Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel *Dreissena polymorpha*

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Abstract

The pharmaceutical fluoxetine, a selective serotonin reuptake inhibitor (SSRI), is often detected in municipal wastewater treatment plant effluents and surface waters within the ng/l range. There is, however, insufficient research evaluating potential hazards of fluoxetine in aquatic organisms at environmentally relevant concentrations. Taking into account that several SSRIs (fluoxetine, fluvoxamine) act as spawning inducers in bivalves, this study aimed at investigating the effects of fluoxetine exposure in the zebra mussel (*Dreissena polymorpha*) by assessing its potential to induce spawning at environmentally relevant concentrations (20 and 200 ng/l), as well as alterations of endogenous levels of testosterone and estradiol. Histological analyses of female and male gonads showed a concentration dependent decrease of oocyte and spermatozoon density, with a reduction in the number of oocytes per follicle of 40% to 70%, and spermatozoa density of 21% to 25%, relative to controls, following exposure to 20 and 200 ng/l of fluoxetine for 6 days, respectively. There was also a significant increase (1.5-fold) in the endogenous levels of esterified estradiol in organisms exposed to 200 ng/l fluoxetine. Overall, the study shows that exposure to low levels of fluoxetine may effectively induce gamete liberation in the zebra mussel as well as alter endogenous levels of estradiol, and evidences the need of further investigating the potential of fluoxetine to alter the endocrine system of molluscs at environmentally relevant concentrations.

Keywords: Fluoxetine; spawning; zebra mussel; estradiol esters; oocytes; spermatozoa.

1. Introduction

In recent years an increased occurrence of pharmaceuticals have been reported in surface waters, seawater, effluents from municipal wastewater treatment facilities and even groundwater (Fent et al., 2006). A growing body of literature has emerged describing the potential of these compounds to adversely affect aquatic organisms. The main danger of pharmaceuticals arises from their environmental persistence and high bioactivity (Daughton and Ternes, 1999; Bringolf et al., 2010). Moreover, its continuous influx into the aquatic environment results in chronic exposure of aquatic organisms, especially of those residing in effluent-dominated ecosystems (Brooks et al., 2006). Of special concern are drugs which, even at low environmental concentrations, have a negative effect on the nervous or endocrine system of exposed organisms. One of the most studied is ethinylestradiol, an endocrine disrupter that produces adverse effects on fish, gastropod and mussel populations at concentrations between 1 and 50 ng/l (Jobling et al., 2004; Ciocan et al., 2010; Cubero-Leon et al., 2010).

Other pharmaceuticals of special environmental concern are the selective serotonin reuptake inhibitors (SSRIs), including fluoxetine, the active compound in Prozac®. Fluoxetine, like other SSRIs, is a high-prescription-volume drug in the United States and several other countries, used for the treatment of depression and certain compulsive disorders (RxList, 2009). As a consequence, many industrialized countries with large metropolitan areas have detectable quantities of SSRIs and their metabolites in their surface waters (Kwon and Armbrust, 2006). Fluoxetine is metabolized in the human body to norfluoxetine glucuronide and it is primarily excreted via urine containing approximately 2–11% of the administered dose as unchanged parent compound (Hiemke and Härtter, 2000). Kolpin et al. (2002) reported concentrations of fluoxetine in streams of the United States as high as 12 ng/l while other sources mention

77 concentrations up to 99 ng/l in sewage effluents in Canada (Metcalfe et al., 2003), as
78 well as in brain tissue samples of fish (1.58 ± 0.74 ng/g) found in an effluent-dominated
79 stream in North Texas, United States (Brooks et al., 2005). Fluoxetine has also been
80 detected in biosolids and sediments in the United States at average concentrations of
81 37.4 and 1.84 µg/kg, respectively (Furlong et al., 2004). Even though SSRIs and their
82 metabolites are usually found in low concentrations (ng/l) in the aquatic environment,
83 their biological effects at relevant environmental concentrations have seldom been
84 assessed on aquatic organisms.

85 SSRIs block the reuptake of serotonin (5-HT) from the pre-synaptic nerve cleft
86 resulting in an increased 5-HT neurotransmission in humans. As such, they can mimic
87 the action of 5-HT (Brooks et al., 2003). Exposure of goldfish (*Carassius auratus*) to
88 fluoxetine (540 ng/l) lead to a disruption of its reproductive physiology and energy
89 metabolism, altering neuroendocrine hormones involved in steroidogenesis, spermiation
90 and carbohydrate metabolism (Mennigen et al., 2010a,b). *In vitro* studies or injection of
91 fluoxetine stimulated ovarian and testicular development and increased the size of
92 ovaries and oocytes in the decapod crustacean *Uca pugilator* (Kulkarni and Fingerman,
93 1992; Sarojini et al., 1993). Such findings suggest that fluoxetine indirectly induced
94 gonad development and oocyte maturation in crustaceans, probably via a putative 5-HT
95 ovarian and testicular stimulating factor released from the thoracic ganglia. Recently,
96 De Lange et al. (2006) reported that 100 ng/l fluoxetine reduced the locomotion activity
97 of the amphipod *Gammarus pulex.* In bivalve molluscs, such as *Dreissena polymorpha*
98 and *Macoma balthica*, several reproductive events like spawning and parturition are
99 regulated by serotonin and can be induced or potentiated by exogenous administration
100 of SSRIs (Fong, 1998; Fong et al., 1998; Honkoop et al., 1999). Fong (1998) reported
101 that water fluoxetine levels of 34 to 340 µg/l induced spawning in male zebra mussels

D. polymorpha. In a later study, Fong et al. (2003) showed that SSRIs induced spawning in mussels and clams by increasing serotonin activity. In addition, there is evidence that fluoxetine at concentrations of 300 and 3000 µg/l induced the release of non-viable glochidia in the endangered freshwater naiad *Elliptio complanata* (Bringolf et al., 2010). As the reproductive cycle of this species includes an obligatory parasitic form (McMahon and Bogan, 2001), the viability of the glochidia is of crucial importance (Faria et al., 2010). Thus, a spawning inducing compound like fluoxetine may potentially interfere with the reproductive cycle of endangered species and significantly reduce their reproductive success.

Nevertheless the above mentioned studies conducted in bivalves, despite of providing some mechanistic information on the mode of action of SSRIs, were not performed at realistic environmental concentrations. Likewise in fish and crustaceans, SSRIs may also disrupt the reproductive axis in sexually mature bivalves (Kulkarni and Fingerman, 1992; Sarojini et al., 1993; Mennigen et al., 2010a,b) which share similar steroidogenic pathways with vertebrates (Porte et al., 2006). Thus, 17β-estradiol for example is involved in the regulation of the immune response (Canesi et al., 2004), the induction of Ca^{2+}-dependent NO production through activation of a signalling pathway at the cell surface (Stefano et al., 2003), and the modulation of the lysosomal function, as well as lipid and glucose metabolism in mussel hepatopancreas (Canesi et al., 2007). It has also been suggested that 17β-estradiol may play an important role in reproductive events and sexual maturation and differentiation in bivalve molluscs (Janer et al., 2005; Gauthier-Clerc et al., 2006; Croll and Wang, 2007). In addition, there is evidence that mussels possess the ability to maintain stable levels of endogenous estradiol through its conjugation with fatty acids, a metabolic process that considerably reduces the biological activity of free steroids (Janer et al., 2005).

This study aimed at determining the potential effects of exposure to low levels of fluoxetine (20-200 ng/l) on female and male gonad structure and gamete release as well as on the levels of endogenous steroids (testosterone and estradiol) in the freshwater mussel *D. polymorpha*.

2. Material and methods

2.1 Sample collection

Zebra mussels (*D. polymorpha*) mean weight: 0.3 ± 0.05 g; mean length: 22 ± 1 cm were collected in April 2008 from the Ebro River, from an unpolluted site near the town of Riba Roja (Catalonia, Spain). Animals were transported in buckets of approx. 40 L filled with local water to the animal-holding facilities (IDAEA, Barcelona) and kept in 20 L tanks at 20 ± 1 °C. Before exposure, mussels were acclimatized for a period of 10 days to ASTM reconstituted water of similar hardness (170 mg/l $CaCO_3$) and conductivity (600 µS/cm) as local Ebro River water.

2.2 Experimental design

Environmental conditions, i.e. temperature, conductivity of ASTM water and photo-period simulated the original conditions of mussels. Animals were fed daily with a suspension 1:1 of algae *Scenedesmus subspicatus* and *Chlorella vulgaris* (10^6 cells/ml). Water was renewed every day. After acclimatization, ~400 mussels were selected for the experiments. They were placed on fishing nets suspended in 20 L glass aquaria (~90 individuals per aquaria). Dissolved oxygen was maintained constant by continuous aeration using filtered compressed air through glass diffusers.

151 Mussels were exposed to different concentrations of fluoxetine (20 and 200 ng/l)
152 for 6-days added to the rearing water. There were two sets of controls: non-exposed
153 mussels (C) and mussels exposed to 0.002% triethylene glycol, used as a carrier solvent
154 (SC). Water was changed daily and a fresh dose of fluoxetine was added; mussels were
155 fed every 48 hours adding food 15-30 min before water renewal. After 6-day exposure,
156 mussels were dissected, the gills separated from the whole body and stored at −80 ºC.
157 For histology, 20 organisms per exposure group were dissected, placed into cassettes
158 and fixed in 4% buffered formaldehyde (0.1 M phosphate buffer, pH 7.4) for 48 h.
159 Samples were subsequently rinsed with water and stored in 70% ethanol. Prior to
160 paraffin embedding, individuals were transversally cut with a scalpel and both the
161 byssus and the foot removed since they could further interfere during the sectioning
162 process. Both portions were then embedded in paraffin, cut at 7 μm and stained with
163 Hematoxylin-Eosin Y.
164
165 *2.3 Analysis of tissue steroid levels*
166 Tissue levels of free testosterone and estradiol were analyzed as described in
167 Janer et al. (2005). Briefly, tissue samples (0.25 - 0.35 g wet weight; n=12) were
168 homogenized in ethanol, and frozen overnight at −80 °C. Homogenates were then
169 thawed and extracted with 2 ml of ethyl acetate (×3), the organic extracts recombined
170 and reduced under a nitrogen stream. Dry residues were redisolved in 80% methanol.
171 This solution was then washed with petroleum ether to remove the lipid fraction and
172 evaporated to dryness. The dry residue was redisolved in 4 ml Milli-Q water and passed
173 through a C18 cartridge (Isolute, International Sorbent Technology, Mid Glamorgan,
174 UK; 1 g, 6 ml), that had been sequentially pre-conditioned with methanol (4 ml) and
175 Milli-Q water (8 ml). After finishing the concentration step, cartridges were washed

with Milli-Q water (8 ml), dried and connected to a NH_2 cartridge (Sep-Pack® Plus; Waters, Milfold, MA, USA). The C18-NH_2 system was then washed with 8 ml n-hexane and the steroids eluted with 9 ml dichloromethane: methanol (7:3). This fraction was collected and evaporated to dryness.

Total testosterone and estradiol (free + esterified) were measured as described by Gooding et al. (2003), with some modifications. Tissue, homogenized as for free steroid determination (see above), was extracted with ethyl acetate (3×2 ml). The organic extract was evaporated under nitrogen, resuspended in 1.0 ml methanol containing 1% KOH, and incubated at 45 °C for 3 h. After the saponification step, Milli-Q water (4.0 ml) was added, and the sample extracted with dichloromethane (3×3 ml).

The efficiency of the extraction and delipidation procedure was 74 ± 3% for testosterone and 80 ± 3% for estradiol (Morcillo et al., 1999). The recovery for the purification step (SPE cartridges), evaluated with radiolabelled steroids was in the range 95 – 97% for both testosterone and estradiol (Janer et al., 2005). Dry extracts (tissue and water samples) were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine, and assayed for testosterone and estradiol concentration using commercial RIA kits (Radim, Rome, Italy). Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection were 25 pg/g for testosterone (T) and 20 pg/g for estradiol (E2). Intra-assay coefficients of variation were of 6.1% (T) and 3.3% (E2). Inter-assay coefficients of variation were of 9.3% (T) and 3.5% (E2).

2.4 Quantitative histology using image processing techniques

Histological preparations were first examined under a light microscope to determine sex and gonadal maturation stage following conventional histological

procedures according to the method described by Juhel et al. (2003). For quantitative histological analysis only individuals in the spawning stage were selected, due to their high incidence (85-90%). Other stages of maturation were not considered in this study due to their low presence (10-15%) and thus the difficulty to make a separate and accurate evaluation. All tissue images were captured using a Digital Sight-Ri1 (Nikon) digital camera mounted on an Eclipse 90i (Nikon) light microscope. A photo ocular lens of 10x was used with an objective of either 4x (females) or 10x (males) resulting in a magnification of 40x and 100x, respectively. The image analysis methods used in this study are adapted from those already described by Arimoto and Feng (1983) and Heffernan and Walker (1989) as a sensitive assessment of the gonadal gamete content in mussels with modifications aimed to improve accuracy and reproducibility. Gonadal gamete content was assessed in the follicle (females) and tubule lumen (males), and only follicles/tubules with a clearly distinguishable wall were selected. Gamete density in female gonads was analyzed as the number of oocytes per follicle area (μm^2) of 10 follicles in three randomly chosen sections per individual gonad (30 follicles per individual). Follicle area was determined with the aid of image analysis software (NIS-Elements AR ver. 3.0, Laboratory Imaging, Nikon). Male gonads were analyzed using ImageJ image analyzing software, using a modification of the protocol described by Toro et al. (2002). Briefly, color images were transformed to 8-bit grey scale images and pictures were then thresholded individually. Tubules were selected and the spermatozoa amount (% inside the tubule lumen) calculated as the ratio between the number of pixels occupied by spermatozoa and the total number of pixels of the tubule area. For each male a total of 10 tubules were selected from 3 randomly chosen sections of the gonad.

226 *2.5 Water analyses*

227 Duplicated water samples were taken from the freshly prepared solutions and
228 after 24 hour exposure to measure oxygen levels, pH and fluoxetine concentrations in
229 water. Dissolved oxygen concentration (DO) was measured using a polarographic
230 oxygen electrode coupled to a CyberScan DO 300 EUTECH model meter (Lab Process
231 Distributions, Alella, Barcelona, Spain). Water pH was measured using an epoxy-body
232 combination electrode, coupled to a Crison micro pH 2001 meter and calibrated with
233 standard pH buffer solutions (Sigma, Madrid, Spain). Measurement of fluoxetine levels
234 was limited to the highest tested concentration (200 ng/l) as follows: 1000 ml of water
235 were pre-concentrated in Oasis 60 mg SPE cartridges which were conditioned with 10
236 ml of methanol followed by 10 ml of water; water was pre-concentrated at a flow rate of
237 10 ml/min and eluted with 2x4 ml of methanol. This extract was then reduced under
238 nitrogen to incipient dryness and reconstituted with 500 µl of the mobile phase.
239 Fluoxetine was analyzed by High Performance Liquid Chromatography coupled to
240 Diode Array Detection (HPLC-DAD) with an Agilent Technologies 1200 series diode
241 array detector G1315D; 50 µl of the extract were injected on a 50 x 4.6 mm XBridge
242 TM C18 column of 3.5 µm particle diameter (Waters, USA). A gradient elution starting
243 at 70:30 water (pH 4.0) acetonitrile to 100% acetonitrile in 20 min was used at a flow
244 rate of 0.8 ml/min. Acquisition was done at a wavelength of 226 nm. Under those
245 conditions, fluoxetine eluted at 4.15 min and had recoveries from water samples
246 determined using an external standard of 99 ± 3% (n=4).

247

248 *2.6 Statistical analyses*

249 Differences in steroid levels were determined by one-way ANOVA, followed by
250 Tukey's post-hoc multiple comparison tests ($P<0.05$). Significant differences in the

number of oocytes per follicle area and spermatozoa density of tubules across treatments were analyzed using a nested one-way ANOVA with individual mussels nested within treatments, followed by Tukey's post-hoc multiple comparison test ($P<0.05$). Prior to all analyses requirements for parametric testing were determined and if not met data was log transformed to improve normality and homoscedasticity of variance.

3. Results

3.1 Water analyses

During the entire experiment, oxygen levels were 90% of saturation and pH was within 7.8 - 8.1. Measured fluoxetine levels in freshly prepared water were close to nominal values (mean ± SEM, n=4) 180 ± 30 ng/l, decreasing to 110 ± 60 ng/l after 24 h exposure.

3.2 Histological analyses

Of the 80 analyzed organisms, 50 were females and 30 males. Most individuals were found in the spawning stage (stage 3). Within females 87% of individuals from the control (C), solvent control (SC) and 20 ng/l fluoxetine treatments, and 100% of those exposed to 200 ng/l of fluoxetine had gonads in stage 3. Within males 86%, 88%, 83% and 78% of individuals from the C, SC, 20 ng/l and 200 ng/l fluoxetine treatments had gonads in stage 3, respectively (Fig. 1). Female gonads were characterized by having the entire peripheral area and parts of the central lumen of the follicle occupied by mature oocytes (Fig. 1A). In addition a few pedunculated oocytes were located at the walls of the pre-spawning follicles. The connective tissue was almost completely

reduced and formed a discontinuous layer surrounding the follicles. Male gonads were characterized by a high amount of spermatozoa with visible tails closely packed in the center of the star-shaped lumen of the seminiferous tubules. Larger precursor germ cells (spermatocytes) were located at the periphery while a thin layer of connective tissue served as separation between adjacent tubules (Fig. 1C).

Exposure to fluoxetine dramatically decreased the number of oocytes per follicle and spermatozoa density in tubules (Fig. 1B, D). In exposed females, the central lumen was mostly empty, only a few residual oocytes were found at the periphery of the follicles (Fig. 1B). Exposed males showed a reduction in the number of spermatozoa in the center of the lumen concomitant with a thickening of the connective tissue layer (Fig. 1D). Also the typical star shaped alignment of the spermatozoa in the center of the lumen was much less apparent in males exposed to fluoxetine. In female gonads, nested one-way ANOVA results performed on 1200 follicles indicated that despite the existence of significant ($P<0.05$) differences among individual mussels within treatments ($F_{38, 1158} = 13.9$), fluoxetine exposure significantly ($F_{3, 42} = 30.4$, $P<0.05$) affected the number of oocytes per follicle area. Results depicted in Fig. 2A clearly showed decreasing numbers of oocytes per follicle with increasing exposure to fluoxetine. The decrease in the number of oocytes per follicle area was approximately 40% and 70% for 20 and 200 ng/l fluoxetine, respectively, compared to the control group. In testes, nested one-way ANOVA results performed on 250 tubules indicated also the existence of significant ($P<0.05$) differences among individual mussels within treatments ($F_{21, 225} = 25.7$), but fluoxetine exposure levels significantly ($F_{3, 21} = 12.4$, $P<0.05$) reduced the tubule area covered by spermatozoa (Fig. 2B). The decrease in spermatozoa density was of 21% and 25% for 20 and 200 ng/l fluoxetine, respectively.

301 *3.3 Tissue steroid levels*

302 Steroid levels were measured in the soft tissue of control and fluoxetine exposed
303 organisms. Levels of free/unconjugated testosterone were in the range of 0.08 - 0.14
304 ng/g wet weight, and levels of free estradiol were of 0.04 - 0.10 ng/g wet weight. No
305 significant ($P<0.05$) differences were observed between control and exposed organisms
306 (Fig. 3A). After saponification of the samples, the ester bonds of the steroid metabolites
307 were cleaved and total steroids (including both free and esterified forms) were
308 determined. Esterified steroids were determined by subtraction of free steroids from
309 total steroid content. Both testosterone and estradiol were predominately found in their
310 esterified form (97.6% to 98.9%). Esterified testosterone was in the range of 3.39 - 4.38
311 ng/g wet weight and esterified estradiol ranged from 5.77 to 12.79 ng/g wet weight.
312 After six day exposure, esterified estradiol levels increased significantly ($P<0.05$) 1.5
313 fold in the group exposed to 200 ng/l fluoxetine. Interestingly, no alterations were
314 detected in esterified testosterone levels (Fig. 3B).

315

316 **4. Discussion**

317 The histological analyses of fluoxetine-exposed zebra mussel gonads clearly
318 showed a significant decrease in the number of oocytes within the follicles and of
319 spermatozoa density within the male seminiferous tubules, suggesting the ability of
320 fluoxetine to induce spawning at rather low concentrations (20-200 ng/l). The
321 apparently lower gamete decreases reported for males than for females (Fig. 2) is related
322 to the fact that mature testes include simultaneously several types of germinal cells
323 (spermatocytes and spermatozoa) and that mainly spermatozoa located in the center of
324 the star-shaped lumen of seminiferous tubules were released after exposure to fluoxetine
325 as depicted in Fig. 1. Unfortunately due to the small size and huge number of

326 spermatozoa it was impossible to count them. Instead changes in germinal cell density
327 were evaluated. On the contrary, mature ovarian mostly had mature oocytes that were
328 released after fluoxetine exposure (Fig. 1). Fong (1998) reported that fluoxetine at
329 concentrations of 34 and 340 µg/l induced spawning in male and female zebra mussel in
330 just a few hours. In the present study, we demonstrated that spawning may be inducible
331 at even much lower fluoxetine concentrations following exposure for a period of several
332 days. The observed decrease in the number of gametes in the gonads of exposed
333 organisms may be caused by a cumulative effect of the compound directly on the
334 gonads or through the accumulation of fluoxetine also in other peripheral tissues other
335 than the gonad. The concentration of fluoxetine in water markedly decreased after 24
336 hours of exposure, suggesting an uptake of the compound by the test organisms. Several
337 studies performed on different aquatic organisms indicated that fluoxetine
338 bioaccumulates in significant amounts and that such accumulation is pH dependent.
339 Estimated bioaccumulation factors of fluoxetine at neutral pH ranged from 80 to 3100
340 in the Japanese medaka *Oryzias latipes* (Nakamura et al., 2008; Paterson and Metcalfe,
341 2008). Bringolf et al. (2010) reported bioconcentration factors of fluoxetine over 1000
342 in the freshwater mussel *Elliptio complanata* inhabiting a contaminated effluent
343 channel.

344 Testosterone and estradiol extracted from the whole body of *D. polymorpha*
345 were predominantly found in their esterified form, agreeing with previous results
346 reported for other molluscs (Gooding and LeBlanc, 2004; Janer et al., 2006). Our
347 experiment showed that short term exposure to fluoxetine did not significantly alter free
348 steroid levels, but led to a significant increase (1.5 fold) in esterified estradiol in zebra
349 mussels exposed to the highest fluoxetine concentration (200 ng/l). The mechanism
350 behind the observed increase in esterified estradiol is unknown. Generally, esterification

of fatty acids significantly increases their lipophilicity, so that they can be stored in the lipoidal matrices of the organism while concurrently reducing their bioactivity and bioavailability (Borg et al., 1995). Interestingly, we observed levels of esterified estradiol of 8-14 ng/g w.w. and testosterone 3-8 ng/g w.w. in zebra mussel during the spawning stage, followed by a significant increase to 38 ng/g of esterified estradiol and 14 ng/g of esterified testosterone after the spawning phase, when gonads are in a reabsorbing stage (Lazzara et al., unpublished results). Thus, it is likely that a stimulation of gamete release by fluoxetine will consequently modulate levels of endogenous steroids; however, only estradiol was modulated whereas no effect on testosterone was detected.

Several studies have shown that serotonergic agents can modify estrogen titers and *vice versa* (Maswood et al., 1999; Raap et al., 2000). Rats injected with fluoxetine revealed that concentrations of 0.5 - 5 mg/kg may significantly alter circulating estrogen levels (Taylor et al., 2004). Moreover estradiol titers increased in Japanese medaka exposed to 0.1 and 0.5 mg/l fluoxetine in water, whereas testosterone levels remained unaffected (Foran et al., 2004). The mechanisms involved in the modulation of estradiol levels by fluoxetine are unclear, but Rehavi et al. (2000) speculated that in rats SSRIs may inhibit the release of the luteinizing hormone which results in decreased ovarian release of estrogens in females.

Moreover, fluoxetine can mimic the action of 5-HT which is implicated in different functions in both vertebrates and invertebrates. 5-HT regulates several biological functions, like heartbeat rhythm, feeding, and locomotion in the gastropod mollusc *Ilyanassa obsoleta* (Couper and Leise, 1996) and spawning and oocyte maturation in the bivalves *Spisula solidissima* and *S. sachalinensis* (Hirai et al., 1988). Interestingly, 5-HT receptors were found on the membrane of oocytes and spermatozoa

of the clam *Spisula solidissima* and were involved in the release of gametes (Kadam et al., 1991). These receptors are regulated by 17β-estradiol which may potentiate 5-HT induced gamete release through increasing receptor sensitivity (Osada et al., 1998; Croll and Wang, 2007). However, it is unclear whether SSRIs directly increase 5-HT levels in bivalves or act as 5-HT receptor ligands (Fong et al., 2003). Nevertheless, an increased stimulation of 5-HT receptors could be to some extent counteracted by an increased esterification of estradiol, which would lead to reduced receptor sensitivity. Thus, one may hypothesize that the observed increase in esterified estradiol levels without a concomitant increase in esterified testosterone titers, would act as a mechanism of defense against an increased stimulation of 5-HT receptors by fluoxetine.

Overall, this study showed a concentration-dependent increase in gamete release in both females and males under fluoxetine exposure. This confirms the role of fluoxetine as a spawning inducer in zebra mussel, as reported in earlier studies with much higher fluoxetine concentrations. In the present study, spawning occurred at concentrations of fluoxetine as low as 20 ng/l. Concentrations of fluoxetine of 99-841 ng/l in waste water treated effluents and of 12-30 ng/l in contaminated surface waters have often been reported (Corcoran et al., 2010). Therefore, it is likely that exposure to fluoxetine in the low parts-per–trillion levels range may have a negative impact on zebra mussel reproduction since in this species fertilization is external and hence spawning has to be highly synchronized because of the short viability of gametes in water (Newell et al., 1982, Ram et al., 1996; Juhel et al., 2003). Thus, this study provides additional evidence that environmentally relevant levels of pharmaceuticals such as fluoxetine are biological active in aquatic organisms (Mennigen et al., 2010a, b). In particular, the present results showed that at ng/l, fluoxetine was able to alter the steroid metabolism and reproduction of mussels.

401

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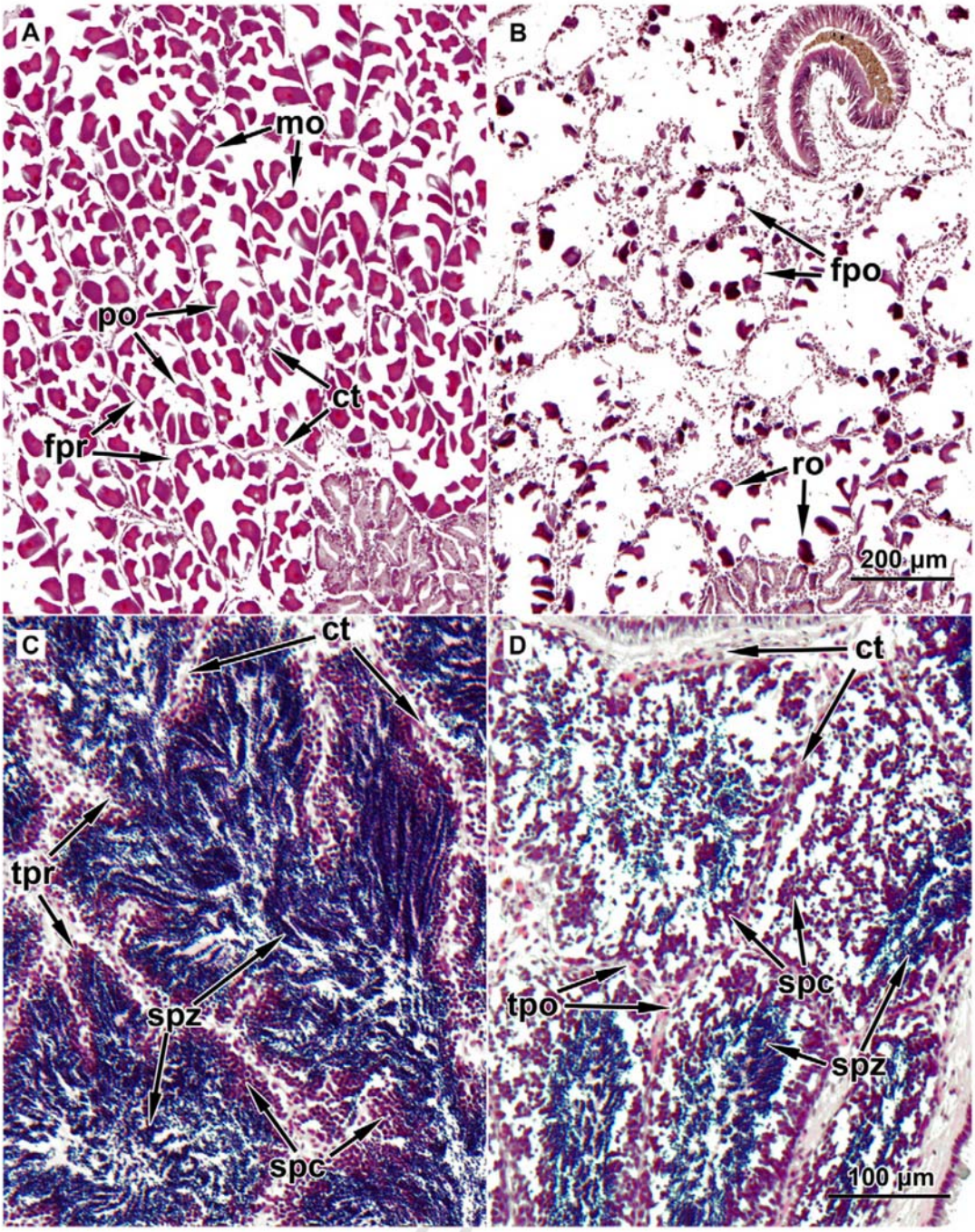
577 Figure 1. Photomicrographs of male and female zebra mussel (*D. polymorpha*) gonads
578 in control and fluoxetine-exposed treatments (200 ng/l). (A) Ovaries of a control female
579 showing follicles filled with mature oocytes; (B) Fluoxetine-exposed female ovaries
580 with mostly empty central lumen and only residual oocytes at the periphery. (C) Control
581 male testes characterized by a dense amount of spermatozoa visible in the center of the
582 star-shaped lumen of the seminiferous tubules; (D) Testes of fluoxetine-exposed male
583 exhibiting the presence of a less dense amount of spermatozoa especially in the center
584 of the lumen. Abbreviations: ct, connective tissue; fpo, follicle post-spawning; fpr,
585 follicle pre-spawning; mo, mature oocyte; po, pedunculated oocyte; ro, residual oocyte;
586 spc, spermatocytes; spz, spermatozoa; tpo, tubule post-spawning; tpr, tubule pre-
587 spawning. Scale bar in B applies to photos of female gonads and D to male gonads.
588
589 Figure 2. Number of oocytes per follicle area in female gonads (A) and of spermatozoa
590 density (% of tubule area covered by spermatozoa) in male gonads (B) of zebra mussels
591 exposed to fluoxetine (20-200 ng/l) for 6 days. Different letters indicate significant
592 ($P<0.05$) differences after nested ANOVA and post-hoc Tukey's test. C, SC are control
593 and solvent control treatments, respectively. Values are mean ± SEM (n=7-11).
594
595 Figure 3. Free and esterified levels of testosterone and estradiol determined in the whole
596 body (without gills) of *D. polymorpha* exposed to fluoxetine (20-200 ng/l) for 6 days.
597 Values are mean ± SEM (n=12). C, SC are control and solvent control treatments,
598 respectively. Different letters indicate significant ($P<0.05$) differences after ANOVA
599 and post-hoc Tukey's test.

600 Figure 1



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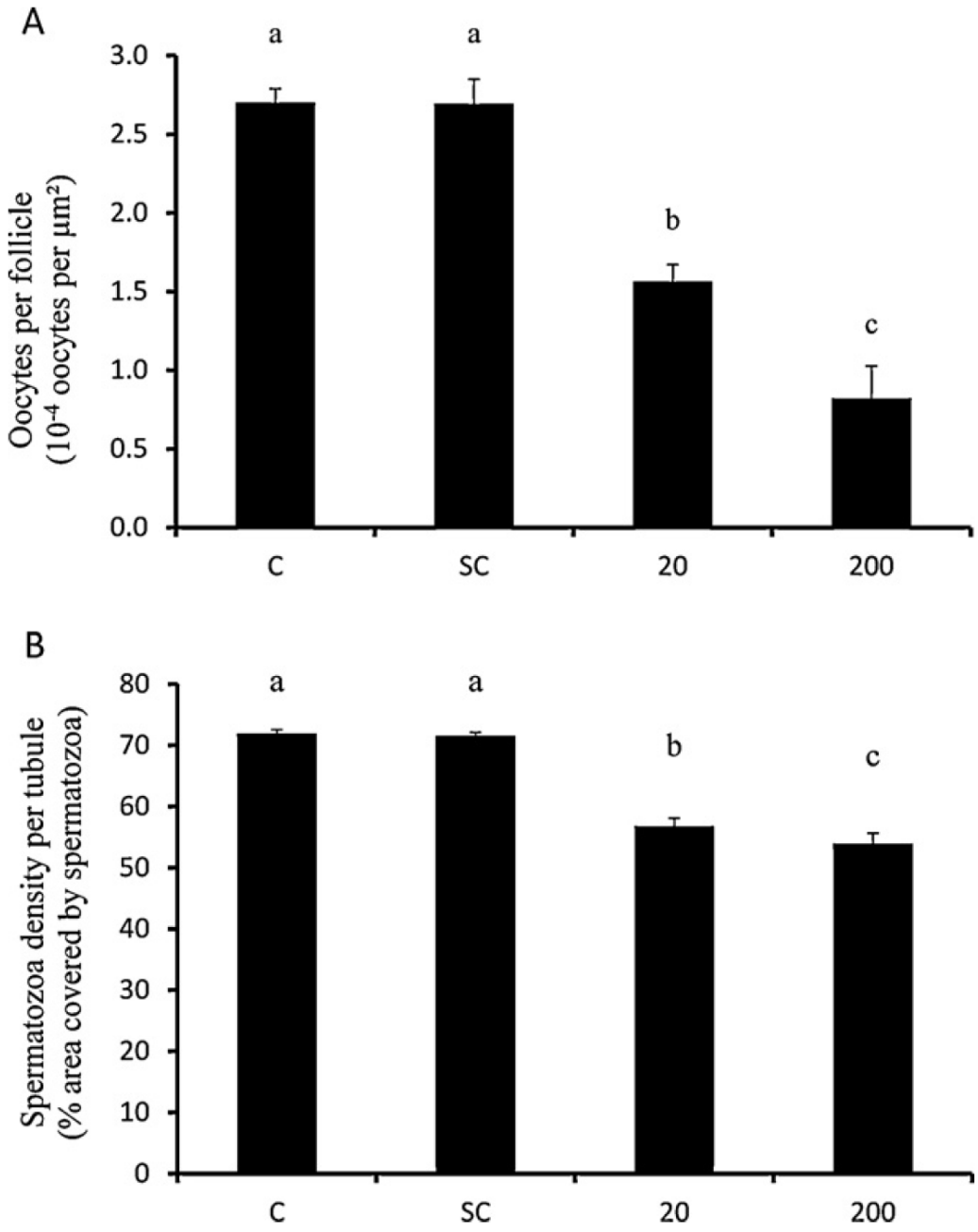
602 Figure 2



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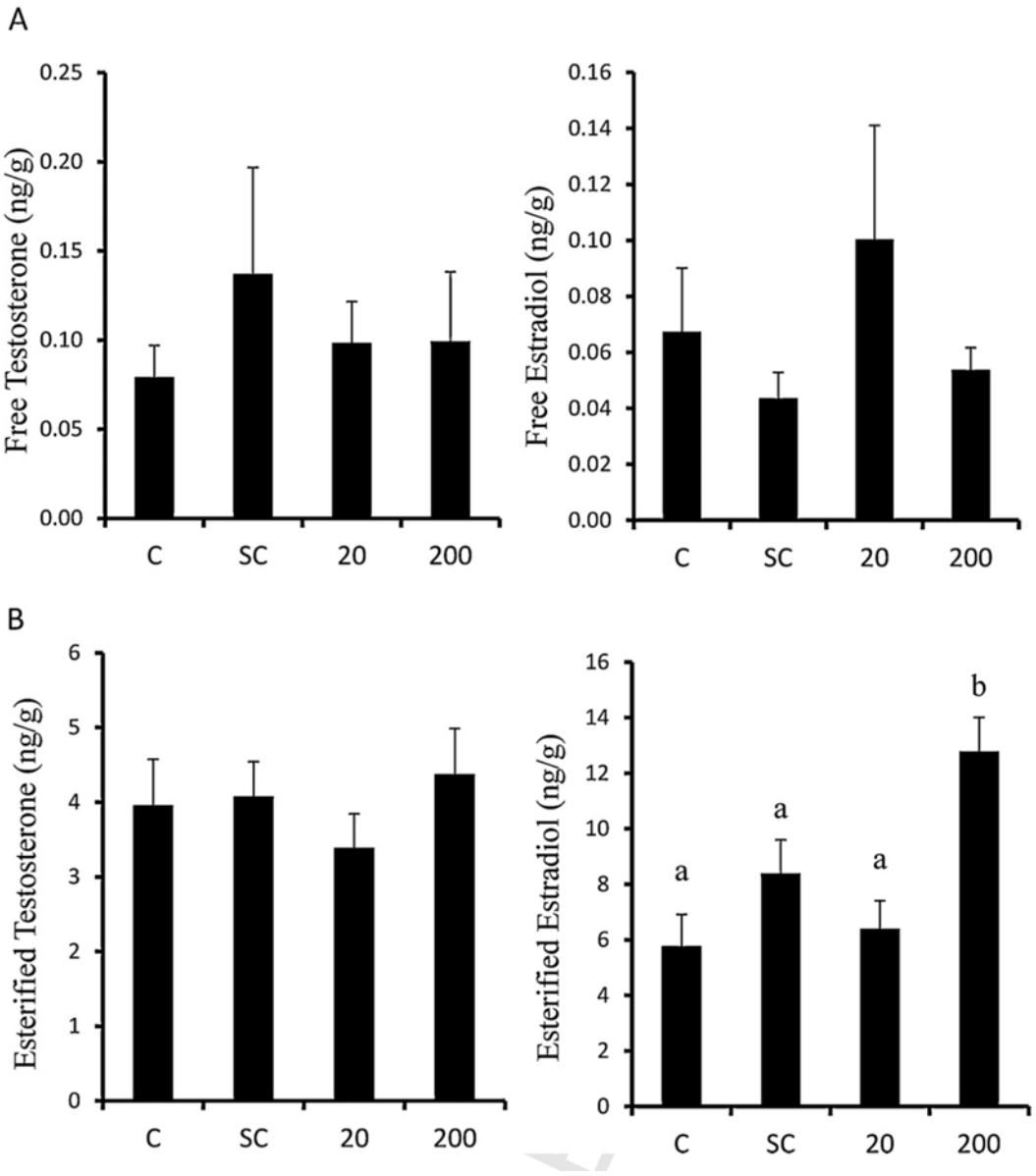
604 Figure 3



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