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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 spermatozoan density, with a reduction in the number of oocytes per follicle of 40–70%, and spermatozoan density of 21–25%, 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 level 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.

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, their 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 concentrations up to 99 ng/l in sewage effluents in Canada (Metcalfe et al., 2003). Brain tissue samples of fish had 1.58 ± 0.74 ng/g fluoxetine in an effluent-dominated stream in North Texas, United States (Brooks et al., 2005). Fluoxetine has also been detected in biosolids and sediments in the United States at average concentrations of 37.4 and 1.84 μg/kg,

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respectively (Furlong et al., 2004). Even though SSRIs and their metabolites are usually found in low concentrations (ng/l) in the aquatic environment, their biological effects at relevant environmental concentrations have seldom been assessed on aquatic organisms.

SSRIs block the reuptake of serotonin (5-HT) from the pre-synaptic nerve cleft resulting in an increased 5-HT neurotransmission in humans. As such, they can mimic the action of 5-HT (Brooks et al., 2003). Exposure of goldfish (*Carassius auratus*) to fluoxetine (540 ng/l) lead to a disruption of its reproductive physiology and energy metabolism, altering neuroendocrine hormones involved in steroidogenesis, spermiation and carbohydrate metabolism (Mennigen et al., 2010a,b). *In vitro* studies or injection of fluoxetine stimulated ovarian and testicular development and increased the size of ovaries and oocytes in the decapod crustacean *Uca pugilator* (Kulkarni and Fingerman, 1992; Sarojini et al., 1993). Such findings suggest that fluoxetine indirectly induced gonad development and oocyte maturation in crustaceans, probably via a putative 5-HT resembling ovarian and testicular stimulating factor released from the thoracic ganglia. Recently, De Lange et al. (2006) reported that 100 ng/l fluoxetine reduced the locomotion activity of the amphipod *Gammarus pulex.* In bivalve molluscs, such as *Dreissena polymorpha* and *Macoma balthica*, several reproductive events like spawning and parturition are regulated by serotonin and can be induced or potentiated by exogenous administration of SSRIs (Fong, 1998; Fong et al., 1998; Honkoop et al., 1999). Fong (1998) reported that water fluoxetine levels of 34–340 μg/l induced spawning in male zebra mussels of *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 providing some mechanistic information on the mode of action of SSRIs, were not performed at realistic environmental concentrations. As 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 of 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. Materials and methods

2.1. Sample collection

Zebra mussels (*D. polymorpha*) with mean weight of 0.3 ± 0.05 g and mean length of 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 photoperiod simulated the original conditions of mussels. Animals were fed daily with a 1:1 suspension 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.

Mussels were exposed to different concentrations of fluoxetine (20 and 200 ng/l), added to the rearing water, for 6 days. There were two sets of controls: non-exposed mussels (C) and mussels exposed to 0.002% triethylene glycol, used as a carrier solvent (SC). Water was changed daily and a fresh dose of fluoxetine was added; mussels were fed every 48 h adding food 15–30 min before water renewal. After the 6-day exposure, mussels were dissected, the gills separated from the whole body and stored at −80 °C. For histology, 20 organisms per exposure group were dissected, placed into cassettes and fixed in 4% buffered formaldehyde (0.1 M phosphate buffer, pH 7.4) for 48 h. Samples were subsequently rinsed with water and stored in 70% ethanol. Prior to paraffin embedding, individuals were transversally cut with a scalpel and both the byssus and the foot removed since they could further interfere during the sectioning process. Both portions were then embedded in paraffin, cut at 7 μm thick sections and stained with Hematoxylin–Eosin Y.

2.3. Analysis of tissue steroid levels

Tissue levels of free testosterone and estradiol were analyzed as described in Janer et al. (2005). Briefly, tissue samples (0.25–0.35 g wet weight; $n = 12$) were homogenized in ethanol, and frozen overnight at −80 °C. Homogenates were then thawed and extracted with 2 ml of ethyl acetate (3×), the organic extracts recombined and reduced under a nitrogen stream. Dry residues were redissolved in 80% methanol. This solution was then washed with petroleum ether to remove the lipid fraction and evaporated to dryness. The dry residue was redissolved in 4 ml Milli-Q water and passed through a C18 cartridge (Isolute, International Sorbent Technology, Mid Glamorgan, UK; 1 g, 6 ml), that had been sequentially pre-conditioned with methanol (4 ml) and 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