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Evidence That Bivalve Burrowing Is Mediated by Serotonin Receptors: Activation of Foot Inflation and Protrusion by Serotonin, Serotonergic Ligands and SSRI-Type Antidepressants in Three Species of Freshwater Bivalve

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Evidence That Bivalve Burrowing Is Mediated by Serotonin Receptors: Activation of Foot Inflation and Protrusion by Serotonin, Serotonergic Ligands and SSRI-Type Antidepressants in Three Species of Freshwater Bivalve

Abstract

Foot inflation and protrusion in bivalve mollusks is part of a sequence of events necessary for burrowing into the substrate. Although this process been observed after exogenous exposure to the neurotransmitter serotonin [5-hydroxytryptamine (5-HT)] and by some selective serotonin reuptake inhibitor (SSRI) type antidepressants, no study has shown that foot inflation and protrusion as a precursor to burrowing is under serotonergic control. In order to elucidate the physiological mechanism mediating this response, we tested over 2,000 freshwater bivalves in three species by exposing them to 5-HT, two 5-HT receptor agonists, two receptor antagonists and five antidepressants. Fingernail clams (Sphaerium striatinum), Asian clams (Corbicula fluminea) and unionid mussels (Elliptio complanata) all showed significant foot inflation or foot protrusion when exposed to 5-HT and the serotonergic agonists 8-OH-DPAT and alpha-methyl 5-HT. Some SSRI-type antidepressants (fluoxetine and fluvoxamine) also significantly induced these responses in all three species but were not as potent at the concentrations tested. In S. striatinum and C. fluminea, a 2-h exposure to the 5-HT2 receptor antagonists mianserin and cyproheptadine effectively blocked foot inflation induced by 5-HT and 8-OH-DPAT. The 5-HT receptor mediating this response is thus sensitive to mammalian 5-HT1 and 5-HT2 receptor ligands, supporting the hypothesis that serotonin mediates the initial step in burrowing.

Keywords

serotonin, bivalve, burrowing, antidepressant

Disciplines

Animal Sciences | Aquaculture and Fisheries | Biology

Evidence that bivalve burrowing is mediated by serotonin receptors: activation of foot inflation and protrusion by serotonin, serotonergic ligands and SSRI-type antidepressants in three species of freshwater bivalve.

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RUNNING HEAD: SEROTONIN REGULATION IN THE BIVALVE FOOT

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ABSTRACT

Foot inflation and protrusion in bivalve molluscs is part of a sequence of events necessary for burrowing into the substrate. Although this process been observed after exogenous exposure to the neurotransmitter serotonin (5-HT) and by some SSRI-type antidepressants, no study has shown that foot inflation and protrusion as a precursor to burrowing is under serotonergic control. In order to elucidate the physiological mechanism mediating this response, we tested over 2,000 freshwater bivalves in three species by exposing them to 5-HT, two 5-HT receptor agonists, two receptor antagonists and five antidepressants. Fingernail clams (*Sphaerium striatinum*), Asian clams (*Corbicula fluminea*) and unionid mussels (*Elliptio complanata*) all showed significant foot inflation or foot protrusion when exposed to 5-HT and the serotonergic agonists 8-OH-DPAT and alpha-methyl 5-HT. Some SSRI-type antidepressants (fluoxetine and fluvoxamine) also significantly induced these responses in all three species but were not as potent at the concentrations tested. In *S. striatinum*and *C. fluminea*, a 2-h exposure to the 5-HT_2 receptor antagonists mianserin and cyproheptadine effectively blocked foot inflation induced by 5-HT and 8-OH-DPAT. The 5-HT receptor mediating this response is thus sensitive to mammalian 5-HT_1 and 5-HT_2 receptor ligands, supporting the hypothesis that serotonin mediates the initial step in burrowing.

INTRODUCTION

In bivalve molluscs, the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) has been identified and localized innervating the gills (Stefano & Aiello, 1975), siphon-mantle muscles (Ram *et al*., 1999), retractor muscles and foot (Vitellaro-Zuccarello *et al*., 1990), and gonads (Masseau *et al.*, 2002; Garnerot *et al*., 2006;). 5-HT mediates actions within these structures such as gill ciliary activity (Carroll & Catapane, 2007), muscle contraction (Ram *et al*., 1999), and oocyte maturation and spawning (Fong *et al*., 1994), in both marine and freshwater bivalves. 5-HT receptors mediating these actions have been identified in several bivalve groups including scallops (Tanabe *et al*., 2010), oysters (Jia *et al*., 2018) and freshwater mussels (Fong *et al*., 1993); see also reviews by Alavi *et al*. (2017) and Tierney (2018).

Foot protrusion and subsequent inflation within the substrate is a necessary component of bivalve burrowing. In a recent paper, Canesi *et al*. (2022) reviewed the physiological systems in bivalves regulated by 5-HT including reproduction, heart function, gill ciliary beating and catch muscles, but no study has shown that foot inflation or protrusion as a precursor to burrowing is under serotonergic control. We have observed these effects on the foot by 5-HT, 5-HT receptor ligands and antidepressants such as fluoxetine ("Prozac") while measuring spawning and oocyte maturation in freshwater bivalves (Fong *et al*., 1993, 1996; Fong, 1998). Exogenous application of these chemicals causes foot inflation, resembling a balloon, often as a prelude to spawning or parturition. In some freshwater bivalves (e.g. *Elliptio complanata*), exposure to these chemicals does not cause foot inflation but causes foot protrusion for several minutes, often leading to the release of conglutinates in females.

In this work, we tested the efficacy of 5-HT, two mammalian 5-HT receptor agonists (8-OH-DPAT and Alpha-methyl 5-HT) and five antidepressants which modulate serotonergic action, and which have been previously shown to affect molluscan behaviour (Fong *et al*. 1993, 2003) on foot inflation and protrusion in three species of freshwater bivalve, Fingernail clams *Sphaerium striatinum* (Fingernail clams), *Corbicula fluminea* (Asian clams) and the unionid mussel *Elliptio complanata*. To show that the response was receptor mediated, we tested the effect of two 5-HT receptor antagonists (mianserin and cyproheptadine) on blocking foot inflation. The possible effect of antidepressants was tested because of their occurrence in aquatic environments (Sehonova *et al*., 2018), and thus having an ecotoxicological effect.

MATERIAL AND METHODS

Animal collection and maintenance

Sphaerium striatinum, *Corbicula fluminea* and *Elliptio complanata* were collected by hand from Marsh Creek, Adams Co., PA, USA, (39.83°N, 77.23°W) in May and June 2021. In the lab, animals were maintained in c. 19 aquaria containing c. 9.5 l of a 50:50 mixture of creek water and dechlorinated tap water for 1 day. Thereafter, they were kept in dechlorinated tap water until testing which occurred within 2–3 days of collection. Bivalves were not fed prior to testing.

Serotonin (Sigma-Aldrich), alpha-methyl serotonin (Santa Cruz Biotechnology), 8-OH-DPAT (Sigma-Aldrich), sertraline (AK Scientific), venlafaxine (AK Scientific), citalopram (R & D Systems), mianserin (AK Scientific) and cyproheptadine (AK Scientific) were solubilized in dechlorinated tap water. Fluvoxamine (R & D Systems) was solubilized in 100% ETOH. Tested concentrations of 5-HT, 8-0H-DPAT and alpha- methyl-5-HT, the blockers mianserin and cyproheptadine, and the antidepressants were selected based upon their efficacy at inducing and blocking foot-related

locomotory behaviours in freshwater mussels Fong *et al*., 1993; Fong, 1998) and fingernail clams (Fong *et al*., 1996, 2003).

Experimental procedure

All experiments were conducted at room temperature (22 ˚C). *Sphaerium striatinum* (1 cm shell length) and *C. fluminea* (1.5–2.0 cm shell length) were tested in 20 ml scintillation vials (one clam per vial). Each clam initially received 9.0 ml of dechlorinated tap water and 1.0 ml of drug at a 10× higher concentration than the final concentration. In the case of 8-OH-DPAT and Alpha-methyl 5-HT, 4.5 ml of dechlorinated tap water and 0.5 ml of drug was added. Individuals of *E. complanata* (2.0-7.0 cm, shell length) were tested in scintillation vials (shell length of 2–3 cm), 100 ml beakers (shell length 4–5 cm) or 5 cm finger bowls (shell length of 6–7 cm), with final volumes ranging from 10–100 ml depending upon shell length (one mussel per container). Each mussel received dechlorinated tap water for 10 min, thereafter, the appropriate volume of drug at a 10× higher concentration than the final concentration was added. All three bivalve species were observed over a 4-h period for foot inflation (in clams) or foot protrusion (in mussels), and none was tested more than once. Foot inflation and protrusion (FIP) was defined as an increase in fluid pressure in the foot causing a balloon-like appearance and extension from the shell for at least 30 min. During this time, the foot remained outside of the shell, extended a greater distance from the shell compared with control clams, was not tucked under the shell in a burrowing position and was never withdrawn back into the shell for the duration of the 4-h experiment. Control mussels sometimes extended their feet, but only a short distance from the shell, tucked their feet under the shell in an attempt to burrow and withdrew their feet back into the shell.

To show that the foot inflation response was mediated by a serotonin receptor, we prtreated individuals of *S. striatinum* and *C. fluminea* with the 5-HT_2 receptor antagonists mianserin and cyproheptadine, then applied either 5-HT or 8-OH DPAT. Mianserin and cyproheptadine have been shown to block 5-HT-stimulated spawning in zebra mussels (Fong *et al*., 1993) and parturition in fingernail clams (Fong *et al*., 1996). For these blocker experiments, we acclimated bivalves in 8.0 ml of dechlorinated tap water for 10 min. Thereafter we added 1.0 ml of either mianserin (1.0 mM) or cyproheptadine (1.0 mM) for 2 h, then added 1.0 ml of 5-HT (1.0 mM) or 8-OH-DPAT (0.1 mM or 1.0 mM) and observed foot inflation over a 4-h period.

Differences in the frequency of FIP between groups was tested by Fisher's exact test. Differences in mean percent foot inflation were tested by unpaired *t*-tests. Null hypotheses were rejected where $P < 0.05$.

RESULTS

Activation of foot inflation and foot protrusion

Exposure to 5-HT and the serotonergic ligands 8-OH-DPAT and alpha-methyl 5-HT significantly induced foot inflation in *Sphaerium striatinum* and *Corbicula fluminea*, and foot protrusion in *Elliptio complanata*. 5-HT induced foot inflation in *S. striatinum* from 50 µM -1 mM, in *C. fluminea* in from 100 µM and 1 mM, and foot protrusion in *E. complanata* in from 100 µM and 1 mM (Fisher's exact test: $P < 0.05$; Fig. 1A). The 5-HT_{1A} receptor agonist 8-OH-DPAT induced foot inflation from 1-µM-to 1 mM (Fisher's exact test: $P < 0.05$), but caused toxicity indicated by shell gaping at the highest concentration in *S. striatinum* (Fig. 1B). The 5-HT_2 receptor agonist, alpha-

methyl 5-HT induced the responses at 1 mM in all three species (Fisher's exact test: $P < 0.05$; Fig. 1C).

By contrast, while some antidepressants induced these responses in all three species, in general, they did not induce as high a percentage of FIP at the concentrations tested compared with 5-HT, 8-OH-DPAT or alpha-methyl-5-HT. For example, fluoxetine significantly induced foot inflation in *S. striatinum* and in *C. fluminea*, but at a lower percentage compared with all nonantidepressants, and only at the highest concentration (10 μM), and was ineffective at inducing foot protrusion in *E. complanata*. (Table 1). Fluvoxamine induced a high percentage of foot inflation in *C. fluminea* and foot protrusion in *E. complanata*, but had no effect on *S. striatinum*. Similar varying degrees of effectiveness was shown by sertraline and venlafaxine, as well as complete ineffectiveness by citalopram (Table 1).

5-HT and the two serotonergic ligands were not only more effective at inducing both foot inflation and protrusion, but they exerted their action faster than did the antidepressants. In all three species, 5-HT, 8-OH-DPAT and alpha-methyl 5-HT induced the fastest responses, with percentages from 70–100% within the first 2 h (Fig. 2). By contrast, both foot inflation and foot protrusion occurred less often and more slowly when bivalves were exposed to antidepressants as opposed to nonantidepressants (Fig. 3). Foot inflation occurred significantly faster in both *S. striatinum* and *C. fluminea* when exposed to nonantidepressants compared with those bivalves exposed to antidepressants over the first hour (Fig. 3A) and second hour (Fig. 3B; *t*-tests: $P < 0.05$).

In *S. striatinum* and *C. fluminea*, a 2-h exposure to the 5-HT_2 receptor antagonist mianserin effectively blocked 5-HT-induced foot inflation (Fisher's exact test: $P<0.05$; Fig. 4A), and to 8-OH-

DPAT (P<0.05; Fig. 4B). Similarly, cyproheptadine blocked 5-HT-induced inflation (Fisher's exact test: P<0.05; Fig. 5A) and 8-OH-DPAT-induced inflation (Fisher's exact test: P<0.05; Fig. 5B).

DISCUSSION

Serotonergic innervation of the bivalve foot has been well documented (Alavi *et al*., 2017) and, because of its presence in pedal ganglia, it is assumed that it regulates actions by the foot. However, no studies are available demonstrating the direct effects of 5-HT on locomotion or burrowing. In this paper, we described two 5-HT-mediated foot responses in freshwater bivalves, namely foot inflation and foot protrusion. Trueman (1966) described the mechanism of burrowing in bivalves as being due to the contraction of adductor muscles, followed by compression of the outer and inner layers of the ligament, with an increase in fluid pressure from the pericardial cavity into the pedal hemocoel, causing dilation. Our observations indicate that exposure to the tested chemicals causes pedal dilation in the form of ballooning and prolonged pedal extension (FIP) for greater than 30 min. We found that 5-HT, 8-OH-DPAT and alpha-methyl 5-HT were the most potent inducers of FIP (Fig. 1). Each of the tested species responded more often and faster when exposed to these chemicals (Fig. 2). The 5-HT receptors that mediate the observed responses were sensitive to 5-HT_{1A} and 5-HT_2 receptor ligands. Similar pharmacological results have been reported for induction of zebra mussel spawning (Fong *et al*., 1993), for fingernail clam parturition (Fong *et al*., 1996) and for germinal vesicle breakdown and the release of intracellular calcium in marine bivalves (Fong *et al*., 1997). FIP was blocked by the 5-HT_2 receptor antagonists mianserin and cyproheptadine, suggesting that these responses were mediated by a 5-HT receptor (Figs 4, 5), a finding similar to that in earlier studies (Fong *et al*., 1993). The receptors mediating this behaviour could be located on the surface of the foot or

alternatively, the chemicals could be internalized into the hemocoel, and then act directly on the foot. Molecular characterization of 5-HT receptors and their subtypes in invertebrates, especially arthropods and molluscs, has been reviewed by Tierney (2018).

Several studies have reported increased foot activity after exposure to SSRI-type antidepressants. Cunha & Machado (2001) observed an increase in foot volume and foot extension when the unionid *Anodonta cygnea* was exposed to the antidepressants fluoxetine and fluvoxamine from 1 µM to 10 mM. Upon exposure to the antidepressants, the foot relaxes, creating favourable conditions for water uptake and extension, with subsequent release of glochidia larvae. Hazelton *et al*. (2013) found that a 1–2 day exposure to fluoxetine at 0.37 and 29.3 µg/l, induced foot protrusion in the unionid *Lampsilis fasciola*. This group of researchers further described swelling and protrusion of the foot in *L. fasciola* exposed to fluoxetine, with the surprising observation that treated animals travelled significantly further, had increased erratic movement and initiated burrowing sooner compared to control mussels (Hazelton *et al*., 2014). The mode of action of the tested SSRI-type antidepressants is by blocking the re-uptake of serotonin at vertebrate synapses. But, amongst molluscs and other invertebrates, these antidepressants may work by binding directly to the serotonin receptor as suggested by Ford & Fong (2016) and demonstrated in *Caenorhabditis elegans* by Kullyev *et al.* (2010).

Our observations that antidepressants induce FIP in the three tested species, and those of other researchers on unionids (Cunha & Machado, 2001; Hazelton *et al*., 2013, 2014) suggest a possible ecotoxicological effect. While our effective concentrations of antidepressants are higher than environmental concentrations (Venkatachalam *et al*., 2023), these drugs have been shown to bioaccumulate in aquatic organisms including the bivalve *Corbicula fluminea* (Burket et al. 2020). Furthermore, both *C. fluminea* and *Sphaerium striatinum* have been reported to use their foot to

pull organic material from sediments into the mantle cavity, where particles are captured on the gill and moved to food grooves (McMahon & Bogan, 2001). Pedal feeding is also known to occur in some unionids (Vaughn & Hakenkamp, 2001; Brendelberger & Klauke, 2009). Juvenile and adult sphaeriids and juvenile *C. fluminea* are also known to use their foot to crawl across the sediment surface, presumably to relocate into more favourable environments (McMahon & Bogan, 2001). It is unknown how antidepressant exposure affects surface crawling behaviour. Future studies are necessary to elucidate how common and widespread these 5-HT-mediated responses are in other freshwater and marine bivalves, and how environmental antidepressants can be important modulators of these actions and on burrowing speed.

Finally, other invertebrate mechanisms similar to FIP, such as the pharyngeal eversion in polychaetes, could be mediated by 5-HT. 5-HT modulates annelid body wall musculature (Walker *et al*., 1993) and has been localized in the supraesophageal ganglia of *Glycera convoluta* (Manaranche & L'Hermite, 1973), in the prostomial nervous system of *Nereis virens* (Marsden *et al.*, 1981) and in the central nervous system of *Nephtys* sp. (Clark, 1966). All of these species have eversible pharynxes, but whether 5-HT mediates this eversion is unknown.

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Figure captions

Figure 1. Percent of *Sphaerium striatinum*, *Corbicula fluminea*) and *Elliptio complanata* with foot inflation or foot protrusion exposed to various concentrations of 5-HT (**A**), 8 OH-DPAT (**B**) or alpha-methyl serotonin (**C**). Sample sizes per group were n = 10–15 for *S. striatinum*, n = 15 for *C. fluiminea* and n = 8 for *E. complanata*. *: Fisher's Exact Test; $P < 0.05$ compared to control.

Figure 2. Cumulative mean percent foot inflation in *Sphaerium striatium* (**A**), *Corbicula fluminea* (**B**) and foot protrusion in *Elliptio complanata* (**C**) over a 4-h period.

Figure 3. Mean (+/- SE) percent foot inflation inz*Sphaerium striatinum* and *Corbicula fluminea* or foot protrusion in *Elliptio complanata* during the first experimental hour (**A**) and the second experimental hour (**B**) between nonantidepressants (5-HT, 8-OH-DPAT and alpha-methyl 5-HT) and antidepressants (fluoxetine, fluvoxamine, sertraline and venlafaxine). Drugs included were those that showed a significant difference compared to control water. *: t-test; $P< 0.05$ comparing nonantidepressants to antidepressants for each species.

Figure 4. Effect of a 2-h pretreatment with the 5-HT_2 antagonist mianserin (100 μM) when combined with 5-HT (100 μM) (**A**) or 8-OH-DPAT (10 and 100 μM) (**B**) on the percent of foot inflation in *Sphaerium striatinum* and *Corbicula fluminea*. Sample sizes are n = 15 per group for *S. striatinum* and n = 18 per group for *C. fluminea*. *: Fisher's exact test; $P < 0.05$ compared with serotonin or 8-OH-DPAT alone.

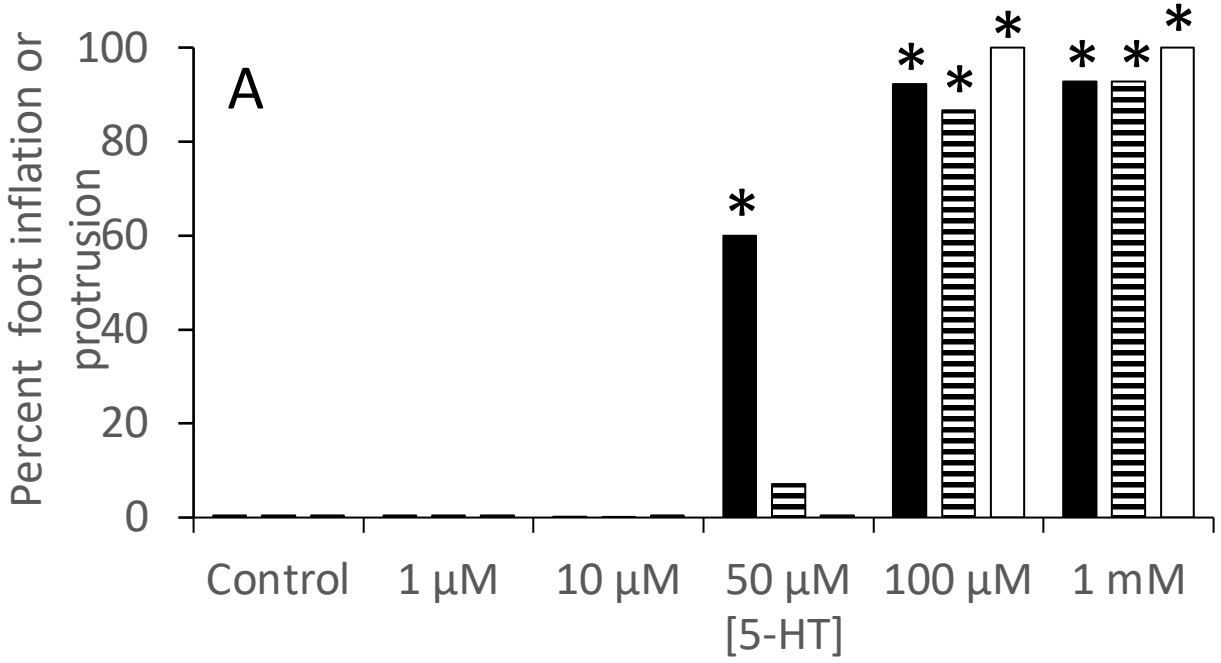
Figure 5. Effect of a 2-h pretreatment with the 5-HT_2 antagonist cyproheptadine (100 μM) when combined with 5-HT (100 μM) (**A**) or 8-OH-DPAT (10 and 100 μM) (**B**) on the percent of foot inflation in *Sphaerium striatinum* and *Corbicula flumine*. Sample sizes are n = 11–15 per group for *S. striatinum* and n = 18 per group for *C. fluminea*. *: Fisher's exact test; $P < 0.05$ compared with serotonin or 8-OH-DPAT alone.

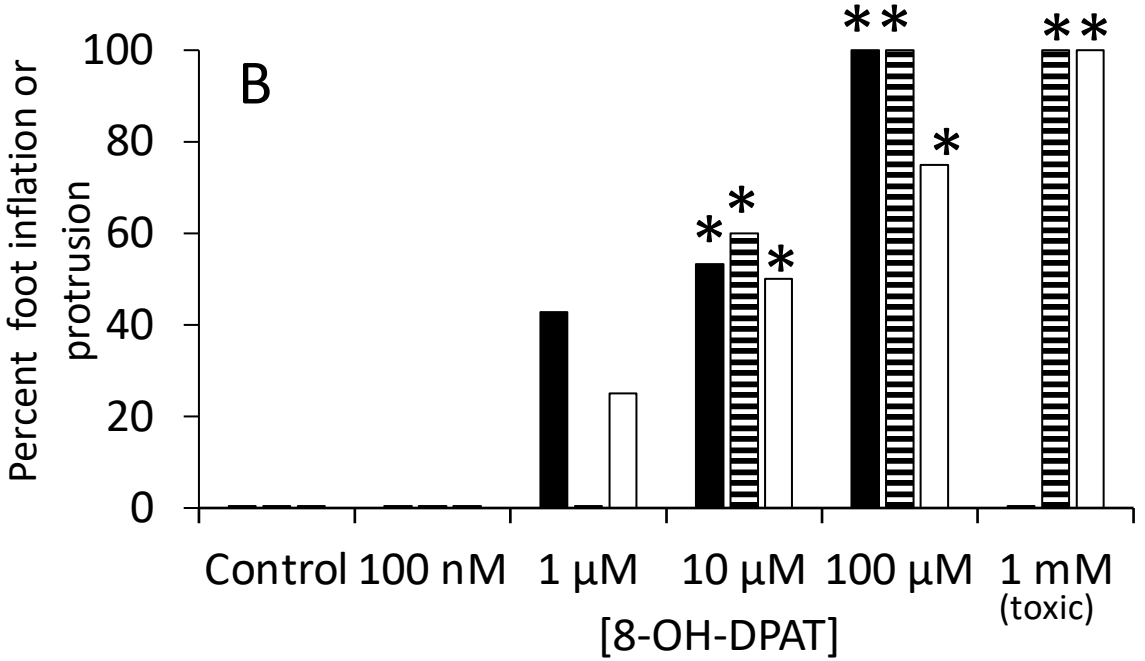
TABLES

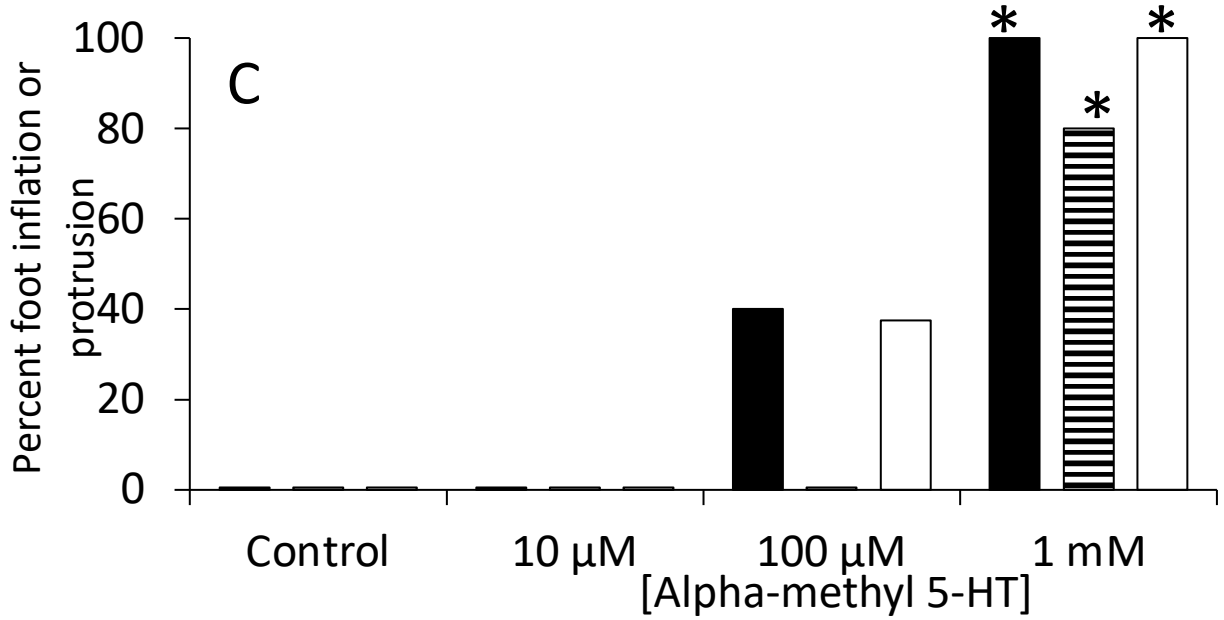
Table 1. Foot inflation in *Sphaerium striatinum* and *Corbicula fluminea*, and foot protrusion in *Elliptio complanata*, showing (number/total tested) induced by various antidepressants.

Antidepressant [µM]	*S. striatinum*	*C. fluminea*	*E. complanata*
Fluoxetine			
10	7/15*	7/15 *	2/8
1	3/15	0/15	0/8
0.1	0/15	0/15	0/8
0.01	0/15	0/15	ND
control	0/15	0/15	0/8
Fluvoxamine			
10	0/15	13/15*	8/8*
1	0/15	4/15*	3/8
0.1	0/15	0/15	1/8
control (0.01%ETOH)	2/15	0/15	0/8
Sertraline			
10	3/15	0/15	0/8
1	4/15*	0/15	0/8
0.1	4/15*	0/15	0/8
control	0/15	0/15	0/8
Venlafaxine			
10	4/15*	2/15	0/8
1	6/15*	0/15	0/8
0.1	0/15	0/15	0/8
control	0/15	0/15	0/8
Citalopram			
10	2/15	0/15	0/8
1	0/15	0/15	0/8
0.1	0/15	0/15	0/8
control	0/15	0/15	0/8

ND indicates no data; an asterisk indicates *P*-values < 0.05 for the Fisher's exact test.

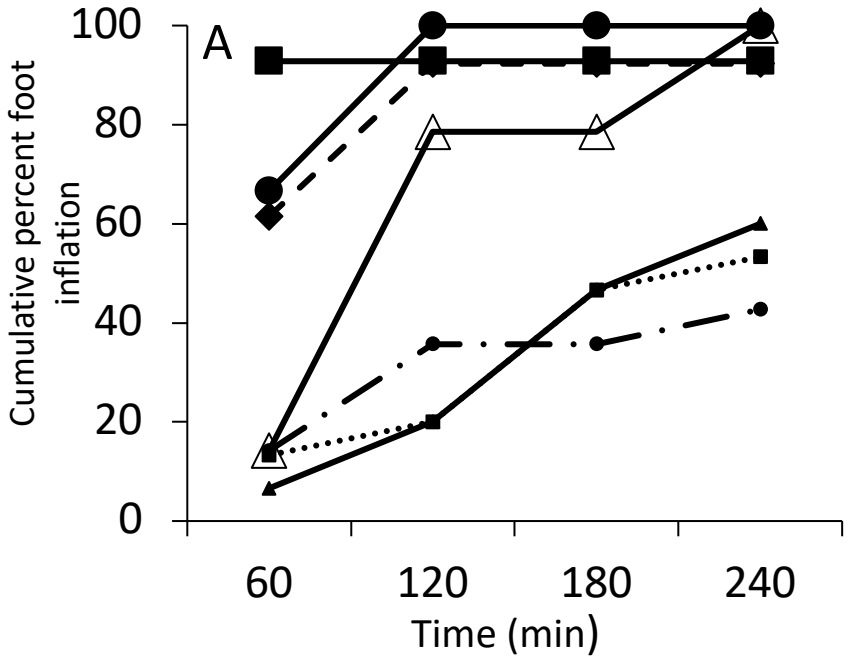

A
Percent foot inflation or protrusion
100
80
60
40
20
0
Control
1 µM
10 µM
50 µM
100 µM
1 mM
[5-HT]

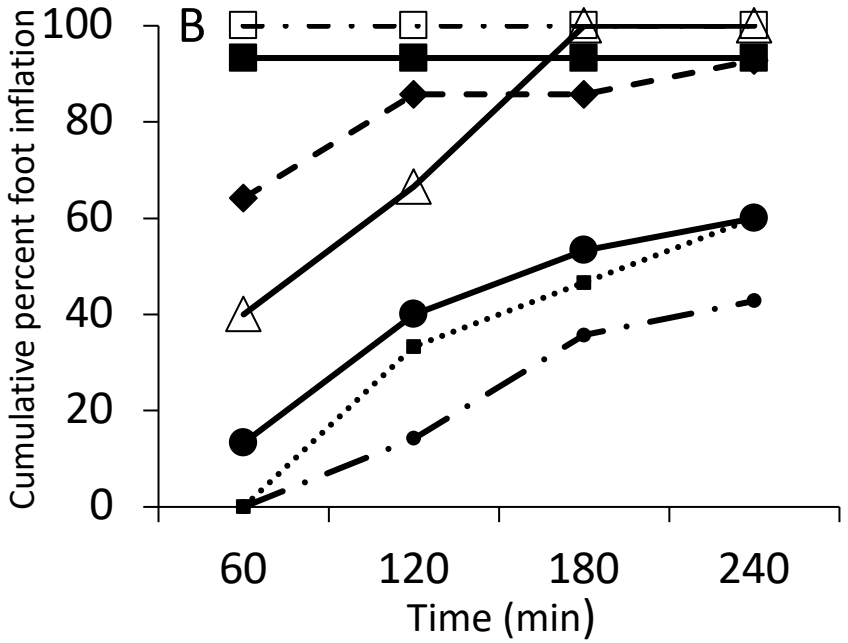

B
Percent foot inflation or protrusion
100
80
60
40
20
0
Control
100 nM
1 µM
10 µM
100 µM
1 mM
(toxic)
[8-OH-DPAT]

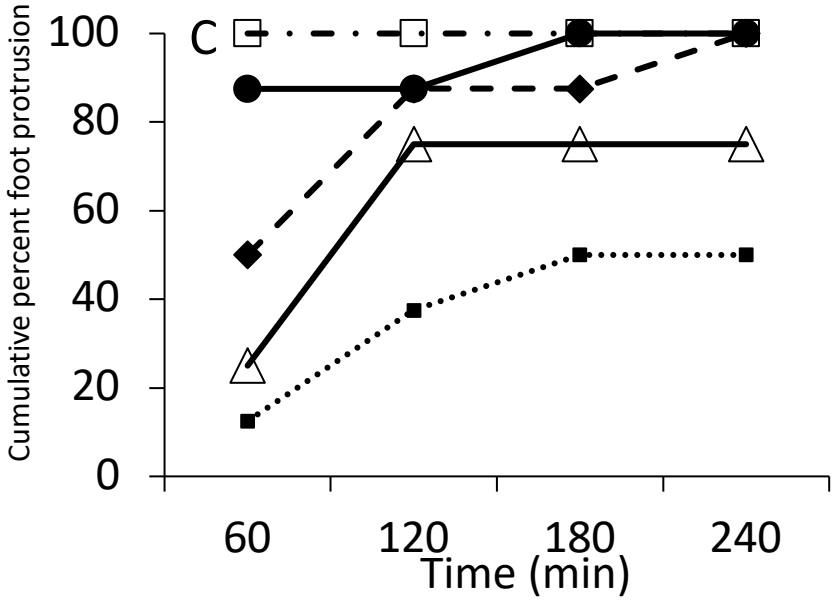

C
Percent foot inflation or protrusion
100
80
60
40
20
0
Control
10 µM
100 µM
1 mM
[Alpha-methyl 5-HT]


Sphaerium
Corbicula
Elliptio

Fig. 1


A
Cumulative percent foot inflation
100
80
60
40
20
0
60
120
180
240
Time (min)


B
Cumulative percent foot inflation
100
80
60
40
20
0
60
120
180
240
Time (min)


C
Cumulative percent foot protrusion
100
80
60
40
20
0
60
120
180
240
Time (min)

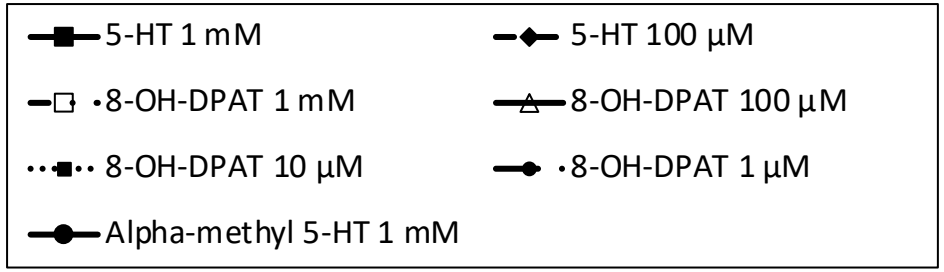

5-HT 1 mM
5-HT 100 µM
8-OH-DPAT 1 mM
8-OH-DPAT 100 µM
8-OH-DPAT 10 µM
8-OH-DPAT 1 µM
Alpha-methyl 5-HT 1 mM

Fig. 2

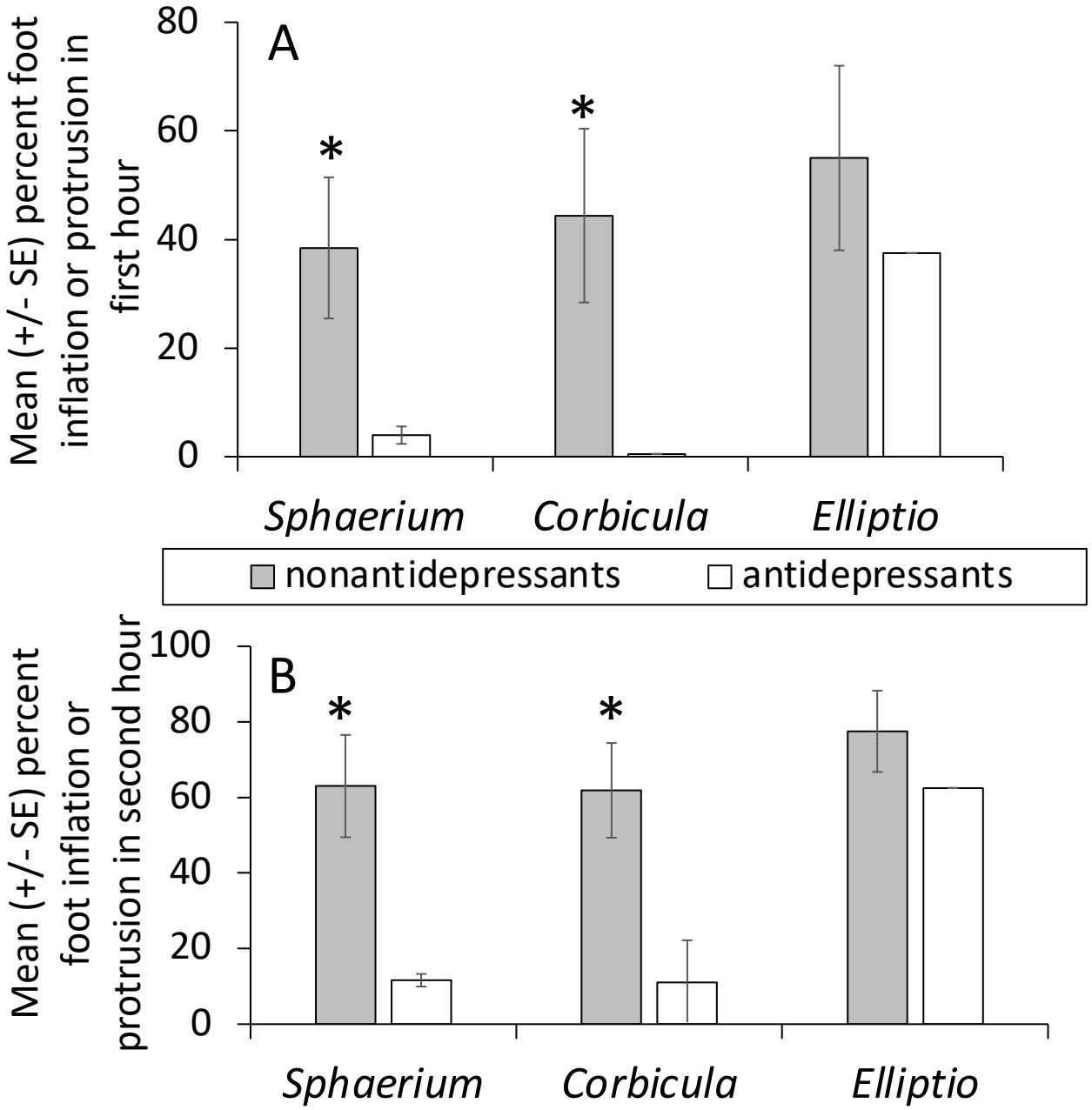

A
Mean (+/- SE) percent foot inflation or protrusion in first hour
80
60
40
20
0
*
*
Sphaerium
Corbicula
Elliptio
nonantidepressants
antidepressants
B
Mean (+/- SE) percent foot inflation or protrusion in second hour
100
80
60
40
20
0
*
*
Sphaerium
Corbicula
Elliptio

Fig. 3

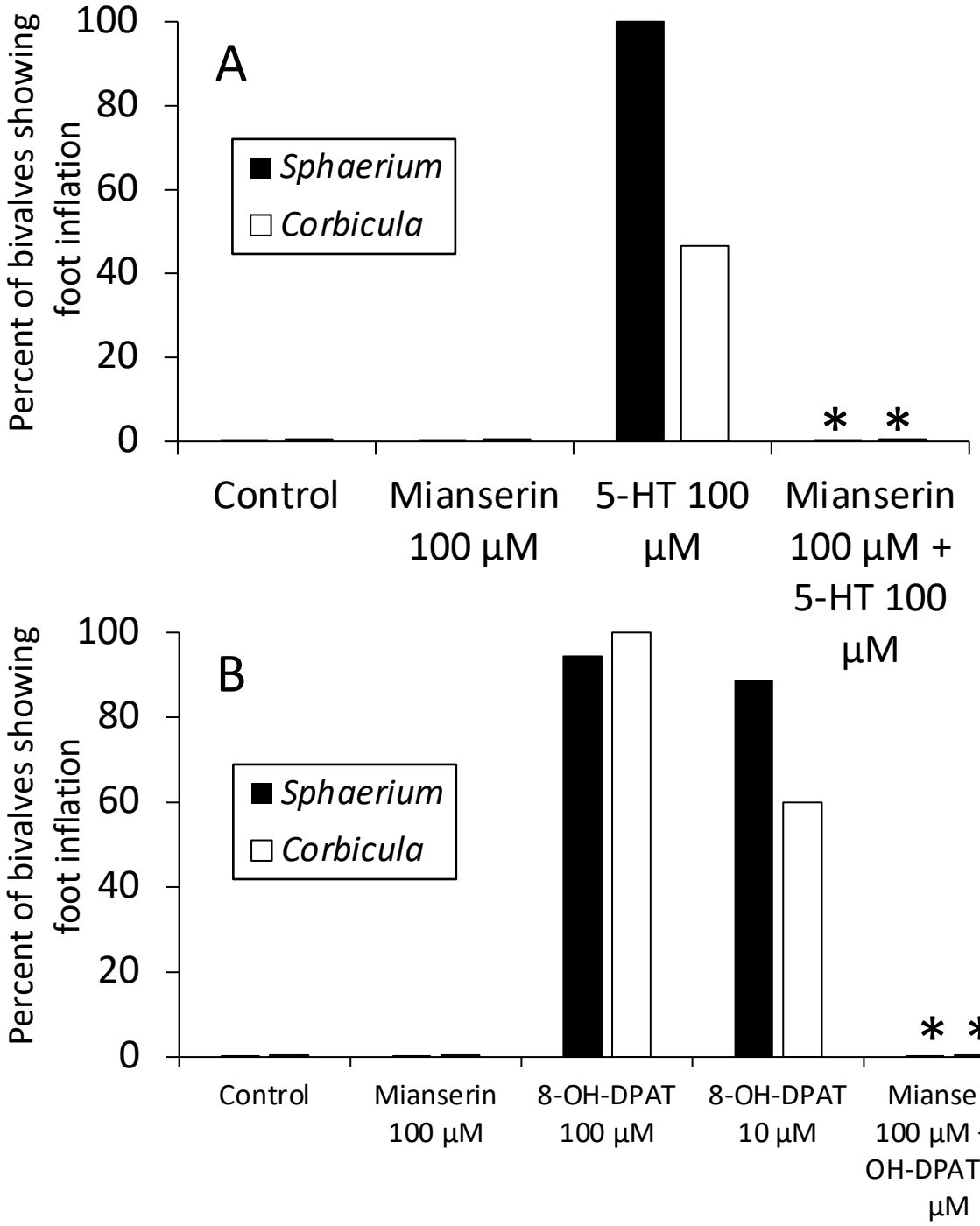

A
Percent of bivalves showing foot inflation
100
80
60
40
20
0
Sphaerium
Corbicula
* *
Control
Mianserin 100 µM
5-HT 100 µM
Mianserin 100 µM + 5-HT 100 µM
B
Percent of bivalves showing foot inflation
100
80
60
40
20
0
Sphaerium
Corbicula
* *
* *
Control
Mianserin 100 µM
8-OH-DPAT 100 µM
8-OH-DPAT 10 µM
Mianserin 100 µM + 8-OH-DPAT 100 µM
Mianserin 100 µM + 8-OH-DPAT 10 µM

Fig. 4

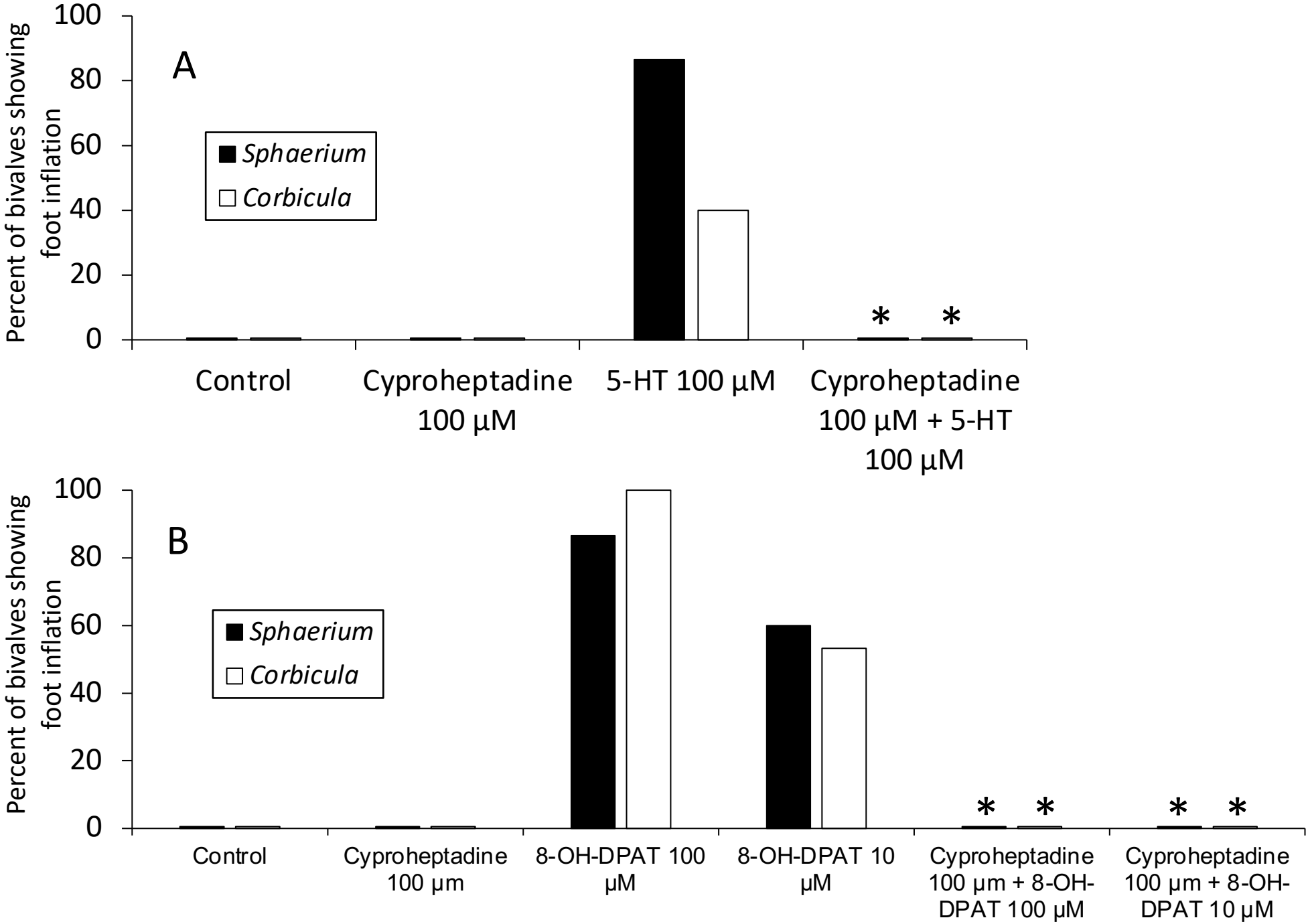

A
Percent of bivalves showing foot inflation
100
80
60
40
20
0
Sphaerium
Corbicula
*
*
Control
Cyproheptadine 100 µM
5-HT 100 µM
Cyproheptadine 100 µM + 5-HT 100 µM
B
Percent of bivalves showing foot inflation
100
80
60
40
20
0
Sphaerium
Corbicula
*
*
*
*
Control
Cyproheptadine 100 µm
8-OH-DPAT 100 µM
8-OH-DPAT 10 µM
Cyproheptadine 100 µm + 8-OH-DPAT 100 µM
Cyproheptadine 100 µm + 8-OH-DPAT 10 µM

Fig. 5