Effects of GABA and epinephrine on the settlement and metamorphosis of the larvae of four species of bivalve molluscs


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Abstract

Competent larvae of different marine bivalve species were treated with GABA and epinephrine at different concentrations and times of exposure to test the ability of the drugs to induce settlement and metamorphosis. GABA induced both settlement and metamorphosis in the mussel *Mytilus galloprovincialis*, the clams *Venerupis pullastra* and *Ruditapes philippinarum* and the oyster *Ostrea edulis*. Maximum induction of settlement (>39%) was achieved after exposure of *V. pullastra* larvae to $10^{-4}$ M GABA; this concentration of GABA also induced the highest percentages of metamorphosis in the four species studied. Epinephrine was identified as an active inducer of settlement and metamorphosis in bivalve molluscs. Exposure to $10^{-5}$ M epinephrine induced significant levels of settlement in *Mytilus*, *Venerupis* and *Ostrea*. In contrast, epinephrine failed to induce settlement behaviour in *Ruditapes*. Maximum induction of metamorphosis was produced by $10^{-5}$ M epinephrine in mussels, clams and oysters; *Ruditapes* showed the highest percentage of metamorphosis (>78%). This is the first report in which the involvement of GABA in the settlement and metamorphosis of bivalve molluscan larvae is demonstrated. It was also recognised that epinephrine plays a role not only in inducing metamorphosis but also in initiating settlement.

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1. Introduction

Researchers and hatchery staff have been trying to develop and improve methods of producing mollusc spat for many years. Difficulties in the culture of bivalve molluscs are mainly associated with settlement and metamorphosis. Planktonic marine molluscan larvae must develop competence and recognize an appropriate exogenous morphogenetic cue before they can settle and metamorphose. Induction of metamorphosis thus depends on both the developmental state of the larva and the perception of a morphogenetic cue...
The molecular mechanisms underlying the acquisition of larval competence and the induction of metamorphosis in marine molluscs are not well understood, although a variety of morphogenetic inducers have recently been identified (Beiras and Widdows, 1995; Boettcher and Targett, 1998; Pires et al., 2000). The planktonic larvae of many marine invertebrates preferentially settle and metamorphose in habitats well suited to subsequent adult life. Early laboratory studies have indicated that the most preferred settlement surfaces are horizontal, of a rough texture and already colonized by adults of the same species. Settlement is also facilitated by abundance of food, illumination and medium to high larval densities (Beiras and Widdows, 1995).

Much of the current research on the subject of larval settlement focuses on the chemical identity of inducers and the signal-transduction mechanisms involved. A wide variety of biotic and chemical substances have been found to be capable of inducing settlement and metamorphosis in larva of different species of marine invertebrates. Several environmental factors may induce invertebrate larval settlement and/or metamorphosis, particularly the nature of substratum, some dissolved compounds or the biofilm colonizing the substratum. Fitt et al. (1990) described the ability of oyster larvae both to initiate settlement behaviour and to complete metamorphosis in response to a variety of bacterial supernatants. Bacterial films have been shown to be involved in inducing settlement of oyster and sea urchin larvae (Tritar et al., 1992; Naidenko, 1996). Satuito et al. (1995) also reported that larvae of *Mytilus edulis galloprovincialis* attached and metamorphosed when exposed to microbial films. It has been shown that mature larvae of the mussel *Mytilus edulis* generally attach to filamentous substrates (Bayne, 1964; Easter and Pechenik, 1987).

Competent molluscan larvae can be induced to settle and begin metamorphosis by functional analogues of these natural inducers. Swimming planktonic larvae of the marine gastropod mollusc *Haliotis rufescens* have been induced to settle and undergo metamorphosis by metabolites of certain red algae and by γ-aminobutyric acid (GABA; Morse et al., 1980; Morse, 1992). GABA also stimulated attachment behaviour and induced metamorphosis in *Haliotis diversicolor* (Bryan and Qian, 1998) and in sea urchins (Rahmani and Ueharai, 2001). Metamorphosis is increased by the catecholamine L-DOPA in the gastropod *Phestilla sibogae* (Pires et al., 2000). Cooper (1982) also showed that mussels and oysters undergo metamorphosis in response to L-DOPA. Coon et al. (1985, 1986) corroborated the inductive activity of L-DOPA in the settlement of the oyster, *Crassostrea gigas* and identified two new inducers, epinephrine and norepinephrine. Furthermore, Estupinan and Waite (1988) reported that a DOPA-containing protein significantly stimulated the settlement of mussel larvae. DOPA induction of settlement in *M. edulis* has also been shown by Dobretso and Qian (2003).

We are interested in the molecular mechanisms underlying the processes of settlement and metamorphosis in bivalve molluscs and the responsiveness of these species to chemical inducers. In this study, we examined the effect of GABA and epinephrine on the settlement and metamorphosis of different marine bivalve species, namely, the oyster *Ostrea edulis* (L.), the clams *Venerupis pullastra* (Montagu) and *Ruditapes philippinarum* (Adams and Reeve) and the mussel *Mytilus galloprovincialis* (Lmk.).

## 2. Materials and methods

### 2.1. Larval culture

Adult mussels (*M. galloprovincialis*), clams (*R. philippinarum*, Adams and Reeve and *V. pullastra*) and flat oysters (*O. edulis*) were collected from Ría de Arousa, Galicia, Spain. The spawning of mussels and clams was induced in the laboratory by first cooling the molluscs to 12±2 °C for 30 min before transferring them to a container filled with seawater at 20±5 °C. The seawater containing sperm and eggs was gently mixed and left undisturbed. After fertilization, the larvae were collected and cultured at 18±2 °C. Oyster larvae were obtained from adult oysters maintained in a container filled with seawater at 18±2 °C for 6–8 weeks. After this treatment, oyster started spawning, and the larvae were released.

Larvae were fed with a mixed diet of *Isochrysis galbana*, *Isocrysis galbana tahití*, *Monocrysis lutheri*, *Chaetoceros calcitraus* and *Skeletonema costatum*.
at a concentration of 100 cells ml\(^{-1}\). In all experiments, veliger larvae were maintained for 14–20 days before harvesting with a 240-µm mesh.

2.2. GABA and epinephrine

Epinephrine and GABA (\(\gamma\)-aminobutyric acid) were obtained from Sigma (St. Louis, MO). Epinephrine was dissolved in 0.005 N HCl and diluted (1:9) in MilliQ sterile water (10\(^{-3}\) M). GABA was dissolved in MilliQ sterile water (10\(^{-3}\) M). Epinephrine and GABA were dissolved in seawater containing the larvae to achieve the final experimental concentration required.

2.3. Metamorphosis assays

Experiments were carried out in triplicate in 100-ml Pyrex tubes in a final volume of 50 ml U.V.-sterilized, 10 µm filtered seawater (FSW). The larval density was 40 larvae ml\(^{-1}\) for \(M.\) galloprovincialis, \(R.\) philippinarum and \(V.\) pullastra and 2 larvae ml\(^{-1}\) for \(O.\) edulis.

To determine the optimum concentration of potential chemical inducers for the production of metamorphosis, larvae were exposed to various concentrations (10\(^{-4}\), 10\(^{-5}\) and 10\(^{-6}\) M) of epinephrine or GABA under airlift, with light and at 15±1 °C, for 24 and 48 h. In each assay, a control without potential chemical inducers was set-up under the same experimental conditions.

Larval metamorphosis was monitored after the relevant exposure time—i.e., 24 and 48 h for \(M.\) galloprovincialis, \(R.\) philippinarum and \(V.\) pullastra and 48 h for \(O.\) edulis—using a binocular microscope (Olympus BX50). The percentage metamorphosis was calculated as 100\(\times\)(total number of larvae metamorphosed/total number larvae). A larva was considered to have undergone metamorphosis when it lost its velum and was using its foot to crawl.

2.4. Settlement assays

Experiments were carried out in triplicate polystyrene 90-mm tissue culture Petri plates in 25-ml final volume of U. V. sterilized, 10 µm FSW, in which were placed 25 veligers of \(O.\) edulis or 500±10 veligers of \(M.\) galloprovincialis, \(R.\) philippinarum or \(V.\) pullastra. Each assay also included a control without potential chemical inducers. The concentrations of compounds and experimental conditions were the same as those described above.

Larvae settlement behaviour was monitored after 48 h with a microscope Nikon MSZ-T2. The numbers of unattached or attached spat were expressed as a percentage of settlement (100\(\times\)total number of larvae settled/total number larvae). Spat were categorized as attached if they could not be dislodged from the substrate with a stream of water.

2.5. Settlement assay in industrial water tanks

The industrial settlement experiments were carried out with \(O.\) edulis in the Remagro factory in Galicia (NW Spain) using 100-l polyethylene water tanks containing U.V.-sterilized, 10-µm FSW. Larvae were induced to settle on a 200-µm mesh on which cockleshell triturate had been placed. The larval density used was 5000 larvae ml\(^{-1}\). The larvae were exposed to 0.5\(\times\)10\(^{-5}\) M epinephrine for 48 h under airlift at 19±1 °C. A control tank without potential chemical inducers was also set-up. The percentages of settlement were calculated as previously described.

2.6. Statistical analysis

Three replicates of each treatment were carried out in each experiment. Results were analyzed by means of SPSS 11.0. Percentages of both metamorphosis and settlement were analysed by ANOVA with Tukey’s HSD. The results were considered to be significantly different when \(p<0.05\).

3. Results

3.1. Visual observations

In response to the chemical inducer, \(M.\) galloprovincialis, \(V.\) pullastra, \(R.\) philippinarum and \(O.\) edulis larvae began to crawl by means of the foot, and the velum was either expended or withdrawn. A few larvae were observed swimming abnormally and simply rotating. After 24 h, the velum had completely disappeared, and individuals crawled with the foot. The number of fully developed larvae increased
continuously for 48 h. Other morphological features, such the presence of an eye-spot and/or shell size, were important indicators of the competence of metamorphosis in both mussel and oyster larvae.

3.2. Effect of concentration and exposure time on settlement

The values of the percentage settlement of *M. galloprovincialis*, *V. pullastra*, *R. philippinarum* and *O. edulis* obtained under the different chemical treatments after 48 h are shown in Figs. 1 and 2. GABA and epinephrine were identified as active inducers of settlement for all of the bivalve species under study.

At a concentration of $10^{-4}$ M, GABA was a particularly active inducer of settlement for *M. galloprovincialis* ($p<0.05$), *V. pullastra* ($p<0.05$) and *R. philippinarum* ($p<0.05$; Fig. 1). The percentage settlement of *V. pullastra* larvae (39%) was the highest among all treatments. Larvae of *O. edulis* showed maximum percentages of settlement with both $10^{-4}$ and $10^{-5}$ M GABA ($p<0.05$).

At a concentration of $10^{-5}$ M, epinephrine induced significantly higher settlement behaviour than at the other concentrations tested in *M. galloprovincialis*, *V. pullastra* and *O. edulis* ($p<0.05$; Fig. 2). A significant increase in the percentage settlement was also induced by $10^{-4}$ M epinephrine in *Venerupis* and *Ostrea*. Approximately 21% of the *V. pullastra* larvae were induced to settle at $10^{-5}$ M ($p<0.05$). However, when exposed to epinephrine, the larvae of *R. philippinarum* did not exhibit significantly higher settlement rates than the control larvae.

The results obtained for *O. edulis* in the industrial water tanks using $0.5 \times 10^{-5}$ M epinephrine were similar to those obtained in the laboratory assays (15% attached larvae, $n=2$).

3.3. Effect of concentration and exposure time on metamorphosis

Results varied quantitatively between experiments but were qualitatively consistent. The results presented here are representative of most experiments.

Fig. 3 shows the percentage of larvae induced to metamorphose by continuous exposure to various concentrations of GABA after 24 and 48 h. GABA induced a higher percentage of metamorphosis than in larvae exposed to seawater only (larval control). The larval control showed percentages of metamorphosis of less than 24% for *M. galloprovincialis* and *R. philippinarum* and 53% for *V. pullastra*. Maximal numbers of mussels and clams were induced to metamorphose with $10^{-4}$ M GABA. After 24 h (Fig. 3), $10^{-4}$ M GABA induced highest larval metamorphosis, with percentages of 52% for mussels ($p<0.05$) and approximately 53% for clams ($p<0.05$). Approximately 70% of *R. philippinarum* ($p<0.05$) and 76% of *V. pullastra* ($p<0.05$) larvae were induced to metamorphose with $10^{-4}$ M GABA after 48 h. Similar percentages were obtained by using $10^{-5}$ M GABA. Molar concentrations of $10^{-4}$, $10^{-5}$ and $10^{-6}$ M GABA induced high percentages of metamorphosis in *M. galloprovincialis* ($p<0.05$).

The percentages of *M. galloprovincialis*, *V. pullastra* and *R. philippinarum* larvae induced to metamorphose as a function of the time they were exposed to different concentrations of epinephrine are shown in Fig. 4. Maximum metamorphosis was induced by $10^{-5}$ M epinephrine in both mussels and clams.
However, $10^{-4}$ M epinephrine induced the highest percentage of metamorphosis in *V. pullastra* larvae after 24 h (68.26%), which differed significantly from the larval control ($p < 0.05$). After 48 h, $10^{-4}$, $10^{-5}$ and $10^{-6}$ M epinephrine induced very similar percentages of metamorphosis in mussels, significantly higher than the control ($p < 0.05$). The highest percentage of metamorphosis occurred in *R. philippinarum*, i.e., 78% ($p < 0.05$), with $10^{-5}$ M epinephrine although there was no significant difference to that of *V. pullastra*.

Approximately 15% of control larvae of *O. edulis* had undergone metamorphosis after 48 h (Figs. 3 and 4). The highest percentages of metamorphosis were induced by $10^{-4}$ M GABA and $10^{-5}$ M epinephrine: 59% ($p < 0.05$) and 55.86% ($p < 0.05$), respectively. Other concentrations of both GABA and epinephrine induced lower number of larvae to metamorphose.

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**Fig. 2.** Percentage of *M. galloprovincialis, V. pullastra* and *R. philippinarum* and *O. edulis* larvae induced to settle in response to sea water only (control) or to continuous exposure to $10^{-4}$, $10^{-5}$ and $10^{-6}$ M epinephrine. Experiments lasted 24 and 48 h; all points represent the mean±S.D. of triplicate determinations for each, $n=3$. Asterisks indicate significant difference (*$p<0.05$) from control.

**Fig. 3.** Percentage of *M. galloprovincialis, V. pullastra* and *R. philippinarum* and *O. edulis* larvae induced to metamorphose in response to sea water only (control) or to continuous exposure to $10^{-4}$, $10^{-5}$ and $10^{-6}$ M GABA. Experiments lasted 24 and/or 48 h; all points represent the mean±S.D. of triplicate determinations for each assay, $n=3$. Asterisks indicate significant difference (*$p<0.05$) from control.
The effect of exposure time on the metamorphosis was the same in all the different bivalve larvae used and with the two compounds tested. Exposure to GABA or epinephrine for 48 h always increased the percentages of metamorphosis in comparison to that after 24-h exposure.

The percentages of metamorphosis induced by GABA and epinephrine did not differ significantly except when using a $10^{-6}$ M solution with *V. pullastra* ($p < 0.05$) and a $10^{-4}$ M solution with *M. galloprovincialis* ($p < 0.05$).

### 4. Discussion

Using the terminology previously described by Coon et al. (1985), we can differentiate between settlement and metamorphosis behaviour of competent larvae, as used in this study. Settlement is a reversible response of competent larvae that are searching for an appropriate substratum. The settlement process includes extension of the food, crawling on the substratum and finally the fixation of the larvae to the substratum. Metamorphosis commences with the reception of stimuli produced by an appropriate substratum and continues with morphogenetic processes and is irreversible. Metamorphosis includes loss of the larval feeding organ, development of the gills and production of the adult shell. The transformations that occur during metamorphosis are thought to be controlled by a combination of neuronal and neuroendocrine activities. Several authors have shown that the apical sensory organ of gastropod veliger larvae is involved in the perception of cues for settlement and metamorphosis (Hadfield et al., 2000). The role played by the nervous system during metamorphosis of the nudibranch mollusc, *P. sibogae*, has also been studied by Leise and Hadfield (2000). The range of chemical cues that induce settlement and metamorphosis in many larval invertebrates, including molluscs, appear to be as varied as the number of species that respond to them (Jackson et al., 2002; Lau et al., 2003).

In the present study, we have demonstrated that larvae of bivalve molluscs can be induced to settle and metamorphose by exposure to GABA and the catecholamine, epinephrine. This investigation confirms...
and expands earlier reports of the ability of GABA and epinephrine to induce settlement and metamorphosis in *Haliotis* (Degnan and Morse, 1995) and *Crassostrea* (Coon et al., 1985, 1986; Beiras and Widdows, 1995), respectively, although no recent studies of this kind have been carried out with clams and mussels. In the present study, inductive activity was observed when compounds were added in solution, although previously reported studies found this method of exposure to be ineffective (Naidenko, 1996). The present study is the first to show that the neuroactive compound GABA is effective at inducing settlement and metamorphosis in bivalve molluscs.

GABA was found to be an active inducer of settlement in the four bivalve species studied (Fig. 1). *V. pullastra* showed the highest percentage of settlement, around 39%. High levels of settlement have also been observed in *H. rufescens* after exposure to 10⁻⁶ M GABA (Morse et al., 1980). Epinephrine was also identified as a settlement cue in mussels, clams and oysters except for *R. philippinarum* (Fig. 2). This result is not consistent with previously reported observations where the catecholamines epinephrine and norepinephrine failed to induce oyster larvae to settle but were effective in inducing metamorphosis (Coon et al., 1985, 1986). The percentages of settlement in mussels and oysters obtained in the present study were lower than those obtained by other authors in *M. galloprovincialis* and *C. gigas* by using bacterial films (Fitt et al., 1990; Tritar et al., 1992; Satuito et al., 1995). There may be several explanations for these differences; for example, the use of plastic plates appears to decrease the number of settled larvae; the use of other surfaces may have induced higher settlement rates than the plastic plates used in the present study. However, the percentages of settlement induced in *O. edulis* by GABA and epinephrine are consistent with those induced in *C. gigas* and *O. edulis* by using bacterial films (Tritar et al., 1992) and in *Pinctada margaritifera* by using GABA (Doroudi and Southgate, 2002). Epinephrine failed to induce settlement in *P. margaritifera* (Doroudi and Southgate, 2002).

In the present study, GABA and epinephrine were also identified as active inducers of metamorphosis. The experiments with *O. edulis* (Fig. 3) showed that 10⁻⁴ M GABA induced the highest percentage of metamorphosis (60%). This result is not consistent with that of a previous study in which GABA failed to induced metamorphosis in *C. gigas*, but the neurotransmitter L-DOPA (10⁻⁵ M) was found to be an active inducer (46%; Beiras and Widdows, 1995). GABA has been shown to be an inducer of metamorphosis in gastropods such as *Haliotis* (86% by 10⁻⁶ M GABA: Morse, 1992) and *Strombus gigas* (60–70% by 5×10⁻⁵ M; Boettcher and Targrett, 1998); these were similar to the percentages of mussel and clam larvae induced to metamorphose in the present study (Fig. 3).

The catecholamine epinephrine has also proved to be an active inducer of metamorphosis in *M. galloprovincialis*, *V. pullastra* and *R. philippinarum* (Fig. 4). Maximum percentages of metamorphosis were induced by 10⁻⁵ M epinephrine in both mussels and clams, yielding percentages of metamorphosis of 63–79%. Catecholamines also modulated metamorphosis in the gastropod *P. sibogae* (Pires et al., 2000). Lower levels of metamorphosis were observed in *O. edulis* when using 10⁻⁵ M epinephrine as an inducer, approximately 56% after 48 h (Fig. 4). These results are consistent with the percentage metamorphosis obtained by Coon et al. (1986) with *C. virginica* larvae and 10⁻⁴ M epinephrine. However, higher levels of metamorphosis were observed in *C. gigas* with 10⁻⁴ M epinephrine (Coon et al., 1986; Beiras and Widdows, 1995).

Previously reported differences in the efficacy of different chemicals in inducing settlement and metamorphosis may be due to a number of factors, including species differences and also age and qualitative differences amongst batches of cultures of the same species, resulting in a variety of degrees of larval competence and responsiveness to chemical inducers.

The mechanism by which GABA and epinephrine are able to induce metamorphosis is poorly understood. Coon et al. (1986) proposed a model of oyster settlement and metamorphosis which includes induction of settlement by environmental cues and induction of metamorphosis by endogenous neuroactive factors that can be mediated through adrenergic receptors. There is evidence suggesting that the receptors that recognize natural algal inducers are the same receptors that recognize GABA in the induction of settlement and metamorphosis (Morse, 1992). It has also been suggested that the neuroactive
compartes could act at the apical sensory membrane of chemosensory cells, at the basal signalling membrane of these cells, at cells deeper within the nervous system or even on the target cells for metamorphosis signals from the nervous system (Cragg, 2004).

We have a particular interest in the study of the processes of settlement and metamorphosis, which are two crucial stages in the commercial culture of bivalve molluscs. The identification of chemical cues able to induce attachment and metamorphosis will allow large populations of synchronized spat to be obtained for studying the processes of metamorphosis.

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References