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Effects of chemical cues on larval settlement of the flat oyster (*Ostrea edulis* L.): A hatchery approach

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ABSTRACT

The flat oyster *Ostrea edulis* (L.) is a species native to Europe and a highly valued product for consumption. Rearing the oyster larvae, through the settlement and metamorphosis processes, is extremely critical for researchers and the hatchery industry. Settlement and metamorphosis in marine invertebrates typically occur in response to environmental, chemical and/or physical cues. The effect of several environmental factors, bacteria biofilms and analogues of natural inducers on settlement and metamorphosis of different bivalve larvae has been reported. However, there are no studies about the effect of chemical inducers on the settlement and production of flat oyster larvae under laboratory and hatchery conditions.

In the present study, competent larvae of the flat oyster *O. edulis* were induced to settle by exposure to the neurotransmitter GABA, L-DOPA, the catecholamines epinephrine and norepinephrine, and IBMX, under laboratory conditions. After 24 h, maximum percentages of settlement were induced by 10^{-4} M IBMX, 10^{-4} M L-DOPA, 10^{-5} M GABA and 10^{-6} M epinephrine. Exposure to IBMX and norepinephrine also induced significant levels of settlement in *Ostrea* after 48 h. In contrast, 10^{-4}, and 10^{-5} M epinephrine and 10^{-6} M L-DOPA failed to induce significantly higher settlement rates than the control larvae. GABA was found to be the most effective inducer. Maximum settlement rate was achieved by 10^{-4} M GABA (>64%), four times higher compared to the control larvae. Exposure to 10^{-5} M and 10^{-6} M GABA also induced a significantly higher larval settlement. Mortality of *O. edulis* larvae was not affected by the chemical inducers. In hatchery conditions, GABA was an active and fast inducer of settlement and metamorphosis. 10^{-6} M GABA promoted synchronisation of larvae and positively affected the spat growth.

1. Introduction

The flat oyster *Ostrea edulis* (L.) is a species native to Europe and traditionally an attractive and highly valued product for consumption in various European countries. However, available supplies of *O. edulis* are limited because of a decline in fishery brought about by recruitment failure and diseases, which are mainly caused by parasites and environmental stress. According to the FAO statistical database, from 2000 to 2005, flat oyster production in Europe was 5–6000 tonnes, which represents only 3–5% of the production of the Pacific oyster *Crassostrea gigas* (Thunberg, 1793), a more easily cultured introduced species. The average price for *O. edulis* is commonly three to five times greater than the cheaper *C. gigas*. Spain and France are the largest producers of flat oyster; in 2009, 51% of the production was in Spain (2600 tonnes) and 30% in France.

The irregular supply of wild spat in Europe could be compensated by aquaculture. The development and improvement of bivalve spat production have been a challenge over the years for researchers and hatchery staff. Due to a highly variable settlement success, a lack of synchronised settlement and a variable post-settlement growth and survival, rearing oyster larvae, through the settlement and metamorphosis processes, is extremely critical for the hatchery industry in order to generate a healthy cohort of juveniles. The European Commission's 7th framework-founded SETTLE project focused on these key events during hatchery production of two European native bivalves: the great scallop *Pecten maximus* and the flat oyster, *O. edulis*.

Metamorphosis is closely associated with the process of settlement, in which the larva abandons its pelagic habitat and joins the benthos as a juvenile. Settlement and metamorphosis in marine invertebrates typically occur in response to environmental, chemical and/or physical cues and involve extremely rapid and irreversible changes in both morphology and habitat (Hadfield et al., 2001).

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There has been almost no research into the mechanisms by which signals from the environment access the metamorphosis control mechanisms. There is evidence that some mechanisms are widely shared whereas others may be relatively and taxonomically restricted. For example, G-protein-mediated signal transduction of the inductive molecule GABA was inferred for the abalone *Haliotis rufescens* and for the scyphozoan *Cassiopea andromeda*, while no evidence of G-protein function was found in the polychaete *Hydroides elegans* or in the bryozoans *Bugula stolonifera*. The same neurotransmitter can function as an inhibitor of metamorphosis in one taxon, while having precisely the opposite function in others (for review see Bishop et al., 2006).

The larval nervous system of marine invertebrates plays an essential role in coordinating metamorphic events and in integrating them with the environment. Data from a few selected species suggest that little *de novo* gene transcription is required during this process and that mechanisms of signal transduction appear to rely primarily on cell–cell conductance. Recent evidence indicates that hormones also regulate the development leading to metamorphosis in a variety of marine invertebrates. Chemosensory pathways, which can frequently be activated by various neurotransmitters and non-specific compounds, are the mediators between settlement cues and subsequent metamorphic changes (Heyland and Moroz, 2006). It has been suggested that the apical sensory organ has a sensory function and is involved in the detection of external metamorphic cues and in the possible transduction of an environmental signal which initiates metamorphosis in molluscs (Croll, 2009).

The effect of several environmental factors on the settlement and metamorphosis of bivalve larvae has been previously studied. It has been reported that the pearl oysters *Pinctada fucata* (Gould, 1850) and *Pinctada mazatlanica* (Hanley, 1856) and the American oyster *Crassostrea virginica* (Gmelin, 1791) preferred to settle on poorly-illuminated areas (Alagarswami et al., 1983; Michener and Kenny, 1991; Saucedo et al., 2005). Settlement in the Pacific oyster *C. gigas* is also facilitated by medium to high larval densities and by the abundance of food (Beiras and Widdows, 1995).

Bacterial films have been shown to be involved in inducing the settlement of both *O. edulis* and *C. gigas* larvae (Fitt et al., 1990; Tritar et al., 1992). Competent larvae can be induced to settle and to begin metamorphosis by functional analogues of the natural inducers. Catecholamines have also been implicated in many of the physiological processes that occur during the larval development of molluscs such as settlement (Croll et al., 1997). Many pharmacological compounds such as GABA (Doroudi and Southgate, 2002; García-Lavandeira et al., 2005; Morse et al., 1979, 1980), L-DOPA (Coon et al., 1985; Dobretsov and Qian, 2003; Pires et al., 2000), epinephrine (Coon et al., 1986; García-Lavandeira et al., 2005), norepinephrine (Beiras and Widdows, 1995; Coon et al., 1986) and choline (Beiras and Widdows, 1995; Hirata and Hadfield, 1986) have been shown to be effective inducers of settlement and metamorphosis in bivalves and other molluscs. Larvae of the blacklip pearl oyster *Pinctada margaritifera* were induced to settle by GABA but catecholamines had no effect (Doroudi and Southgate, 2002). However epinephrine, norepinephrine and choline have been shown to be effective inducers in *C. gigas* larvae (Beiras and Widdows, 1995; Coon et al., 1985, 1986). To date, no chemical cues have been reported to improve the yields of flat oyster larvae under laboratory and hatchery conditions, except some preliminary results obtained with GABA and epinephrine by our research group (García-Lavandeira et al., 2005).

Inducing a more rapid settlement or a greater degree of settlement and metamorphosis of *O. edulis* larvae with chemical cues will significantly improve and expand the commercial hatchery production of this valuable species. Our aim is to investigate the responsiveness of the flat oyster (*O. edulis*) to chemical inducers such as the neurotransmitter γ-amino-n-butyric acid (GABA), L-3, 4-dihydroxyphenylalanine (L-DOPA), the catecholamines epinephrine (EPI) and norepinephrine (NOREPI), and 3-isobutyl-1-methylxanthine (IBMX) at different concentrations and times of exposure, in order to improve the hatchery production of this species. In the present study we carried out a series of controlled laboratory and hatchery-based experiments to investigate: (1) whether *O. edulis* larvae were induced to settle by those chemical compounds and (2) whether the chemicals affect the mortality of the flat oyster larvae.

2. Materials and methods

2.1. Larval culture

Flat oysters (*O. edulis*) were collected from Ría de Noia, Galicia, Spain. Oyster larvae were obtained from adult oysters conditioned in the hatchery of Cofradía San Bartolomé de Noia at 18 ± 2 °C for 4 weeks. After this treatment the oyster started spawning, and the larvae were released. Larvae were fed with a mixed diet of *Isochrysis galbana*, *Isochrysis galbana tahití*, *Monochrysis lutheri*, *Tetraselmis suecica*, *Rhodomonas salina* and *Chaetoceros calcitrans*, at a concentration of 100 cells μl^{-1} *Isochrysis* equivalents. In all the experiments, veliger larvae were maintained for 12–14 days before harvesting with a 300-μm mesh, counted and measured. Larvae were considered competent for settlement at around 12–14 days after spawning when they developed eye and a foot, displayed crawling behaviour and were larger than 330-μm.

2.2. GABA, L-DOPA, EPI, NOREPI, and IBMX

GABA, L-DOPA, EPI, NOREPI and IBMX were obtained from Sigma Chemical Co. (St. Louis, MO). Epinephrine and norepinephrine were dissolved in 0.005 N HCl and diluted (1:9) in MilliQ sterile water to a concentration of 10^{-3} M. GABA, L-DOPA and IBMX were dissolved in MilliQ sterile water (10^{-3} M). These chemical compounds were dissolved in seawater containing the larvae to achieve the final experimental concentration required. All the neuroactive compounds were freshly prepared to avoid oxidation of the chemicals prior to the experiments.

2.3. Settlement assays

Nine experiments were carried out from February to November 2010, in triplicate polystyrene 90-mm tissue culture Petri plates in 25 ml final volume of U.V.-sterilised, 10 μm filtered sea water (FSW), in which 25 veligers of *O. edulis* were approximately placed. For each experiment, only larvae from a single batch rearing were used. Triplicate Petri plates for the control and for each concentration of the chemicals were used. Food and aeration were not provided during these experiments. To determine the optimum concentration of potential chemical inducers for the induction of settlement, larvae were exposed to different concentrations (10^{-4}, 10^{-5} & 10^{-6} M) of GABA, L-DOPA, EPI, NOREPI and IBMX in dark and at 18 ± 2 °C for 24 h and 48 h. To standardise the data across experiments, a new set of controls was used for each batch cohort under the same experimental conditions. Larvae settlement behaviour was monitored after 24 and 48 h with a microscope Nikon SMZ-2T. Mortality was recorded to determine the toxicity effects of chemical compounds on oyster larvae.

The numbers of unattached, attached or dead larvae were expressed as the percentage of settlement (100 × total number of settled larvae / total number larvae) or the percentage of mortality (100 × total number of dead larvae / total number larvae). Larvae were categorised as settled only if they were alive and attached to the bottom of the Petri plate. Larval attachment was confirmed if they could not be dislodged from the plate with a stream of water. Unattached larvae either were swimming in the Petri plate or were removed from the plate without any resistance. Larval metamorphosis was monitored using the microscope. A