# INDUCTION OF SETTLEMENT AND METAMORPHOSIS IN THE TROPICAL OYSTER, *CRASSOSTEA BELCHERI* (SOWERBY), BY NEUROACTIVE COMPOUNDS

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***ABSTRACT*** Larvae of the tropical oyster *Crassostrea belcheri* set and metamorphosed when exposed to epinephrine (EPI), norepinephrine (NE), L-3,4-dihydroxyphenylalanine (L-DOPA) and gamma-amino butyric acid (GABA). The optimum concentration of these neuroactive compounds was $10^{-5}$ M when larval set increased from 20% in the controls to between 60–80%. Larval setting performances were generally depressed when exposure was extended to 24 hours compared to 1 hour. These results extend out knowledge of induced settlement in tropical oyster species and provides a useful technique for enhancing seed production in tropical bivalve hatchery operations.

***KEY WORDS:*** Induction, settlement, metamorphosis, oyster, neuroactive compounds, *Crassostrea belcheri*

## INTRODUCTION

Swimming larvae of many benthic marine bivalves remain in the plankton for a period of time before undergoing metamorphosis into the juvenile stage. This metamorphic event usually occurs in conjunction with settlement.

The settlement response in numerous bivalve larvae is regulated by intrinsic and extrinsic factors, including heredity, age, nutritional history of the larvae as well as physical and chemical characteristics of available substrate (Hatfield 1984).

Previous studies on oyster larvae have shown that neuroactive compounds like epinephrine, norepinephrine, L-3,4-dihydroxyphenylalanine and gamma-aminobutyric acid have influenced setting and metamorphosis in *Crassostrea gigas* (Coon et al. 1985, 1986, Cooper 1983, Cooper and Shaw 1984), *C. virginica* (Walch et al. 1988) and *Ostrea edulis* (Shpigel et al. 1989). However, nothing has been reported on the effects of neuroactive compounds on the setting of the tropical oyster *Crassostrea belcheri*. The aim of this study was to test the setting response of *C. belcheri* larvae towards neuroactive compounds, as an attempt to optimize the setting performance of tropical oyster larvae in the hatchery.

## MATERIALS AND METHODS

Larvae of *C. belcheri* were obtained using standard culture techniques described in Wong et al. (1989). Eyed larvae of *C. belcheri* were cultured using 1 μm filtered seawater (18 ppt). Culture media were changed on alternate days. A prescribed diet of *Isochrysis galbana* (Tahitian strain) was provided daily. Metamorphic competence was ascertained by the presence of well-developed eyes and a predilection to metamorphose on the surfaces of the holding tanks or on cultch provided.

Competent eyed larvae collected in a 253 μm nitex sieve were used in all experiments. The neuroactive compounds tested were epinephrine (EPI), norepinephrine (NE), L-3,4-dihydroxyphenylalanine (L-DOPA) and gamma-amino butyric acid (GABA) at concentrations of $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$ M.

The experiments were conducted in plastic tissue culture plates (24 wells; Falcon #3047) using 1 μm filtered seawater mixed with fresh water (salinity: 18 ppt) at room temperature (26–28°C). EPI and NE were dissolved in 0.0005 N hydrochloric acid while L-DOPA and GABA were prepared as 10 × stock solutions in distilled water. For experiments, the stock solutions were diluted (1:9) in seawater to achieve the desired final experimental concentrations.

All test treatments were conducted in triplicate. In each experimental replicate, 50–100 larvae were placed in a total volume of 2 mL of test solution. A few washed marble chips measuring 3–4 mm in diameter were placed at the bottom as cultch. Larvae were exposed to various concentrations of the test compounds for 1 hour or 24 hours, then removed, rinsed and placed in fresh 18 ppt medium for the remainder of the observation period (24–48 hours). Food was not provided during the experiment. Controls were kept in 18 ppt with the same number of marble chips. In all experiments, the larvae were examined regularly with a dissecting microscope to monitor behaviour patterns or transient-responses to the test compounds.

At the end of each experiment, all wells were examined to determine the percentage of the total number of larvae which had set and/or metamorphosed. A larva was categorized as metamorphosed if it showed significant new shell growth. Spat that could not be washed off by using a water jet from a Pasteur pipette were recorded as "cemented." The experimental procedures were modified from those reported in Coon et al. (1985).

All statistical comparisons were conducted using one-way analysis of variance at a 95% level of significance. If statistical significance was noted, then a Duncan's Multiple Range Test was conducted to determine which treatments were significantly different.

## RESULTS

Observations on the behavioural responses of *C. belcheri* eyed larvae when exposed to the various test compounds showed that they were similar to those reported by Coon et al. (1985) for *C.*

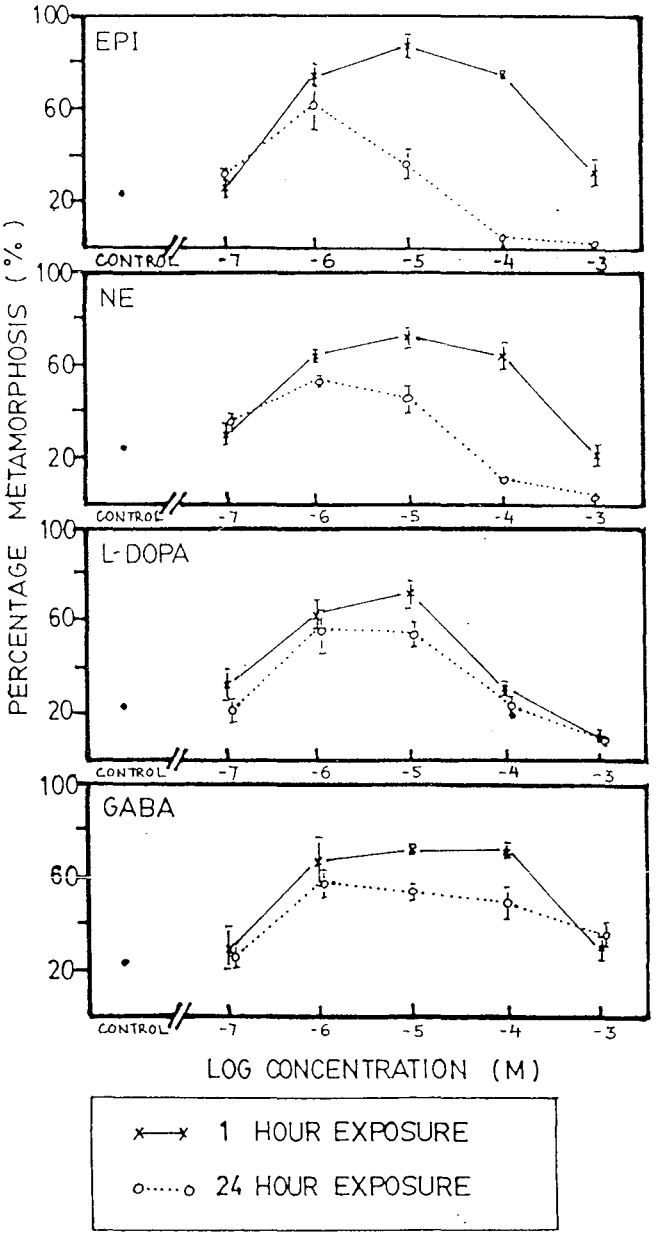


**Figure 1. The percentage of *Crassostrea belcheri* eyed larvae which metamorphosed in response to 1 hour and 24 hour exposures to various concentrations of test compounds.**

*gigas*. On exposure to EPI and NE, the larvae sank to the bottom of the well and immediately began morphogenetic differentiation. On the other hand, larvae exposed to both L-DOPA and GABA swam with their foot extended followed by crawling along the substratum. Subsequently, they cemented themselves and underwent metamorphosis.

Figure 1 shows the percentage of eyed larvae which had metamorphosed (when scored 4 days later) after 1 hour and 24 hours exposure to various concentrations of the test compounds. Setting performance was generally depressed when larvae were exposured for 24 hours, sometimes to levels lower than controls. In the case of 1 hour exposure, all 4 compounds increased the portion of larvae setting, with the best concentration at $10^{-5}$ M.

Details of statistical analysis on the setting performances for 1 hour and 24 hours exposure are summarised in Tables 1 and 2 respectively. The highest larval setting occurred in EPI at $10^{-5}$ M (87.0 ± 5.3%). This was significantly higher ($P > 0.05$) than those recorded for NE, L-DOPA and GABA at the same concentration (70.1–71.8%). Concentrations of L-DOPA above $10^{-4}$ M caused a variety of toxic effects including reduced larval activity, withdrawal of all tissues into the larval shell and increased mortality.

Figures 2 and 3 show the relative proportion of spat which were cemented after 1 hour and 24 hours exposure to various concentrations of the test compounds. EPI at $10^{-4}$ M yielded the highest percentage of free (cultchless) spat followed by EPI at $10^{-5}$ M and NE at $10^{-4}$ M. In comparison, the yield of free spat was negligible with L-DOPA and GABA. Once again, exposure for 24 hours resulted in lower number of free spat.

## DISCUSSION

The result of this study demonstrates that larvae of the tropical oyster, *C. belcheri* can be induced to set by exposure to the compounds EPI, NE, L-DOPA and GABA. Exposure for 1-hour period increased larval setting success up to 3.5 times that controls, but prolonged exposure to some of these resulted in reduced effects sometimes to levels below controls.

The optimum concentration of EPI for larval settlement in *C. belcheri* is lower than the $10^{-4}$ M reported for *C. iredalei* (Leu 1990), *C. gigas* and *C. virginica* (Coon et al. 1985, 1986) as well as *C. lugubris* and *Saccostrea commercialis* (Jarayabhand, pers. comm.). However, the optimum concentration of NE ($10^{-5}$ M) for *C. belcheri* is comparable to those reported for *C. lugubris* and *S. commercialis* (Jarayabhand, pers. comm.) though lower than

**TABLE 1.**

**Summary of statistical analysis on mean setting percentage when larvae were exposed to different concentrations of the test compounds for 1 hour.**

| Concentration (M) / Treatment | Percentage Setting (%) (m ± s.d.) | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | Control |
| EPI | 26.7 ± 3.5[b,c] | 74.0 ± 4.5[a] | 87.0 ± 5.3 | 74.4 ± 1.2[a] | 32.8 ± 5.1[b] | 24.5 ± 1.5[c,e,f,j] |
| NE | 30.1 ± 5.7[e] | 64.3 ± 2.6[d] | 70.6 ± 5.8[d] | 64.4 ± 5.5[d] | 21.8 ± 4.3[f] | |
| L-DOPA | 32.4 ± 6.7[h] | 63.7 ± 6.5[g] | 71.4 ± 5.9[g] | 31.6 ± 2.3[h] | 11.3 ± 3.1 | |
| GABA | 29.5 ± 9.1[j] | 66.9 ± 11.1[i] | 71.8 ± 2.3[i] | 71.8 ± 3.4[i] | 29.8 ± 5.7[i] | |

Data sets bearing the same letters are not significantly different ($P < 0.005$).

**TABLE 2.**

**Summary of statistical analysis on mean setting percentage when larvae were exposed to different concentrations of the test compounds for 24 hours.**

| Concentration (M) / Treatment | Percentage Setting (%) (m ± s.d.) | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | Control |
| EPI | 31.9 ± 1.8[a] | 61.8 ± 9.9 | 36.4 ± 6.7[a] | 4.3 ± 2.0 | 0.9 ± 0.1 | 24.5 ± 1.5[e,h] |
| NE | 35.7 ± 4.3[c] | 52.4 ± 3.3[b] | 44.7 ± 6.7[b,c] | 10.1 ± 1.1 | 3.7 ± 1.0 | |
| L-DOPA | 21.9 ± 5.4[e] | 56.0 ± 9.8[d] | 54.4 ± 6.0[d] | 23.9 ± 5.4[e] | 9.4 ± 2.3 | |
| GABA | 26.1 ± 5.6[g,h] | 58.1 ± 5.5[f] | 54.4 ± 3.1[f] | 49.8 ± 6.1[f] | 35.7 ± 4.8[g] | |

Data sets bearing the same letters are not significantly different ($P < 0.005$).

the $10^{-4}$ M reported for *C. iredalei* (Leu, 1990), *C. gigas* and *C. virginica* (Coon et al. 1985, 1986).

Prolonged exposure to EPI and NE at concentrations higher than $10^{-5}$ M resulted in reduced metamorphic induction, reduced rates of morphogenetic differentiation and increased mortality. Similar observations were reported for *C. iredalei* (Leu 1990), *C. lugubris* and *S. commercialis* (Jarayabhand, pers. comm.). These observations differ from those of Coon et al. (1985, 1986) who

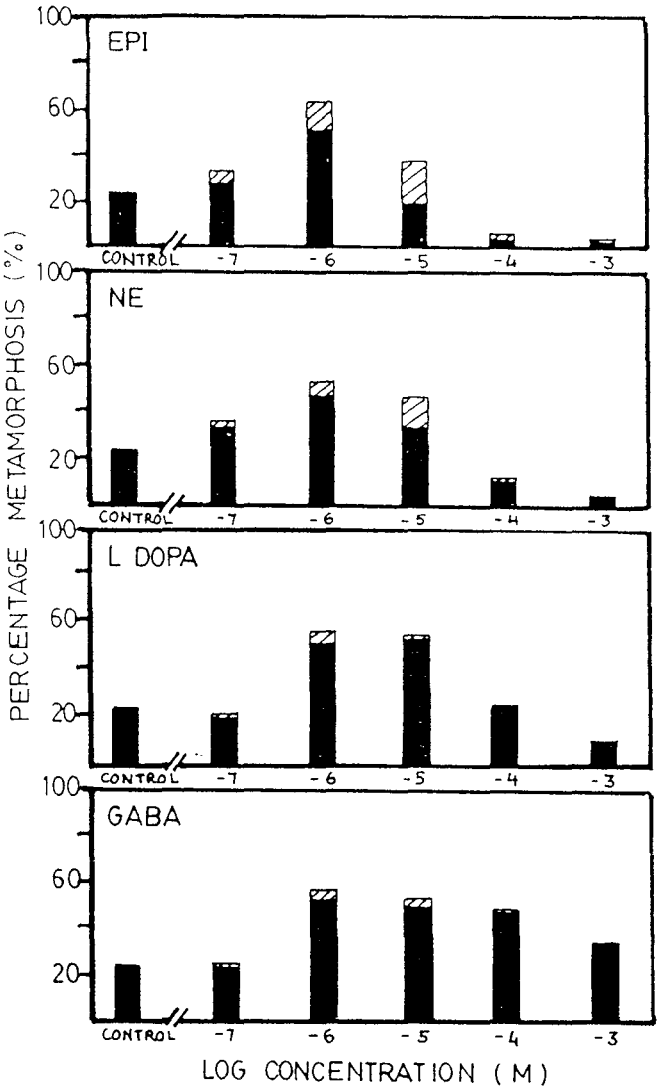


**Figure 2. The relative proportion of *Crassostrea belcheri* spat which were cemented (■) and free (□) in response to a 1 hour exposure to various concentrations of test compounds.**

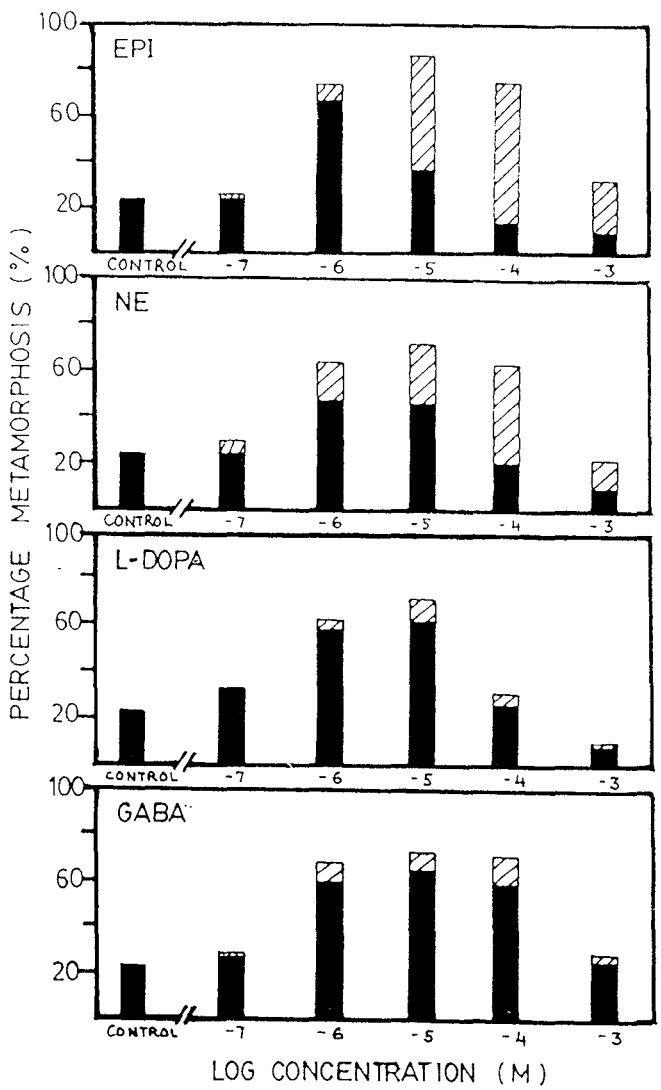


**Figure 3. The relative proportion of *Crassostrea belcheri* spat which were cemented (■) and free (□) in response to 24 hour exposure to various concentrations of test compounds.**

reported that in *C. gigas* prolonged exposure to EPI and NE at $10^{-4}$ M resulted in increased percentage set from 80 to 90% and 50 to 80%, respectively.

The percentage of free spat resulting from EPI and NE treatment (10–80%) was comparable to that reported for *C. gigas* (85–90%) and *C. virginica* (65%) (Coon et al. 1986). However, only 38.7% of *C. iredalei* spat was reported as cultchless by Leu (1990). According to Coon et al. (1986), the mechanism through which EPI and NE are able to induce metamorphosis independent of settlement behaviour is mediated through vertebrate type alpha-adrenergic receptors. Because EPI triggers metamorphosis without the settlement behaviours that normally preceded it, these receptors presumably function later, or on a different pathway in the chain of events that comprise settlement and metamorphosis, than the events that culminate in attachment.

Setting performance of *C. belcheri* eyed larvae can also be enhanced via a 1-hour exposure to L-DOPA at $10^{-4}$ to $10^{-7}$ M with highest setting percentage at $10^{-5}$ and $10^{-6}$ M. Prolonged exposure to L-DOPA at $10^{-3}$ M resulted in a lowering of the percentage set in *C. belcheri*. In general, the response of *C. belcheri* eyed larvae to L-DOPA paralleled those reported for *C. lugubris* and *S. commercialis* (Jarayabhand, pers. comm.), *C. gigas* (Cooper 1983, Coon et al. 1985) and *C. iredalei* (Leu 1990). However, L-DOPA was not effective in inducing metamorphosis in *Chlamys hastata* (Hodgson and Bourne 1988). The same authors reported that when higher concentrations of the drug were tested ($10^{-4}$ and $10^{-5}$ M) they were found to be toxic to the larvae. Prolonged exposure to L-DOPA caused complete withdrawal of all tissues into the larval shell. Thus, even when L-DOPA induced metamorphosis, prolonged continuous exposure to the drug reduced the rate of morphogenetic differentiation.

The mechanism of induction as well as a model of natural settlement and metamorphosis has been proposed by Coon et al. (1985) based on the observations of the response of *C. gigas* to EPI, NE and L-DOPA. Since L-DOPA induces behaviours exhibited by the larvae in response to natural environmental stimuli and results in a complete response including both settlement and metamorphosis, L-DOPA or some L-DOPA-mimetic molecules could be sufficient environmental stimuli to initiate settlement. However, there is presently no evidence that L-DOPA, EPI or NE occurs in the $10^{-4}$ to $10^{-5}$ M range in nature.

The results presented here also show that GABA is effective in inducing *C. belcheri* larvae to set and metamorphose. Larvae exposed to $10^{-4}$ to $10^{-6}$ M GABA showed higher setting percentages (70–71%) compared to the controls (24.5%); whereas there was no significant difference at $10^{-3}$ and $10^{-7}$ M compared to controls. The setting performance of *C. belcheri* eyed larvae exposed to $10^{-3}$ M GABA for 24 hours was enhanced. This may be explained according to the hypothesis proposed by Morse et al. (1980), where metamorphic responses of the abalone larvae, *Haliotis* sp. to GABA was slow due to the lack of suitable receptors. Unlike EPI, NE and L-DOPA, GABA did not exhibit toxic effects on *C. belcheri* larvae during prolonged exposure (24 hours). However, GABA was reported to be ineffective in causing bivalve larvae such as *C. gigas* (Coon et al. 1985), *C. hastata* (Hodgson and Bourne, 1988), *Perna viridis* (Baylon 1988) and *C. iredalei* (Leu 1990) to set or metamorphose.

Though post-test survival was not studied here, Coon et al. (1985) and Shpigel et al. (1989) have demonstrated that EPI induced spat of *C. gigas* and *O. edulis*, respectively, had appeared completely normal after 3 weeks to 8 months growth.

The reasons for the differences between *C. belcheri* and *C. gigas* and *C. virginica* as well as other tropical species such as *C. iredalei, C. lugubris* and *S. commercialis* in both the magnitude and consistency of their response to the neuroactive compounds (EPI, NE, L-DOPA and GABA) are not clear. Further research is needed to determine whether the differences in the induction of metamorphosis of bivalve larvae by neuroactive compounds is governed by species-specific factors.

## ACKNOWLEDGMENTS

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