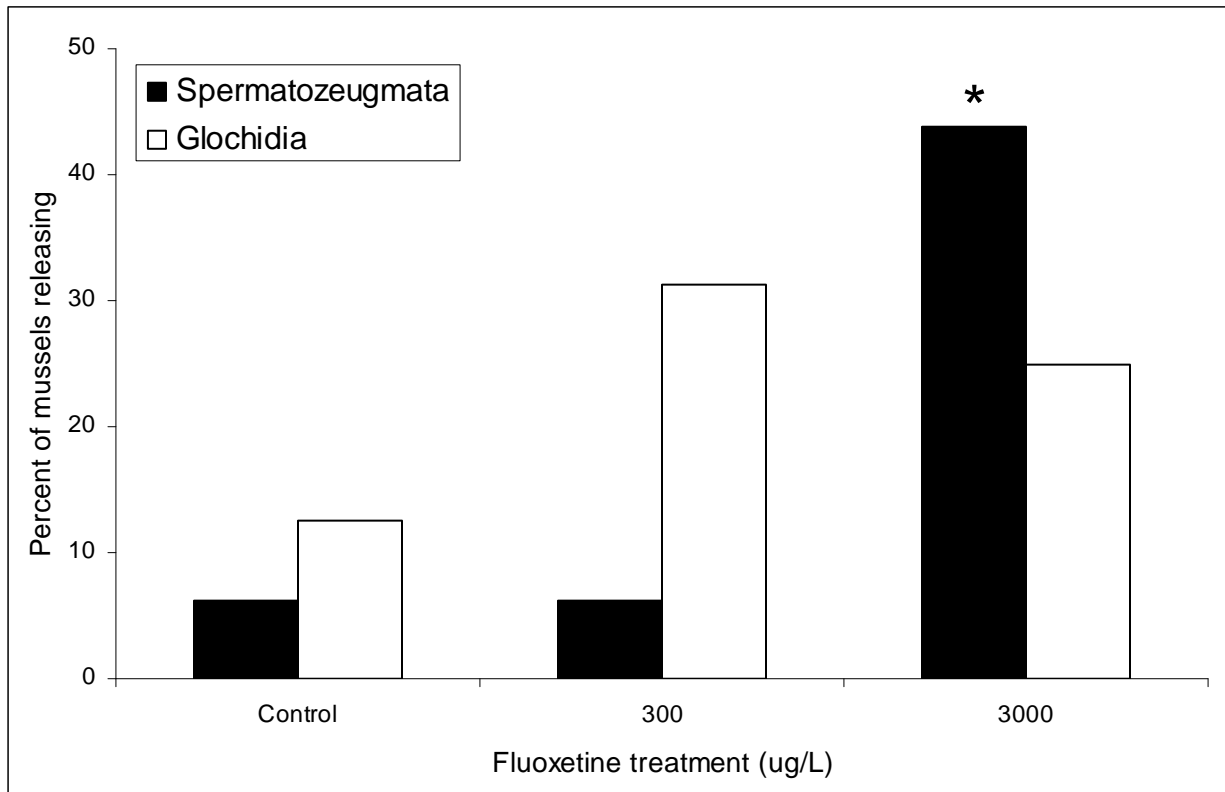


Figure 3. Percent of mussels (N = 16) of unknown gender that released spermatozeugmata or glochidia during 24-h exposure to fluoxetine (0 – 3000 $\mu\text{g/L}$). Error bars indicate \pm one standard deviation. An asterisk (*) indicates a significant difference compared to other treatments (Chi Square; $P \leq 0.05$).

4. DISCUSSION

The ecological effects of an ill-timed release of larval mussels (glochidia) or gametes caused by environmental fluoxetine exposure could be potentially devastating to localized mussel populations. Likewise, the inability of a female mussel to attract a suitable fish host through altered or ill-timed mantle flap (fish lure) display behavior such that she would not be able to successfully infest a fish with glochidia could also result in reproductive failure and devastate local mussel populations. Our results demonstrate that fluoxetine has the potential to disrupt reproduction of native mussel species by inducing release of spermatozuogmata and glochidia as well as altering mantle flap display behavior. The lowest observed effect concentration (LOEC) of fluoxetine in our study (300 µg/L) is greater than those measured to date in the environment (maximum 0.1 µg/L; Metcalfe, 2003); however, our test durations were relatively short (48 ≤ 96 h) for all experiments. Currently little is known about the effects of chronic, low-level fluoxetine exposure (consistent with surface waters receiving treated wastewater effluent) on mussel reproduction.

Serotonin is a widely occurring biogenic monoamine that regulates a variety of reproductive processes in mollusks including oocyte maturation (Fong et al., 1994), spawning (Fong et al., 1993; Ram et al., 1993; Ram et al., 1996), and parturition (Fong and Warner, 1995) and has been used for enhanced culture of economically important freshwater and marine bivalves. However, high concentrations of serotonin are required to induce spawning and parturition and the concomitant expense can be prohibitive for use in developing countries where aquaculture is becoming increasingly important. As a result, fluoxetine and other SSRI drugs have been investigated as potential alternatives to serotonin and have indeed been reported to induce spawning (Fong, 1998) and parturition (Cunha and Machado, 2001; Fong, 1998) at concentrations that are orders of magnitude lower than required for serotonin and therefore may be more economically feasible than serotonin. The mechanism by which SSRI drugs induce reproductive effects has not been fully elucidated and may involve SSRIs acting as ligands at post-synaptic receptors (acting in a manner similar to serotonin), rather than as inhibitors of reuptake transporters, or may indeed involve blocking reuptake of endogenous serotonin in the post-synaptic cleft.

The LOEC for release of nonviable glochidia by *E. complanata* in the present study was 300 µg fluoxetine/L, but the next lowest test concentration was 30 µg/L, thus the true threshold for effects (in acute tests) lies somewhere between 30 and 300 µg/L. Mussels in all treatment groups released mature glochidia, suggesting that some mussels brought into the lab were on the verge of releasing glochidia in the stream before they were collected and transported to the laboratory. The mussels were collected during June and July when gravid *E. complanata* are observed in North Carolina streams (C. B. Eads, North Carolina State University, Raleigh, NC, personal communication), therefore stress from handling and transport likely stimulated the release of mature glochidia in those individuals. However, the release of immature, nonviable glochidia was related to fluoxetine exposure in a concentration-dependent manner.

Fluoxetine and other SSRI drugs have been used previously to induce spawning and parturition in mussels. Cunha and Machado (2001) used fluoxetine and fluvoxamine, another SSRI drug, to control timing and intensity of glochidia release in *Anodonta cygnea*, for aquaculture purposes. Significant numbers of viable glochidia were released by gravid mussels exposed to 309 and 3090 µg/L of fluoxetine during a 24-h exposure period in their study. *Elliptio complanata* in the present study appeared to be similarly sensitive to fluoxetine (i.e., the LOEC for glochidia release was 300 µg/L). Additionally, consistent with reports by Cunha and Machado (2001) for *A. cygnea*, we observed a strong increase in volume of the foot in *E. complanata* during early stages of exposure to fluoxetine (300 and 3000 µg/L) and serotonin. Cunha and Machado (2001) attributed the increase in foot size to relaxation of foot muscles and the resulting favorable conditions for uptake and storage of water.

To our knowledge this is the first report of reproductive effects with male unionid mussels exposed to an SSRI drug. Male *E. complanata* exposed to 3000 µg fluoxetine/L in the present study released spermatozuogmata (aggregates of hundreds to thousands of spermatozoa) but those exposed to the next lowest concentration, 300 µg/L, did not. Fong (1998) reported induction of spawning in zebra mussels exposed to fluoxetine, fluvoxamine and paroxetine. The lowest concentration of fluoxetine required to induce spawning in zebra mussels was 155 µg/L for males and 1545 µg/L for females in his study. The effects of fluoxetine on viability of spermatozuogmata or individual spermatozoa after release from the sperm sphere have not been elucidated.

In humans, fluoxetine is used for treatment of not only depression, but also for treatment of obsessive –compulsive behaviors associated with Tourette’s syndrome (Eapen et al., 1996) as well as convulsive seizures (Pasini et al., 1996), so it is not surprising that it affected mussel behavior in the present study. Consistent with other studies of mollusks in which fluoxetine stimulated reproductive processes (Avila et al., 1996; Fong, 1998; Uhler et al., 2000), fluoxetine exposure resulted in a concentration-dependent stimulation of mantle lure display behaviors by *L. fasciola* and *L. cardium*. It is currently unclear if such behavioral effects would preclude successful attraction of a suitable host fish for glochidia infestation. Additionally, it is not known if fluoxetine (or other SSRIs) would induce display behaviors in female mussels that are not gravid (i.e., out of season), or prematurely induce displays in those that contain immature or nonviable glochidia. Likewise, additional research is required to elucidate the effects of fluoxetine on other ecologically-relevant behaviors such as burrowing and feeding. Because SSRIs are most commonly associated with municipal wastewater effluents, they generally exist as mixtures rather than individual chemicals in surface waters. It would be prudent to evaluate the potential for surface waters to disrupt neuroendocrine pathways based on total SSRI concentration and activity rather than focus on individual chemicals because the combined total SSRI activity may better estimate effects on critical biological endpoints.

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