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Inhibition and sex specific induction of spawning by serotonergic ligands in the zebra mussel *Dreissena polymorpha* (Pallas)

P. P. Fong, J. Duncan and J. L. Ram

Department of Physiology, Wayne State University, Detroit (Michigan 48201, USA)
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Abstract. Serotonin (5-hydroxytryptamine, 5-HT) stimulates spawning in the zebra mussel (*Dreissena polymorpha*), a macrofouling European bivalve that has recently invaded North America. To develop methods of controlling zebra mussel spawning, two vertebrate serotonin antagonists, methiothepin and metergoline, known to bind with high affinity to snail 5-HT receptors, were tested for their ability to block 5-HT-induced spawning in zebra mussels. Methiothepin inhibited 5-HT-induced spawning at concentrations as low as 10^{-6} M. Metergoline (10^{-4} M) inhibited 5-HT-induced spawning; however, at lower concentrations (10^{-8} to 10^{-5} M), metergoline by itself significantly induced spawning in male, but not female zebra mussels. Metergoline (10^{-5} M)-induced male spawning was inhibited by 10^{-5} M methiothepin. Thus, methiothepin is the most effective inhibitor and metergoline the most powerful inducer of spawning yet tested in zebra mussels.
Key words. Zebra mussel; *Dreissena polymorpha*; serotonin; spawning; receptor; methiothepin; metergoline.

The zebra mussel, *Dreissena polymorpha* (Pallas), is ubiquitous in freshwater environments in Europe, and has recently been introduced accidentally into the Great Lakes region of North America[1]. It is a biofouling pest species that attaches with byssal threads to hard substrates, forming dense aggregations on water intakes, navigation buoys, and hard-shelled animals including each other. Furthermore, its high fecundity, planktonic larval form, and ability to attach to boats has enabled it to spread rapidly. It spread quickly from the Black Sea area throughout Europe after an extensive canal system was built in the nineteenth century[2], and in North America, it has spread to the Mississippi River basin and Hudson River watersheds in less than 10 years[3,4]. Increased knowledge about factors regulating its reproduction may be useful in developing methods to control or mitigate the impact of this bivalve.

Reproduction in bivalve molluscs may be regulated by a number of environmental and chemical factors. Temperature, photoperiod, salinity, and food availability have salient effects on the rate of gametogenesis, establishing conditions whereby spawning may occur[5-7]. Spawning itself may be under similar environmental control and many factors such as temperature[6,8-10], lunar periodicity[11,12], and phytoplankton blooms[13] acting alone or synergistically may contribute to its occurrence. In some species, chemical signals may initiate synchronized spawning. The biogenic monoamine serotonin (5-hydroxytryptamine, 5-HT) is a potent inducer of spawning in a number of bivalve species[14-20]. Since 5-HT is found in bivalve ganglia and gonads, it has been implicated as a neurohormone which controls bivalve spawning[1,21]. Furthermore, since externally applied 5-HT can induce spawning in both male and female zebra mussels[20], 5-HT or related compounds released with gametes may mediate chemical synchronization of spawning between individuals. However, little is known about the receptors that mediate the spawning response elicited by 5-HT.

Previous studies on zebra mussels, *Dreissena polymorpha*, have shown that spawning can be induced by 5-HT or the 5-HT_1 receptor agonists 8-OH-DPAT, TFMPP, and 1-1-naphthylpiperazine[22]. Spawning in zebra mussels can be inhibited by the 5-HT_2 receptor antagonists cyproheptadine and mianserin and the 5-HT_1 antagonist NAN-190, but is unaffected by the 5-HT_2 receptor antagonist ketanserin. These results suggested that spawning is controlled by a receptor with a mixed 5-HT_1/5-HT_2 pharmacology[1,20,22]. In the freshwater snail *Lymnaea stagnalis*, a gene for a 5-HT receptor has recently been cloned and expressed in COS-7 cells[23]. Competition binding studies revealed two compounds which outcompeted [^{3}H]LSD at concentrations 1000 to 10,000 times lower than NAN-190, 8-OH-DPAT, or 5-HT, namely methiothepin (1-[10,11-dihydro-8-(methylthio)dibenzo[b,f]thiepin-10-yl]-4-methylpiperazine mesylate) a vertebrate 5-HT_1/5-HT_2 receptor antagonist[24] and metergoline (1-methyl-8-beta-carbobenzyloxy-aminomethyl-10-alpha-ergoline), a vertebrate 5-HT_{1C}/5-HT_{1D} receptor antagonist[25]. These data from a 5-HT receptor of a freshwater mollusc suggested to us that methiothepin and metergoline might be effective antagonists in blocking 5-HT-induced spawning in zebra mussels. Our experiments with these two drugs revealed the most powerful 5-HT agonist and antagonist mediating spawning yet described for zebra mussels.

Materials and methods

Zebra mussels were collected by hand on 28 May 1993 from western Lake Erie at Monroe, Monroe Co., Michigan, USA (41° 54 'N, 83° 23 'W). Animals were immediately transported to the laboratory and maintained in an aquarium of recirculating water at 12 °C until used. Animals ranged from 13–25 mm shell length. Serotonin creatinine sulfate (Sigma Chemical Co., St. Louis, Missouri, USA) was dissolved in aquarium water. Methiothepin (Research Biochemicals Inc., Natick, Massachusetts, USA) was initially dissolved at pH 5.8 in aquarium water, then serially diluted to achieve desired concentrations (final pH = 7.02). Previous experiments have shown that pH 7 is not inhibitory to spawning[20]. Metergoline (Farmitalia) was initially dissolved in 100% ethanol and then serially diluted. Control experiments, described in 'Results', tested whether ethanol, at the concentrations used, would inhibit 5-HT-induced spawning.

All spawning assays were carried out in 20 ml glass vials (1 mussel/vial). To test inhibitory effects of methiothepin and metergoline on 5-HT-induced spawning, zebra mussels were initially acclimated in 4.0 ml aquarium water for 10–15 min. Each mussel then received 0.5 ml of either methiothepin or metergoline for 2 h. Thereafter 0.5 ml of 5-HT (10^{-4} or 10^{-3} M) was added to each vial. Thus, the final concentrations were 10-fold less than the added concentrations. Animals were exposed to 5-HT for 4 h, during which time they were observed for release of sperm or oocytes. Spawning was confirmed by microscopic analysis of water. To test the effect of methiothepin or metergoline alone, mussels were exposed to the agent for 6 h. Non-spawning animals were dissected at the end of the experiment to determine sex and reproductive maturity[20]. Results were analyzed statistically using Fisher's Exact Test[26], and null hypotheses were rejected where $p < 0.05$.

Results

Two-hour pre-treatments with methiothepin (10^{-4} M) completely inhibited spawning in response to both 10^{-4} and 10^{-3} M 5-HT ($p < 0.00001$ for 10^{-3} M, $p < 0.0007$ for 10^{-4} M; fig. 1). Methiothepin alone did not cause spawning. Moreover, 10^{-5} M methiothepin reduced spawning induced by 10^{-3} M 5-HT by 50% ($p < 0.03$, one-tailed; fig. 2). Both 10^{-6} and 10^{-5} M methiothepin significantly inhibited spawning in 10^{-4} M 5-HT ($p < 0.05$ for both comparisons; fig. 2).

Metergoline inhibited spawning at high concentrations (10^{-4} M), but induced spawning in male zebra mussels when applied at low concentrations (10^{-8} to 10^{-5} M). The vehicle (1% ethanol) did not induce spawning itself, nor did it reduce 5-HT-induced spawning; however, 10^{-4} M metergoline significantly reduced spawning induced by 10^{-3} M 5-HT ($p < 0.01$; fig. 3). At lower concentrations, metergoline stimulated spawning in males. Induction of spawning in response to metergoline was tested at concentrations ranging from 10^{-10} up to 10^{-4} M. A high percentage of males spawned in response to 10^{-8} to 10^{-5} M metergoline (10^{-8} M, $p < 0.02$; 10^{-7} M, $p < 0.00001$; 10^{-6} M, $p < 0.0004$; 10^{-5} M, $p < 0.00001$, compared to the no-metergoline controls,

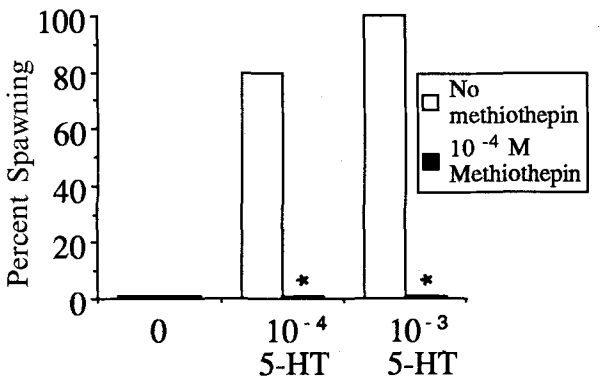

Figure 1. Effect of 10^{-4} M methiothepin on 5-HT (10^{-4} and 10^{-3} M)-induced spawning in zebra mussels. 0 group = aquarium water. Each group contained 10 animals. *$p < 0.0007$, comparing 10^{-4} M methiothepin to no methiothepin.

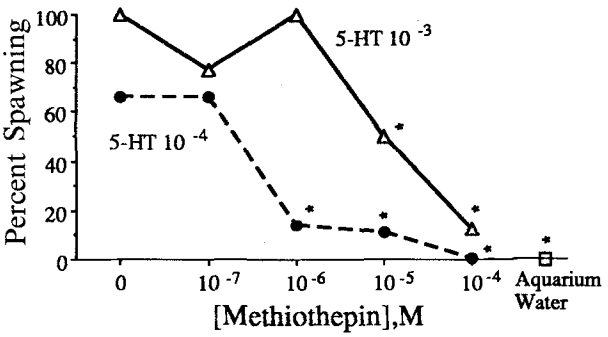

Figure 2. Dose response curves for zebra mussel spawning in 10^{-4} and 10^{-3} M 5-HT in the presence of varying concentrations of methiothepin. Each group contained 10 animals. *$p < 0.05$, compared to 5-HT alone.

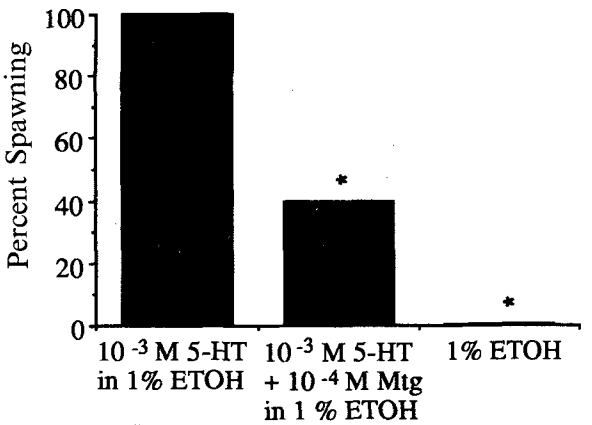

Figure 3. Effect of 10^{-4} M metergoline (Mtg, dissolved in 1% ethanol) on 5-HT (10^{-3} M)-induced spawning in zebra mussels. Each group contained 10 animals. *$p < 0.01$, compared to 5-HT in 1% ethanol.

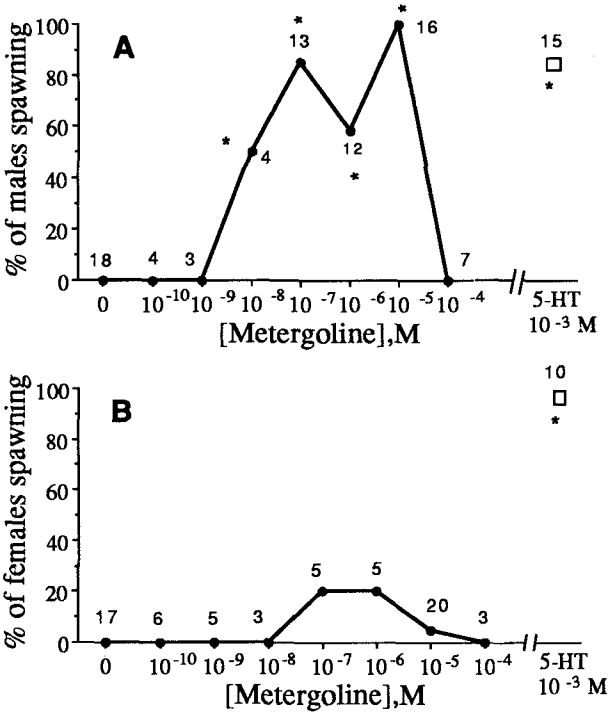

Figure 4. Percent of males spawning (A) and females spawning (B) in varying concentrations of metergoline. Metergoline (10^{-4} M) was initially dissolved in 1% ethanol, then serially diluted to achieve desired concentrations. Data are pooled from 3 experiments. Numbers adjacent to each point show the total number of animals of that sex tested at the given concentration, out of 10–40 animals (of both sexes, since zebra mussel gender cannot be determined prior to spawning or post-experiment dissection) initially tested in each treatment. Percent spawning in 5-HT (10^{-3} M) tested simultaneously is given for comparison. *$p < 0.05$, compared to no drug (group 0).

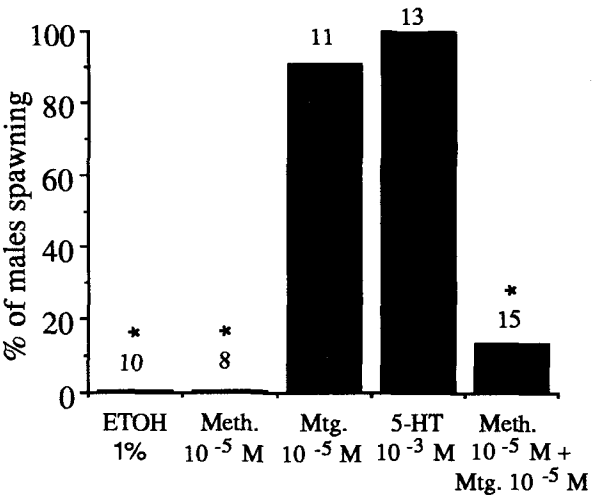

Figure 5. Effect of 10^{-5} M methiothepin (Meth.) on 10^{-5} M metergoline (Mtg.)-induced male spawning. Methiothepin was present 2 h before and during the 4-h treatment with metergoline. No females spawned. Metergoline (10^{-5} M) was dissolved in 0.1% ethanol. 5-HT (10^{-3} M) was dissolved in 1% ETOH. Numbers above each bar indicate the number of males out of a total of 25 animals initially tested in each group. *$p < 0.0002$ compared to 10^{-5} M metergoline alone.

data pooled for 3 experiments; fig. 4A). No mussels spawned in response to 10^{-4} M metergoline, and only 3 of 32 (9.3%) females spawned in metergoline at concentrations ranging from 10^{-8} to 10^{-5} M in 3 experiments. Metergoline (10^{-5} M)-induced male spawning was significantly reduced by 10^{-5} M methiothepin ($p < 0.0002$; fig. 5).

Discussion

This study showed that both methiothepin and metergoline have effects on zebra mussle spawning at much lower concentrations than has previously been demonstrated for 5-HT or other serotonergic ligands. Methiothepin inhibited spawning at concentrations as low as 10^{-6} M, whereas, metergoline had agonist effects at concentrations as low as 10^{-8} M. In previous studies[20,22], 5-HT and 8-OH-DPAT were tested at concentrations as low as 10^{-5} M, and both required at least 10^{-4} M to induce spawning. Previously tested inhibitors (NAN-190, cyproheptadine, mianserin) required at least 10^{-4} M to inhibit spawning. Thus, methiothepin is the most effective blocker of 5-HT-induced spawning yet tested, and metergoline is the most powerful inducer to spawning in male zebra mussels.

5-HT receptor types and subtypes are generally classified on the basis of their binding affinity to agonists and antagonists in rodent brain membranes[27,28]. However, comparatively little is known about invertebrate 5-HT receptors. Recently, a gene for a gastropod serotonergic receptor (5-HTlym) has been cloned and expressed in COS-7 cells[23]. In our study, the relative efficacy of methiothepin and metergoline agrees somewhat with the competitive binding data for 5-HTlym. Methiothepin, which effectively inhibited 5-HT-induced spawning at concentrations 2 orders of magnitude less than any previously tested inhibitor, has an affinity for 5-HTlym 4 orders of magnitude greater than 5-HT. Metergoline induced spawning in male zebra mussels at concentrations 4 orders of magnitude less than 5-HT. Its affinity for 5-HTlym is approximately 2 orders of magnitude greater than 5-HT. Almost complete inhibition of metergoline-induced spawning by an equal concentration of methiothepin indicates that methiothepin may have a greater affinity for the spawning receptor. Previous studies by this laboratory showed approximately equal efficacy of 8-OH-DPAT and 5-HT at inducing spawning[20] and no inhibitory effects of either ketanserin or propranolol on spawning[22]. This agrees with the approximately equal affinity of 5-HT and 8-OH-DPAT for 5-HTlym and the lower affinity of ketanserin and propranolol than 5-HT for 5-HTlym[23]. The apparent relative potencies for spawning and binding may be affected by oxidation, metabolism, or penetration, so the moderately good agreement with the binding data for 5-HTlym supports the contention that the receptors may be related.

This is the first study to demonstrate a sex specific induction of zebra mussel spawning at a time when both sexes are capable of spawning. All experiments included a positive control group in which animals were exposed to 5-HT, demonstrating that both sexes were capable of spawning, yet metergoline selectively induced males. The observation that metergoline induced spawning at low concentrations, but inhibited spawning at high concentrations may be attributed to mixed agonist/antagonist properties of this drug. Previously, male-only responses have been obtained with cold-stored, late season, or early season zebra mussels[20,29], a result attributed to lack of readiness of females to respond. In the present study, females were competent to respond. These results suggest that the 5-HT receptors in females are either less accessible to metergoline (the need for ethanol to solubilize metergoline indicates it is more hydrophobic than 5-HT) or that the effective 5-HT receptors for inducing spawning differ between males and females. Some of these questions may be resolved by in vitro investigations of the spawning mechanism and by investigating the structure and binding properties of 5-HT receptors in zebra mussels.

The presence of novel 5-HT receptors mediating germinal vesicle breakdown (GVBD) in bivalve oocytes has been suggested[30–32]. Previous studies in our laboratory[33] have demonstrated that GVBD occurs in zebra mussel ovaries prior to spawning in response to 5-HT. Radioligand binding assays and pharmacological characterizations with surf clams (*Spisula*)[31,32] demonstrated novel 5-HT binding sites (5-HT_5) on oocyte plasma membranes and vitelline envelopes. Thus, GVBD and spawning in *Dreissena* and GVBD in *Spisula* may be mediated by a new class of 5-HT receptors, possibly similar to 5-HTlym.

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