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Settlement and metamorphosis of *Styela canopus* Savigny larvae in response to some neurotransmitters and thyroxin

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Abstract

The larvae of ascidian *Styela canopus* Savigny were treated with epinephrine, norepinephrine, L-DOPA, GABA and thyroxin to test the ability of these compounds to induce or inhibit larval settlement and metamorphosis. The results showed that epinephrine, norepinephrine and L-DOPA at the concentration of 1 μmol/dm^3 induced larval settlement and metamorphosis in *S. canopus*, with short exposure (1 h) to 1 μmol/dm^3 of L-DOPA inducing rapid settlement. In contrast, GABA at the concentrations of 0.1 ~ 100.0 μmol/dm^3 significantly inhibited the settlement and metamorphosis of *S. canopus* larvae. In addition, thyroxin at 1 ~ 50 μg/dm^3 had no effect on larval settlement and metamorphosis in *S. canopus*. These results suggest the importance of neurotransmitters in the settlement and metamorphosis of *S. canopus* larvae.

Key words: *Styela canopus*, larvae, settlement, metamorphosis, neurotransmitter, thyroxin

1 Introduction

As important steps in the life cycles of most marine benthos, settlement and metamorphosis of their planktonic larvae influence directly the population distribution and fluctuation of benthos. Research on larval settlement and metamorphosis of marine benthos, related to aquaculture (Ke et al., 2000; Liu et al., 1998) and marine antifouling (Huang, Feng, et al., 2003), is popular in marine biology. The influences of various chemicals on larval settlement and metamorphosis have been studied in many species of marine benthos. Among these investigations, the inductive effects of various neurotransmitters on the settlement and metamorphosis of larvae have been found in many marine benthos, of which the most outstanding ones were choline on *Phestilla sibogae* (Hirata and Hadfield, 1986), L-DOPA on *Crassostrea gigas* (Coon et al., 1985) and GABA on *Haliotis rufescen* (Morse et al., 1979).

Ascidians (Urochordata) are an important marine benthic organism and have special significance in animal evolution and classification because the ascidian larva is considered a basic model of vertebrate morphogenesis (Huang, Ke, et al., 2003). To date, the mechanisms regulating the settlement and

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metamorphosis of ascidian larvae remain largely unknown. Documentation on the effects of neurotransmitters on larval settlement and metamorphosis in ascidians is few. So far, only *Herdmania momus* (Degnan et al., 1997), *Ascidia malaca* (Patricolo et al., 1981) and *Ciona savignyi* (Kimura et al., 2003) have been investigated for the influences of some neurotransmitters on larval settlement and metamorphosis. Since these investigations show differences in the responses of larval settlement and metamorphosis to neurotransmitters among the three ascidian species, it is necessary to examine and compare more species to further clarify the significance of the differences in the effects of neurotransmitters on larval settlement and metamorphosis in ascidians.

In the present work, we examined the effects of the neurotransmitters epinephrine, norepinephrine, L-DOPA, GABA and the hormone thyroxin on settlement and metamorphosis of tadpole larvae in *Styela canopus* Savigny, an important macrofouling organisms on the southeast coast of China, to better understand the specific behavioral and morphogenetic responses of ascidian larvae to these neurotransmitters and provide some data for antifouling.

2 Materials and methods

2.1 Animals and larval culture

Adults of *Styela canopus* were collected from submerged rafts at a fish farm near Huoshao Island, Xiamen, and kept in aquaria with little aeration and certain light. Adults of *Styela canopus* were induced to spawn eggs and sperm by the artificial method of intensive aeration. After fertilization, eggs were rinsed three to four times using a mesh of 120 pores per 25.4 mm. Larvae were hatched after 10 h of culture in seawater at 25 ℃. After hatching for 3 h, larvae attained competency to settle and metamorphose, and were used for subsequent testing.

2.2 Treatment of neurotransmitters and thyroxin

All experiments were conducted in Petri dishes, each 6 cm in diameter. At (25 ± 0.5) ℃ in temperature and 27.0 in salinity, seawater is millipore-filtered (0.22 μm). Solutions of epinephrine and norepinephrine were prepared at the concentrations of 0.001, 0.010, 0.100 and 1.000 mmol/dm^3, while solutions L-DAPA and GABA were prepared at the concentrations of 0.1, 1.0, 10.0 and 100.0 μmol/dm^3. Thyroxin was prepared at 1, 5, 10, 30 and 50 μg/dm^3. Filtered seawater (FSW) without added substance was served as the control. Three replicates were monitored in each treatment and in each experimental replicate, 30 ~ 80 competent larvae of *S. canopus* were introduced into each Petri dish containing 10 mL of test solution or FSW. Hatched larvae three hours old were exposed to various concentrations of epinephrine, norepinephrine and L-DOPA for 1 h (from preliminary experiment), then removed and placed in fresh seawater. After another 48 h of exposure to FSW, the number of larvae having settled and the number of larvae having completed metamorphosis were counted. Other larvae of *S. canopus* were exposed to various concentrations of GABA and thyroxin respectively and, after 48 h of continuous exposure, the number of larvae having settled and the number of larvae having completed metamorphosis were counted.

2.3 Effects of L-DOPA on larval settlement and metamorphosis varying with time

According to the results of Section 2.2, L-DOPA at the concentrations of 1 and 100 μmol/dm^3 both had significant influences on larval settlement and metamorphosis in *S. canopus*. So we investigated the variation of settlement/ metamorphosis over time. *Styela canopus* larvae were exposed to 1 and 100 μmol/dm^3 of L-DOPA for 1 h, then removed and placed in FSW. The number of larvae having settled and that of larvae having completed metamor-

phosis were counted at 6, 12, 24, 32 and 48 h after exposure to seawater.

2.4 The biological indices

According to the interrelated article (Svane and Young, 1989) and the biological characteristics of *S. canopus* larvae, the biological indices applied in this study are defined as follows. Larval settlement is confirmed by adhesive papillae of tadpole larvae adhering permanently to substratum. Completed metamorphosis is confirmed by the whole tail being resorbed.

2.5 Statistical analysis

Differences in the settlement and completed metamorphosis percentages of experimental and control treatments were assessed for significance by *t*-test and one-way analysis of variance (ANOVA).

3 Results

3.1 Epinephrine

As shown in Figs 1 and 2, when *S. canopus* larvae were exposed to 1, 10 and 100 μmol/dm^3 of epinephrine for 1 h and then transferred to fresh seawater for 48 h, settlement rates and completed metamorphosis rates were obviously higher than those in the control. This was especially true in the larvae exposed to 1 μmol/dm^3 of epinephrine. Significant increases in the rates of settlement (36.2 % vs 24.9% in the control) and completed metamorphosis (57.7% vs 45.4% in the control) were observed ($P < 0.05$), indicating that 1 μmol/dm^3 of epinephrine induces *S. canopus* larvae to settle and metamorphose. In contrast, when the concentration of epinephrine reached 1 mmol/dm^3, larval settlement was inhibited (3.7% larvae settled vs 24.9% in the control) and metamorphosis was significantly suppressed too (3.1 % vs 45.4 % in the control, $P < 0.01$).

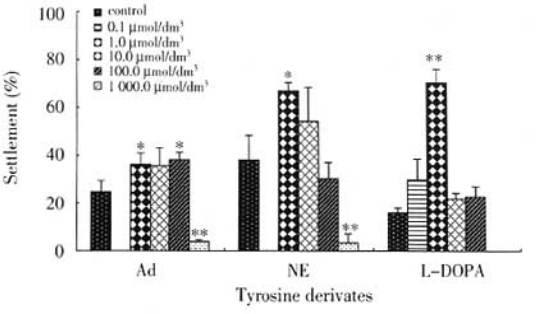

Fig. 1. Percentage of settlement in *S. canopus* larvae after exposure to varying concentrations of epinephrine, norepinephrine and L-DOPA respectively for 1 h and transference to fresh seawater for 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars. * indicates significant difference from the control, $P < 0.05$; * * indicates very significant difference from the control, $P < 0.01$.

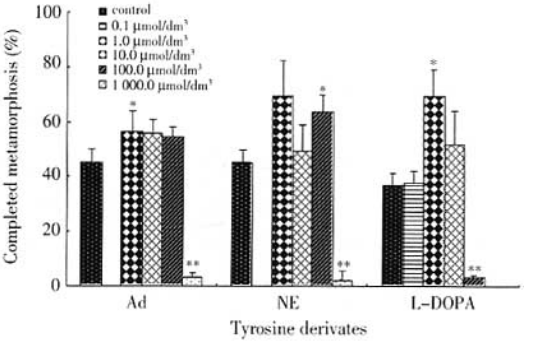

Fig. 2. Percentage of completed metamorphosis in *S. canopus* larvae after exposure to varying concentrations of epinephrine, norepinephrine and L-DOPA respectively for 1 h and transference to fresh seawater for 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars. * indicates significant difference from the control, $P < 0.05$; * * indicates very significant difference from the control, $P < 0.01$.

3.2 Norepinephrine

As shown in Figs 1 and 2, treatment of norepinephrine at 1 μmol/dm^3 markedly increased the rates of settlement (53.6% vs 38.3% in the control) and completed metamorphosis (69.3% vs

45.1% in the control), showing an inductive effect of norepinephrine at 1 μmol/dm^3 on larval settlement and metamorphosis in *S. canopus*. On the other hand, there were no significant differences in the rates of settlement and completed metamorphosis between the treatment of norepinephrine at 10 μmol/dm^3 and the control. Although treatment with 0.1 mmol/dm^3 norepinephrine increased the rate of completed metamorphosis, no marked difference in the rate of larval settlement was noted. Low incidences of settlement (3.4%) and completed metamorphosis (2.1%) were observed at the level of 1 mmol/dm^3 for norepinephrine ($P < 0.01$), indicating the inhibitive effect of high concentrations of norepinephrine on the settlement and metamorphosis of *S. canopus* larvae.

3.3 L-DOPA

The maximum number of settled larvae (70.4% vs 16.2% in the control) and that of metamorphosed larvae (69.3 % vs 36.9 % in the control) were both observed in the treatment with 1 μmol/dm^3 L-DOPA (see Figs 1 and 2). When at treatments with 0.1, 10.0 and 100.0 μmol/dm^3 L-DOPA, settlement and completed metamorphosis rates showed no marked differences from the control, except that the completed metamorphosis rate in the treatment of L-DOPA at 0.1 mmol/dm^3 was lower than that in the control. 1 μmol/dm^3 is the optimal concentration of L-DOPA to induce *S. canopus* tadpole larvae to settle and metamorphose.

As shown in Fig. 3, when *S. canopus* larvae were exposed to 1 μmol/dm^3 of L-DOPA for 1 h and then transferred to fresh seawater for 6 h, settlement and completed metamorphosis rates both had no significant differences from the control but after 12 h showed marked increases above the control ($P < 0.01$). We detected significant increases in the number of settled larvae and metamorphosed larvae from the control only when *S. canopus* larvae were transferred to fresh seawater for at least 12 h after exposure to 1 μmol/dm^3 L-DOPA for 1 h. On the other hand, when *S. canopus* larvae were exposed to 0.1 mmol/dm^3 L-DOPA for 1 h and then transferred to fresh seawater for 24, 32 and 48 h, completed metamorphosis rates were all obviously lower than those in the control but the settlement rates showed no significant differences from the control. We conclude that L-DOPA at 0.1 mmol/dm^3 inhibited *S. canopus* larval metamorphosis only, without affecting settlement rates.

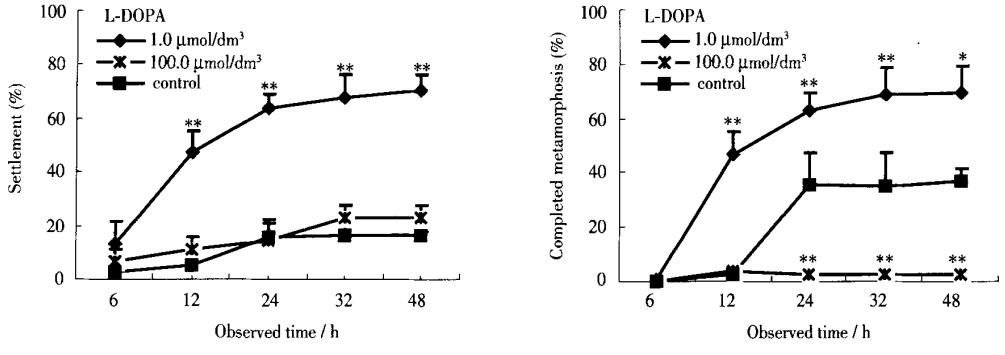

Fig. 3. Percentage of settlement and completed metamorphosis in *S. canopus* larvae after exposure to 1 and 100 μmol/dm^3 L-DOPA respectively for 1 h and transference to fresh seawater for various amounts of time. Data are averages of triplicates, with standard deviations indicated by vertical bars. * indicates significant difference from the control, $P < 0.05$; * * indicates very significant difference from the control, $P < 0.01$.

3.4 GABA

The response of *S. canopus* larvae to the increased external GABA in natural seawater was shown in Figs 4 and 5. Compared with the control, significant reductions in larval settlement and completed metamorphosis rates were observed at the concentrations of 0.1, 1.0, 10.0 and 100.0 μmol/dm^3, showing an inhibitory effect of GABA on larval settlement and metamorphosis in *S. canopus*. Furthermore , no significant differences in settlement and completed metamorphosis rates between concentrations of 0.1, 1.0, 10.0 and 100.0 μmol/dm^3 were detected, indicating that *S. canopus* larvae are inhibited to settle and metamorphose with the similar intensity by GABA at the concentrations over a range of 0.1 ~ 100.0 μmol/dm^3.

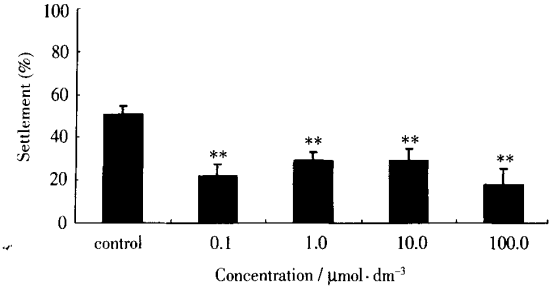

Fig. 4. Percentage of settlement in *S. canopus* larvae in response to continuous exposure to varying concentrations of GABA for 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars. * indicates significant difference from the control, $P<0.05$; * * indicates very significant difference from the control, $P<0.01$.

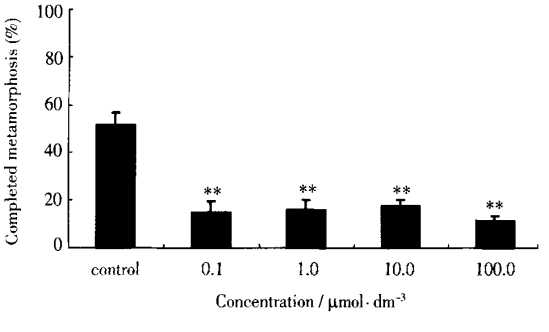

Fig. 5. Percentage of completed metamorphosis in *S. canopus* larvae in response to continuous exposure to varying concentrations of GABA for 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars. * indicates significant difference from the control, $P<0.05$; * * indicates very significant difference from the control, $P<0.01$.

3.5 Thyroxin

Figures 6 and 7 showed respectively the percentages of *S. canopus* larvae having settled and metamorphosed after 48 h of continuous exposure to vari-

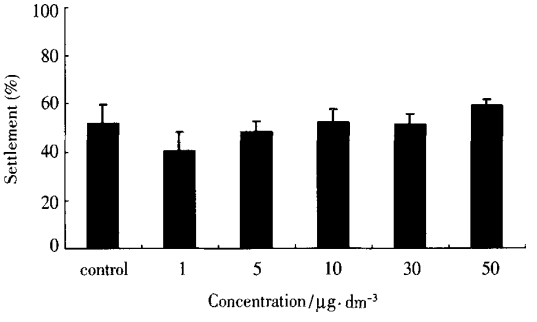

Fig. 6. Percentage of settlement in *S. canopus* larvae in response to continuous exposure to varying concentrations of thyroxin for 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars. * indicates significant difference from the control, $P<0.05$; * * indicates very significant difference from the control, $P<0.01$.

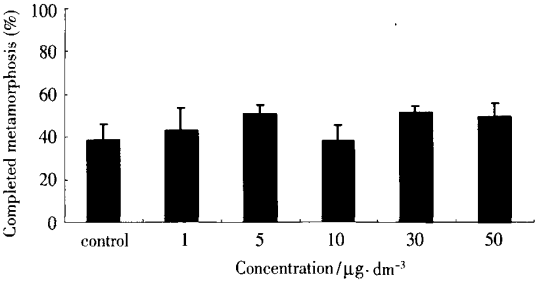

Fig. 7. Percentage of completed metamorphosis in *S. canopus* larvae in response to continuous exposure to varying concentrations of thyroxin for 48 h. Data are averages of triplicates, with standard deviations indicated by vertical bars. * indicates significant difference from the control, $P<0.05$; * * indicates very significant difference from the control, $P<0.01$.

ous concentrations of thyroxin. Compared to the control, neither marked increases nor decreases of larval settlement or completed metamorphosis rates were found at the thyroxin concentrations of 1 ~ 50 μg/dm^3, indicating that thyroxin at the concentrations of 1 ~ 50 μg/dm^3 had no significant effect on the settlement and metamorphosis of *S. canopus* larvae.

4 Discussion

A behavioral settlement process and a morphological metamorphosis process are required for planktonic larvae of most marine benthos to progress to the benthic adult phase. During these two processes, the nervous system plays an important role. Ascidians are a major group of benthic marine organisms, and it is thought that the adhesive papillae of its planktonic larvae receive stimuli from the environment or chemical inductive cues for settlement of larvae (Hirai, 1964). There is evidence suggesting that the adhesive papillae and the brain vesicle are connected with neurons in ascidian planktonic larvae and these nervous structures in ascidian adhesive papillae contribute toward recognition of the substratum and the initiation of metamorphosis (Takamura, 1998). It has also been suggested that neurotransmitter signaling through the nervous system is an integral step in signal pathways for ascidian metamorphosis (Kimura et al., 2003). With regard to the effects of neurotransmitters on the settlement and metamorphosis of ascidian larvae, epinephrine, norepinephrine and acetylcholine have been reported to induce larval settlement and metamorphosis in two ascidian species: *Ascidia malaca* (Patricolo et al., 1981) and *Ciona Savignyi* (Kimura et al., 2003).

In the present work, we examined the influences of epinephrine, norepinephrine, L-DOPA, GABA and thyroxin on the settlement and metamorphosis of tadpole larvae in *Styela canopus*. L-DOPA, epinephrine and norepinephrine are tyrosine derivatives with a variety of biological functions; they act as hormones, neurotransmitters, pigments and adhesive and structural proteins (Rodriguez et al., 1993). Because the oxidation of epinephrine, norepinephrine and L-DOPA occur readily in seawater, the experimental results we obtained are not the effects of epinephrine, norepinephrine or L-DOPA but the effects of a mixture of them and their oxidated products at various concentrations in the course of the experiment (48 h) on the larval settlement and metamorphosis of *S. canopus*. According to our preliminary experiments, the minimum time required for *S. canopus* larvae to be irreversibly affected by each of the three neurotransmitters is 1 h. Therefore, in our experiments, larvae were exposed to various concentrations of epinephrine, norepinephrine and L-DOPA for 1 h, then removed and placed in fresh seawater for 48 h.

The results of our work showed that epinephrine, norepinephrine and L-DOPA at the concentration of 1 μmol/dm^3 induced larval settlement and metamorphosis in *S. canopus*. The inhibitory effects of the three compounds at high concentrations on larval settlement and metamorphosis were also found. However, epinephrine and norepinephrine had little effect on the metamorphosis of *Halocynthia roretzi* (Kimura et al., 2003). And L-DOPA did not promote larval metamorphosis in *Herdmania momus* and *Ciona savignyi* too (Degnan et al., 1997; Kimura et al., 2003). These findings suggest that the effects of epinephrine, norepinephrine and L-DOPA on larval settlement and metamorphosis in ascidians varies with species, indicating that the evolution of settlement/metamorphosis mechanisms in the ascidian is not fully conservative. On the other hand, according to the results of the present work, when *S. canopus* larvae were exposed to 1 μmol/dm^3 of L-DOPA for a short time (1 h) and then transferred to fresh seawater for only 12 h, settlement and completed metamorphosis rates showed significant increases above the

control, indicating that larvae of *S. canopus* can be rapidly induced to settle and metamorphose by short exposure to 1 μmol/dm^3 of L-DOPA. This may result from rapid binding of L-DOPA and its corresponding receptor, which mediates the processes of settlement and metamorphosis. Furthermore, L-DOPA at 0.1 mmol/dm^3 inhibited the larval metamorphosis in *S. canopus* only, not the settlement. This implies that settlement and metamorphosis of *S. canopus* larvae are two processes controlled by different mechanisms and the settlement process exhibits lower sensitivity to high concentrations of L-DOPA. Whether settlement and metamorphosis of *S. canopus* larvae are induced by epinephrine, norepinephrine, L-DOPA or their structural analogs in the field remains to be investigated.

GABA is an inhibitory neurotransmitter and induces hyperpolarization of post-synaptic membranes by means of an increase in membrane permeability to chloride ions (Kuffler et al., 1984; Baloun and Morse, 1984). As far as the influence of GABA on ascidian larval settlement and metamorphosis is concerned, Degnan et al. (1997) found that GABA did not promote larval metamorphosis of *Herdmania momus* and Kimura et al. (2003) reported that GABA had little effect on metamorphosis in *Ciona savignyi* larvae. In the present work, GABA at the concentrations of 0.1 ~ 100.0 μmol/dm^3 significantly inhibited larval settlement and metamorphosis in *S. canopus*. These findings imply that the response of ascidian larvae to GABA is also species-specific.

Thyroxin, one of the iodinated hormones produced, has been reported to induce and mediate many physiological and biochemical functions, especially the development of nervous system. With regard to the influence of thyroxin on the settlement and metamorphosis of ascidian tadpole larvae, Patricolo et al. (1981) and Patricolo et al. (2001) reported that thyroxin induced larvae to metamorphose not only in *Ascidia malaca* but also in *Ciona intestinalis*. But Kimura et al. (2003) demonstrated that thyroxin did not promote larval metamorphosis of *Ciona savignyi*. Our data provided evidence that thyroxin has little effect on the settlement and metamorphosis of *S. canopus* larvae. On the basis of these findings, it is suggested that the response of larvae to thyroxin varies with ascidian species, which may result from the differences in the mechanisms of metamorphosis between ascidian species (Cloney et al., 1971).

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