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Norfluoxetine Induces Spawning and Parturition in Estuarine and Freshwater Bivalves

P. P. Fong · N. Molnar

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Abstract Fluoxetine, a commonly prescribed antidepressant (Prozac), has been detected in sewage effluent. Its active metabolite norfluoxetine is more potent and has been detected in sewage influent and in fish tissues. We tested the effects of norfluoxetine on spawning and parturition in bivalves. Norfluoxetine induced significant spawning in zebra mussels and dark false mussels at concentrations as low as 5 μM. Norfluoxetine induced significant parturition in fingernail clams at 10 μM. Fluoxetine also induced spawning in dark false mussels at concentrations as low as 100 nM. Implications for environmental impacts of norfluoxetine and fluoxetine on native and exotic bivalves are discussed.

Keywords Norfluoxetine · Fluoxetine · Spawning · Bivalves

It is now well established that pharmaceuticals and personal care products (PPCPs) are released into sewage treatment plants (STPs) and discharged in effluent into receiving streams, rivers, and lakes (Daughton 2004). Sources of PPCPs include human domestic use, hospitals, veterinary clinics, and livestock wastes (Daughton 2004). A growing body of literature has identified a large number of PPCPs released in sewage effluent (Kolpin et al. 2002), and in biosolids (Jones-Lepp and Stevens 2007).

One such PPCP, the commonly prescribed selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac) has been detected in streams at concentrations as high as 0.012 μg/L in the United States (Kolpin et al. 2002) and in sewage effluent as high as 0.099 μg/L in Canada (Metcalfe et al. 2003), as well as in tissues from fishes living in an effluent-dominated stream (Brooks et al. 2005; Ramirez et al. 2007). Serotonin mediates or modulates many behaviors in aquatic animals (Fong 1998). Because SSRIs modulate serotonergic systems by blocking reuptake at synapses (Fong et al. 1998), and due to the popularity of Prozac and other SSRIs as antidepressants (Sebastine and Wakeman 2003), several studies have focused on the potential impact of discharged SSRIs on the behavior, physiology, and survival of aquatic animals such as molluscs (Fong 1998; Fong et al. 1998), crustaceans (Henry et al. 2004), and fishes (Semsar et al. 2004). In humans, fluoxetine is metabolized to norfluoxetine by demethylation in the liver (Hiemke and Hartter 2000). About 20%–30% of the fluoxetine ingested by humans remains unchanged in the urine. The remainder is a mixture of metabolites, of which norfluoxetine is the most active (Hartke and Mutschler 1993). While norfluoxetine has not been detected in waters downstream of STPs (Metcalfe et al. 2003), it has been detected in sewage influent (Armbrust 2005) as well as in tissues of fishes living in a sewage effluent-dominated stream (Brooks et al. 2005; Ramirez et al. 2007). These authors found sunfish tissues containing both norfluoxetine and fluoxetine at concentrations as high as 10.27 and 1.58 ng/g, respectively. While norfluoxetine has been shown to be a more potent SSRI than fluoxetine itself (Hiemke and Hartter 2000), no experiments testing the effects of norfluoxetine on the behavior or physiology of aquatic organisms have been performed to date.

We tested the effects of norfluoxetine on spawning in zebra mussels and on parturition in fingernail clams, two reproductive processes known to be induced by SSRIs in

P. P. Fong (✉) · N. Molnar
Department of Biology, Gettysburg College, Gettysburg, PA 17325, USA
e-mail: pfong@gettysburg.edu

these species (Fong 1998; Fong et al. 1998). We also tested both norfluoxetine and fluoxetine on spawning in the estuarine dark false mussel, a bivalve that can live in a variety of aquatic habitats because of its tolerance to a wide range of salinities (Laine et al. 2006). Both zebra mussels and dark false mussels are biofouling pest species in Europe (Verween et al. 2005), thus their exposure to reproduction-activating chemicals flowing downstream from STPs could have important effects on their distribution and abundance, and possibly enhance their biofouling capabilities.

Materials and Methods

Zebra mussels (*Dreissena polymorpha*, 1–3 cm shell length) were collected from shallow (<1 m) water in Lake Erie along the southern shore of Presque Isle, Pennsylvania, USA (42° 17′N, 80° 05′W) in June 2007. They were immediately transported to a field laboratory were experiments were performed at room temperature.

Dark false mussels (*Mytilopsis leucophaeata*, 10–15 mm shell length) were collected from pilings in the Magothy River on the western shore of Chesapeake Bay, Anne Arundel County, MD, USA (39° 05′N, 76° 30′W). Mussels were packed in a cooler and transported (1.5 h) to the lab at Gettysburg College where they were maintained in an aquarium with re-circulating water (7‰) in an environmental chamber at 15°C and 12:12 L:D., and fed powdered algae (Artemia Food, Ocean Star International, Snowville, UT, USA) every other day for the duration of the experiment (10 days).

Fingernail clams (*Sphaerium striatinum*, 7–10 mm shell length) were collected from Marsh Creek, Adams County, PA, USA (39° 50′N, 77° 17′W) in June, 2007. Clams were collected on the day of each experiment and tested within 2 h of collection.

All experiments were performed at room temperature in either 20 mL scintillation vials or 16-well culture plates (one animal per vial or well) with a final volume of 5.0 mL at room temperature (21°C). Zebra mussels were tested with norfluoxetine ranging from 100 nM–50 μM. Dark false mussels were tested with both norfluoxetine (100 nM–50 μM) and fluoxetine (10 nM–50 μM). Fingernail clams were tested with norfluoxetine (100 nM–10 μM). Both norfluoxetine and fluoxetine were purchased from Sigma Chemical Co. (St. Louis, MO., USA).

For experiments on zebra mussels and fingernail clams, drugs were dissolved in control lake or creek water. For experiments with dark false mussels, drugs were dissolved in control 7‰ artificial seawater Coralife, scientific grade marine salt, Energy Savers Unlimited, Inc., Carson, California, USA).

Initially, all animals were acclimated in 4.5 mL of control water for 20–30 min before addition of any drug. After the acclimation period, 0.5 mL of drug was added at a 10× higher concentration than the final concentration. Thus, all concentrations shown are nominal. Animals were observed for evidence of spawning (mussels) or parturition (fingernail clams). Questionable spawnings were confirmed by microscopic analysis of water. Experiments were run for four hours, after which all non-spawners or non-releasers were dissected and their gonads (mussels) or gills (fingernail clams) examined microscopically to determine sex and ability to spawn or give birth (Fong et al. 1998).

For all species tested, gender and reproductive maturity is impossible to determine prior to experiments, thus the number of animals of each gender in various experimental groups varied from experiment to experiment, but each group initially consisted of at least 12 mussels or fingernail clams. Results were analyzed statistically using Fisher's Exact Test and null hypotheses were rejected where $p < 0.05$.

Results and Discussion

Norfluoxetine induced spawning in zebra mussels at concentrations from 1–50 μM (Fig. 1). For males, significant differences in spawning between norfluoxetine and the control were observed at 5, 10, and 50 μM ($p = 0.0007$–0.005). For females, significant differences were observed at 10 μM ($p = 0.02$) and 50 μM ($p = 0.0001$). Similarly, in dark false mussels, norfluoxetine induced spawning in both sexes at concentrations from 1–50 μM (Fig. 2). However, only male mussels spawned at a significantly higher percentage than the control, at 5, 10, and 50 μM ($p = 0.0001$–0.004). Dark false mussels were more responsive to fluoxetine (Fig. 3). Statistically significant male spawning was recorded at concentrations as low as 100 nM as well as at higher concentrations ($p = 0.0001$–0.0004). Females spawned at concentrations as low as 500 nM, but significantly at 1 μM and higher ($p = 0.004$–0.0001). In fingernail clams, norfluoxetine induced statistically significant parturition only at 10 μM compared to the control ($p = 0.02$; Table 1).

The major finding of this study is that external exposure to norfluoxetine, the active metabolite of the antidepressant fluoxetine induces spawning in zebra mussels, dark false mussels, and induces parturition in fingernail clams. A second new result is that fluoxetine also induces spawning in dark false mussels, a euryhaline, biofouling pest. Finally, 10 μM norfluoxetine induced significant parturition in fingernail clams, *Sphaerium striatinum*. In previous laboratory experiments, fluoxetine (1–100 μM) showed no significant effect on parturition in fingernail clams (Fong

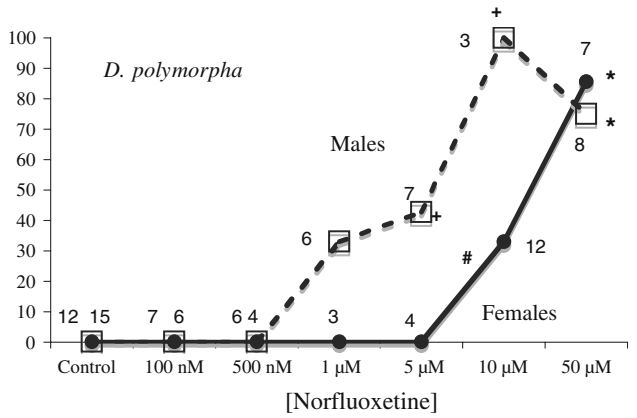

Fig. 1 Percent spawning of zebra mussels (*D. polymorpha*) in various concentrations of Norfluoxetine. Control: lake water. Sample sizes in each group are indicated adjacent to each symbol. $^{*}p = 0.0001$–0.0007, $^{+}p = 0.005$–0.02, $^{\#}p = 0.02$

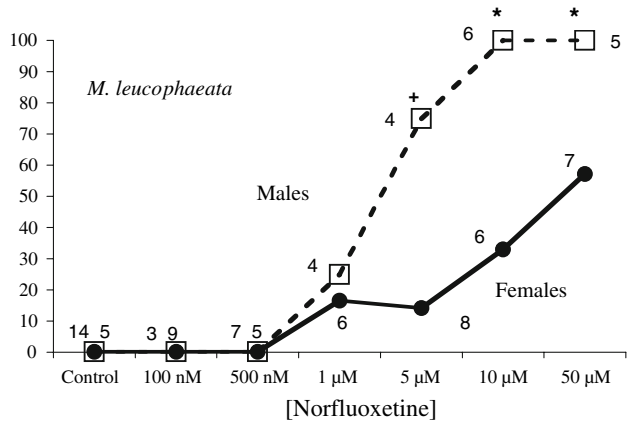

Fig. 2 Percent spawning of dark false mussels (*M. leucophaeata*) in various concentrations of Norfluoxetine. Control: 7‰ water. Sample sizes in each group as in Fig. 1. $^{*}p = 0.0001$, $^{+}p = 0.004$

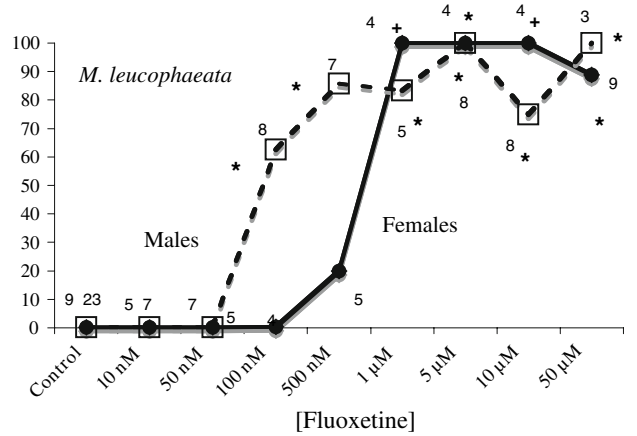

Fig. 3 Percent spawning of dark false mussels (*M. leucophaeata*) in various concentrations of Fluoxetine. Control: 7‰ water. Sample sizes in each group as in Fig. 1. $^{*}p = 0.0001$–0.0004, $^{+}p = 0.001$–0.004

Table 1 Number of *Sphaerium striatinum* which released juveniles/total exposed to norfluoxetine and serotonin (positive control)

Group	# Released/total
Control (creek water)	0/18
Norfluoxetine 10 µM	5/17*
Norfluoxetine 1 µM	1/11
Norfluoxetine 100 nM	0/13
Serotonin 1 mM	7/14**

* $p = 0.02$; ** $p = 0.001$

et al. 1998). Fluoxetine (5 µm) only potentiated serotonin-induced parturition at concentrations as low as 50 nM.

From these and previously published data (Fong 1998), fluoxetine induces spawning in zebra mussels and dark false mussels at a 10–20× lower concentration than norfluoxetine. The lowest concentration of norfluoxetine that induced spawning was 1 µM for both species, but fluoxetine produced an effect in dark false mussels at 100 nM in this study, and 50 nM in zebra mussels (Fong 1998).

The levels of SSRIs such as fluoxetine discharged in sewage effluent and its possible effects on the behavior of aquatic organisms has become the focus of recent studies (Henry et al. 2004; Brooks et al. 2005). While no study to date has detected norfluoxetine in sewage effluent, it has been detected in sewage influent (Armbrust 2005) and in fish tissues (Brooks et al. 2005). The present study shows that similar to its parent compound fluoxetine, norfluoxetine, too, can induce reproductive processes in bivalves.

The discharge of pharmaceuticals can affect reproductive behaviors of aquatic organisms and have important negative environmental effects. Induction of spawning in biofouling pest species like zebra mussels and dark false mussels could possibly increase the chances of successful fertilization by synchronizing spawning, and therefore enhance the spread of these exotic species. Furthermore, if spawning and parturition in native species is induced at the wrong time of the year, for example, when planktonic or benthic food for developing larvae and juveniles is limited, it could cause a higher percentage of early stage mortality. Several species of freshwater mussels (unionids) are endangered in North America due to habitat loss and smothering by exotic species like zebra mussels (Schloesser et al. 1996). This, combined with the recent finding that fluoxetine induces premature release of unionid glochidia (Heltsley et al. 2006) could have serious negative consequences for already endangered unionid populations.

While the drug concentrations necessary to evoke the observed responses are generally higher than environmental concentrations (and in the case of norfluoxetine, very little environmental data exists), the results of this study are still important since aquatic organisms are

exposed to a complex cocktail of pharmaceuticals that may have additive effects (Henry et al. 2004). In addition, the concentrations or effects of pharmaceuticals like fluoxetine, which have been shown to be stable in laboratory waters and persistent in laboratory sediments (Kwon and Armbrust 2006), may accumulate in benthic organisms (Seiler 2002).

Finally, an environmental risk assessment based upon the ratio of predicted environmental concentration and predicted no effect concentration (PEC/PNEC) has been generated for fluoxetine by Sebastine and Wakeman (2003). Their value of 14.2 for fluoxetine supports the contention that this drug may have environmentally important effects on aquatic organisms.

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