Induction of settlement in larvae of the mussel *Mytilus galloprovincialis* using neuroactive compounds

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Abstract

We investigated the effect on Mytilus galloprovincialis larval settlement, as well as the toxicity, of serial concentrations in filtered seawater of acetylcholine (AC), γ-aminobutiric acid (GABA); 3-isobutyl-1-methylxanthine (IBMX); and the potassium ion in the form of potassium chloride (KCl) and potassium sulfate (K2SO4). All the substances assayed induced larval settlement and peak responses were above 90% in exposures to 10− 2 mol L− 1 (M) AC, 10− 4 and 10− 5 M epinephrine, 10− 3 M GABA and 20, 30 and 40 mM KCl. The optimal concentration of K+ varied depending on the anionic component of the compound assayed, and peak settlement response to KCl was higher (100%) than that achieved with K2SO4 (69.7%). The estimated LC50 of the compounds assayed ranged from 9.4 × 10− 6 M (GABA) to 3.1 × 10− 2 M (KCl). GABA, IBMX and K2SO4treatments displayed toxic effects in all the active concentrations. In contrast, AC 10− 5 M, epinephrine 10− 4 and 10− 5 M and KCl 20 mM treatments enhanced larval settlement without an acute short-term effect on mortality. These results provide new insights on the molecular mechanisms controlling settlement in M. galloprovincialis larvae, and yield promising outcomes for the mussel industry to find a reliable method to enhance larval settlement in hatcheries.

Highlights

► We study the effect of neuroactive compounds on mussel larval settlement. ► The larval response to K+ vary depending on the compound trialed (KCl or K2SO4). ► GABA, IBMX and K2SO4 display toxic effects in all the active concentrations. ► AC, epinephrine and KCl can induce larval settlement without acute toxic effect.

Keywords

Mediterranean mussel;

Potassium

1. Introduction

The mussel Mytilus galloprovincialis is mainly cultured in China and in the coastal waters from Galicia (NW Spain) to the northern shores of the Mediterranean. Excluding China, global production of this bivalve species in 2008 was 280 015 tons, of which 64.37% (180 261 tons) was produced in Spain (FAO, 2012). Although it is consumed worldwide, Europe has traditionally been a high-value market. For instance, the price to the consumer of live rope grown mussels in Spain raised from approximately 1000 euro ton− 1 in 2000 (FAO, 2012) to 3000 euro ton− 1 in 2012 (Spanish Government, 2012).

Nevertheless, the seed stocks are variable in space and time (Smaal, 2002) and appear to be decreasing due to overexploitation. Consequently, development of mussel hatchery techniques has become of economic interest, and it was one of the goals of the European Commission's 6th Framework-funded project Blueseed. However, progress in

the mussel culture industry is still hampered because larval settlement and metamorphosis rates are often inefficient and unpredictable (Satuito et al., 1999 ; Young et al., 2011).

Settlement and metamorphosis are critical events for the life cycle of marine invertebrates, by which larvae experiment structural changes and leave their planktonic life to acquire their final benthonic existence. From the end of XXth century, the literature focusing settlement and metamorphosis of marine invertebrate larvae has experimented a tremendous increase (Clare et al., 1998 ; Hadfield, 2011), and several studies have demonstrated that environmental physical and chemical cues play an important role in these processes. Physical cues include surface texture, color and wettability (Gribben et al., 2011; Mason et al., 2011 ; Roberts et al., 1991), water flow (Alfaro, 2005), or shading (Miller and Etter, 2008). Chemical cues include organic and inorganic molecules (Yang et al., 2008), which can be secreted by conspecifics (Porri et al., 2007) or by other organisms that shall potentially provide information about the environment, like bacteria (Alfaro et al., 2011; Bao et al., 2007 ; Ganesan et al., 2010) or algae (Suenaga et al., 2004).

To investigate the chemical cues involved in the transduction pathways controlling larval settlement and metamorphosis, a valuable tool is the pharmacological experimentation, based on the effect of specific ions or molecules on competent (ready to settle) larvae. Using this technique, several neuroactive compounds have already been demonstrated to influence larval settlement and metamorphosis in bivalves. The nature of these compounds is diverse, and among them there would be ions (Dobretsov and Qian, 2003; Yang et al., 2008 ; Yu et al., 2008), amino acids and derivatives (Alfaro et al., 2011), adrenergic hormones (García-Lavandeira et al., 2005; Yang et al., 2008 ; Young et al., 2011), choline derivates (Dobretsov and Qian, 2003; Satuito et al., 1999 ; Urrutia et al., 2004) and organic solvents (Yang et al., 2008). However, the identity and mode of action of these chemicals remain poorly understood.

Although rather scarce, the promising outcomes of previous studies based on pharmacological experiments with mussels (Alfaro et al., 2011; Dobretsov and Qian, 2003 ; Young et al., 2011), including M. galloprovincialis (García-Lavandeira et al., 2005; Satuito et al., 1999 ; Yang et al., 2008) motivated the present study. We examined the effect on M. galloprovincialis competent larvae of the choline derivative, acetylcholine (AC); the vertebrate-type cathecolamine, epinephrine; the amino acid derivative, γ-aminobutiric acid (GABA); the xanthine derivative, 3-isobutyl-1-methylxanthine (IBMX); and the potassium ion in the form of potassium chloride (KCl) and potassium sulfate (K_2SO_4). The objective of this work was to understand the molecular mechanisms underlying M. galloprovincialis settlement, and to provide potential reliable treatments for an enhanced and synchronous rate of larval settlement in hatcheries.

2. Material and methods

2.1. Spawning, fertilization and production of veligers

M. galloprovincialis broodstock used for spawning were collected from culture populations growing on La Atunara harbor, S Spain (N 36° 10′ W 5° 19′). Veligers were obtained from spawning inductions carried out from February to May 2011; gametes were pooled and mixed from at least 12 males and 10 females. Induction by thermal shock, fertilization and production of veligers were carried out as described by Sánchez-Lazo and Martínez-Pita (in press).

2.2. Growth of veligers

Veliger larvae were reared at a stocking density of 20 larvae mL− 1 in 150 L tanks containing 1 μm filtered and UV-treated seawater (FSW), under static conditions. Water was maintained at 22 ± 1 °C, and slight aeration was provided using sterile glass tubes to provide oxygen and to minimize organic matter deposits which might encourage bacterial concentrations. Larvae were fed ad libitum a mixed diet of Isochrysis galbana (clone T-iso), Chaetoceros gracilis and Tetraselmis suecica. The water in tanks was changed twice a week and a freshly harvested microalgal mixture was added. Eyed pediveliger larvae were apparent at around 18–20 days post-fertilization and considered competent for settlement.

2.3. Suspension of competent larvae

18–20 day post-fertilization larvae were sieved and carefully rinsed with FSW. To maximize larval homogeneity in size and development, only the larvae collected between sieve sizes of 180 and 250 μm were used. Several aliquots of larvae were sampled to confirm that they were in the correct developmental stage by its examination under microscope. The rest was transferred into a 5 L glass beaker, and left to stand for ca. 60 min. To separate swimming veligers in the suitable developmental stage from the sunken ones, half the volume of the supernatant was decanted into another beaker, in which the larval concentration was adjusted to 20–30 larvae mL− 1 by adding FSW. These larvae were exposed to treatments within the following 30–60 min.

2.4. Treatments

Treatments consisted in serial concentrations of AC, GABA, epinephrine, IBMX and potassium in the form of KCl and K2SO4. The use of two potassium containing compounds with different anionic compositions responded to the aim of attributing any of the potential effects of the treatments to the cationic (K+) rather than to the anionic component with more confidence. Control exposure consisted in FSW; a new control set was used for each larval batch to standardize the data across experiments.

For each substance, one or more stock solutions were prepared in FSW immediately prior to the bioassays. These stock solutions were used to set up serial concentrations of each compound by consecutive dilutions in FSW in sterile polystyrene Petri plates (55 mm diameter, 14 mm depth). 1 mL of the previously prepared larval suspension (corresponding to 20 to 30 veligers) was subsequently added to each Petri plate. The concentrations tested for KCl were double of these tested for K2SO4, so that potassium molarities assayed were the same for both anionic compositions. Final exposure concentrations used in experiments are shown in Table 1.

Table 1. Stock and final exposure concentrations (M) of the substances assayed.

Treatment	Stock concentration(s)	Exposure concentrations
AC	10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}	10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}
Epinephrine	10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}
GABA	10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}
IBMX	10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}
KCl	10^{-1}	10^{-2}, $2 * 10^{-2}$, $3 * 10^{-2}$, $4 * 10^{-2}$
K_2SO_4	$5 * 10^{-2}$	$5 * 10^{-3}$, 10^{-2}, $1.5 * 10^{-2}$, $2 * 10^{-2}$

All treatments and control sets were conducted with 8 replicates. Petri plates were kept in dark at 22 ± 1 °C over 48 h ± 3 h. After this time, settlement and mortality were analyzed under a dissecting microscope (Nikon SMZ800, Japan) in each Petri plate.

2.5. Records of settlement

Settlement was recorded as a measure of larvae attached to the surface of the Petri plate or to other larvae. Adhesion to substratum was tested by exerting a gentle suction within close proximity to each larva with a micropipette; larvae were considered settled when they offered resistance against suction, or unsettled when they moved freely and/or offered no resistance to suction. Sometimes larvae were found adhered to each other; in these cases only half of the individuals in the larval aggregate were counted as settled. Percent settlement was calculated as the proportion of settled larvae from the total number counted within each plate.

2.6. Records of mortality

Mortality was recorded to test the acute toxicity of the substances assayed on larvae. Records of mortality were carried out subsequently to the settlement checking and using the same individuals. Larvae were considered alive when they showed any movement (swimming, crawling or movements of viscera). Mortality rate was calculated as the proportion of dead larvae out of the total number counted within each plate.

2.7. LC50 calculation

The lethal concentration 50 (LC50) is a helpful tool to compare the toxicity of different compounds, or between different population models. It is defined as the statistically-derived concentration that is lethal to 50% of a modeled population after specified test duration, and can be calculated by means of a Probit transformation of mortality raw data.

Peak mortality of some of the chemicals assayed was too low to provide confidence to the Probit analysis of the data. Thus, LC50 was calculated only on the substances for which this value was above 80%. Raw mortality data for each treatment level were pooled across replicates, and analyses were performed using statistical software developed *ad hoc* by the US Environmental Protection Agency (EPA Probit Analysis Program Used for Calculating LC/EC Values Version 1.5).

2.8. Statistics

Data on percentage of settlement and mortality were arcsine transformed prior to statistical analysis. Values in text and tables correspond to mean ± SD, and in figures correspond to mean ± SE. One-way ANOVAs were used to test the effect of each treatment on larval settlement and mortality. When significant differences were detected among means, a post hoc Tukey pairwise multiple comparison test was performed. The SPSS® 13.0 software package was used to perform these analyses.

3. Results

The effects of the different chemical treatments on larval settlement and mortality are represented in Fig. 1. Percent of settled larvae in the different control trials ranged from 15.5 ± 4.6% to 25.5 ± 8.8%. When comparing with control, all the substances assayed significantly induced settlement on *M. galloprovincialis* larvae at some of the concentrations tested ($p < 0.01$, ANOVA tests, and $p < 0.05$, Tukey tests; Fig. 1).

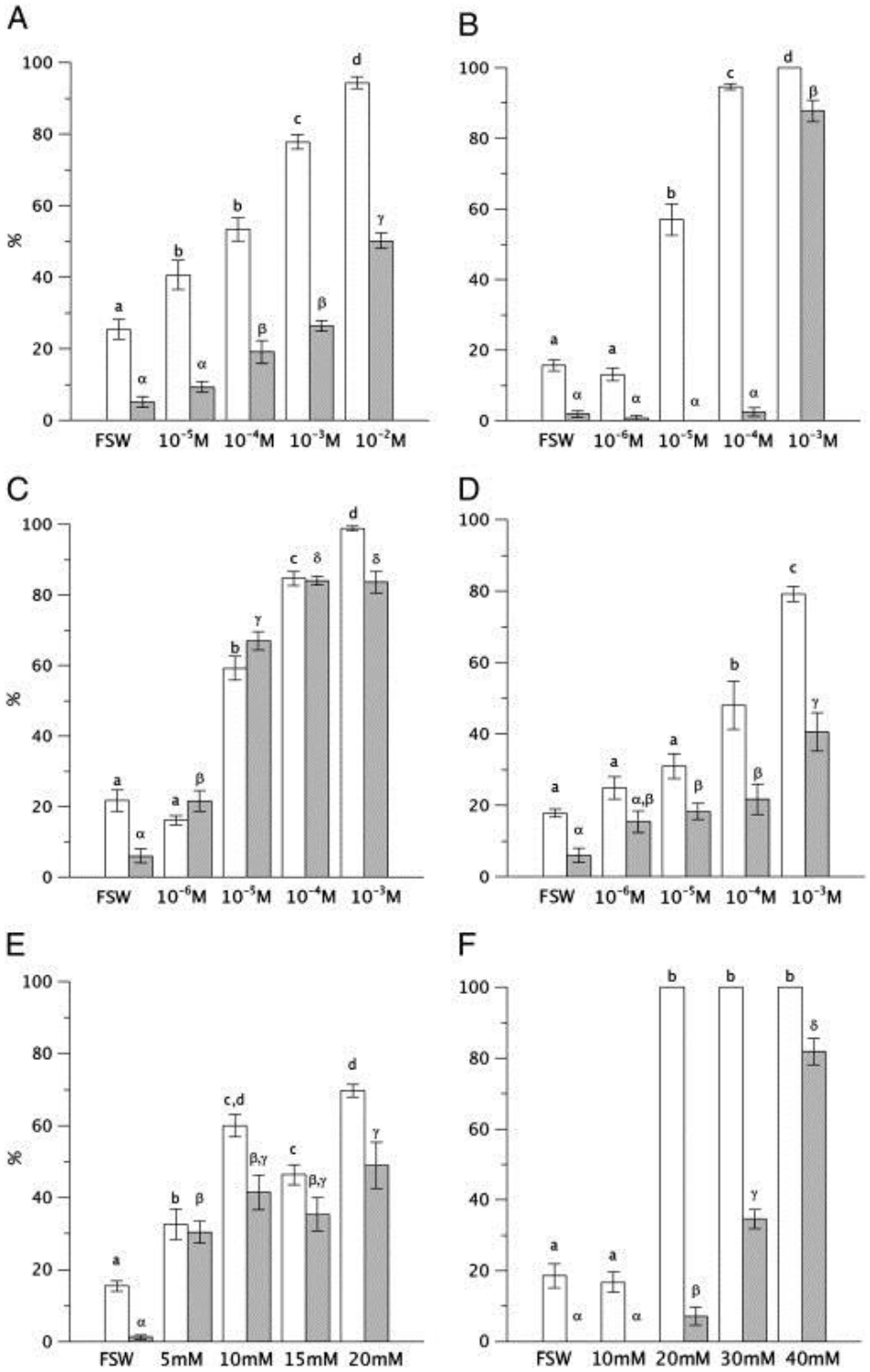

Fig. 1. Settlement (light bars) and mortality (dark bars) of *Mytilus galloprovincialis* larvae after 48 h exposure to serial concentrations of acetylcholine (A), epinephrine (B), GABA (C), IBMX (D), K2SO4 (E) and KCl (F). Data are mean ± SE of 8 replicates. Different superscript letters indicate a significant pairwise difference in settlement; different superscript symbols indicate a significant pairwise difference in mortality ($p < 0.05$, Tukey tests).

The lowest concentrations inducing settlement among the substances assayed were: 10^{-5} mol L^{-1} (M) for AC, epinephrine and GABA, 10^{-4} M for IBMX, 5 mM for K_2SO_4 and 20 mM for KCl. From these minimal active concentrations on, percents of settled larvae increased with the concentration of chemicals, with the only exception of the potassium treatments: in the K_2SO_4 trial, 15 mM K_2SO_4 induced larvae to settle in a lower percentage (46.4 ± 8.1%) than 10 mM K_2SO_4 (60.0 ± 9.0%); in the KCl trial, 100% settlement was recorded and maintained from 20 mM KCl and up.

Peak percentage of settled larvae for all the chemical trials at least tripled settlement in control. This value was above 90% in the AC (94.3 ± 4.9%), epinephrine (100 ± 0%), GABA (98.8 ± 2.2%) and KCl (100 ± 0%) trials, and was lower in the case of IBMX (79.2 ± 6.0%) and K_2SO_4 (69.7 ± 5.1%) trials.

Mortality in the control trials ranged from 0 to 6.0 ± 6.3%. All the chemicals tested in the present study resulted in mortalities significantly higher than mortality in controls at some of the concentrations tested ($p < 0.01$, ANOVA tests, and $p < 0.05$, Tukey tests).

For the substances and final exposure treatments assayed (Table 1), the minimum concentrations which showed a significant increase in mortality with regard to controls were: 10^{-6} M for GABA, 10^{-5} M for IBMX, 10^{-4} M for AC, 10^{-3} M for epinephrine, 5 mM for K_2SO_4 and 20 mM for KCl.

Only in the case of GABA, epinephrine and KCl trials, the peak mortality was above 80%, allowing us to calculate LC50 with confidence. LC50 was 9.4 * 10^{-6} M for GABA, 4.3 * 10^{-4} M for epinephrine and 3.1 * 10^{-2} M for KCl (Table 2). Although its exact value was not assessed, in Table 2 we provide estimations of LC50 for the rest of substances, taking into account the definition of LC50 (see Material and Methods section) and our results on mortality for these substances. Consequently, LC50 shall be around 10^{-2} M for AC, slightly higher than 2 * 10^{-3} M for K_2SO_4, and above 10^{-3} M for IBMX.

Table 2. Highest concentration tested, mortality at the highest concentration tested and estimated LC50 value of settlement inducers tested.

Treatment	Highest concentration tested (M)	Mortality at the highest concentration tested (%)	LC50 (M)
AC	10^{-2}	50.2 ± 6.7	> 10^{-2}
Epinephrine	10^{-3}	87.8 ± 8.5	4.3 * 10^{-4}
GABA	10^{-3}	83.6 ± 8.5	9.4 * 10^{-6}
IBMX	10^{-3}	40.5 ± 15.4	> 10^{-3}
KCl	4 * 10^{-2}	81.9 ± 9.8	3.1 * 10^{-2}
K_2SO_4	2 * 10^{-2}	49.0 ± 18.1	> 2 * 10^{-2}

The only treatment levels inducing settlement without a significant toxic effect were AC 10^{-5} M, epinephrine 10^{-5} M and epinephrine 10^{-4} M. AC 10^{-5} M resulted in a rise of settlement of approximately 15% over the control. Epinephrine 10^{-5} M and 10^{-4} M increased settlement in approximately 40% and 80% respectively over the control. Although KCl 20 mM was slightly toxic for larvae (7.1 ± 6.7% mortality vs 0 mortality in control; $p < 0.05$, Tukey test), it was an extremely effective induction treatment for larvae, resulting in 100% settlement.

4. Discussion

The literature on marine invertebrate larval metamorphosis yields a variety of chemical compounds inducing settlement (Crisp, 1974; Hadfield, 2011 ; Rittschof *et al*., 1998). However, only a few studies of this type dealt with mussels (e.g. Dobretsov and Qian, 2003 ; García-Lavandeira *et al*., 2005). Indeed, the molecular mechanisms underlying this processes remain elusive (Yang *et al*., 2008 ; Young *et al*., 2011). In the current study, we showed that the neuroactive compounds AC, epinephrine, GABA, IBMX, K_2SO_4 and KCl induce *M. galloprovincialis* larval settlement, and that some of these treatments did not display an acute toxic effect for larvae.

Acetylcholine is an ester of acetic acid and choline, which modulates numerous biological processes; for example, it is known that AC is a neurotransmitter involved in neuromuscular nervous signaling (Zhao et al., 2003). However, its mechanism of action on the marine invertebrate larval nervous system is still not clear. AC has been shown to induce metamorphosis and settlement in several bivalve species, including oysters like *Pinctada maxima* (Zhao et al., 2003), *Pinctada fucata martensii* (Yu et al., 2008) and *Crassostrea gigas* (Beiras and Widdows, 1995), the blue mussel *Mytilus edulis* (Dobretsov and Qian, 2003) and the New Zealand endemic mussels *Perna canaliculus* (Young et al., 2011) and *Aulacomya maoriana* (Alfaro et al., 2011). In the present study, settlement and mortality of larvae exposed to AC followed a typical dose response, with a peak induction ahead of 90% after exposure to 10^{-2} M and up to about 50% mortality in the same treatment. Previous to this study, the only outcomes on the effect of AC on *M. galloprovincialis* larvae were reported by Satuito et al. (1999), showing no induction of metamorphosis at concentrations of 10^{-5} M and lower. In contrast, the present study yielded an induction effect of settlement by AC at a concentration as low as 10^{-5} M. These results suggest a different induction ability of AC to settlement (attachment) versus metamorphosis (structural changes), and this observation is consistent with the results by Beiras and Widdows (1995) with *C. gigas* larvae.

Many evidences exist on the role of catecholamines as physiological modulators in mollusks. In bivalve larvae, the presence of vertebrate-type catecholamines has been reported (Bonar *et al*., 1990; Cann-Moisan *et al*., 2002 ; Croll *et al*., 1997) and its implication in settlement and metamorphosis is a research topic of increased interest (Bonar *et al*., 1990; Gohad *et al*., 2010; O'Connor *et al*., 2009 ; Wang *et al*., 2006). Among catecholamines, the effect of epinephrine in competent larvae has been studied in several bivalve species. For example, for various oyster and clam species, epinephrine at 10^{-4} or 10^{-5} M resulted in a rise of the metamorphosis percentage over 50% (Beiras and Widdows, 1995; Coon *et al*., 1986 ; García-Lavandeira *et al*., 2005). In the mussel *M. galloprovincialis*, epinephrine was highlighted as a rapid and effective metamorphosis inducer by Satuito et al. (1999): brief exposure (3 h) of larvae to 10^{-4} M epinephrine yielded high percentages of postlarvae, and nearly 40% larvae metamorphosed in 48 h. More recently, García-Lavandeira et al. (2005) also found high metamorphosis rates in *M. galloprovincialis* larvae exposed to the same and lighter epinephrine exposures (10^{-4} and 10^{-5} M); however, these authors reported a poor induction to settlement by these treatments. By contrast, the current study led to settlement rates over 55% for the same species and treatments, and our results are consistent with those of Yang et al. (2008). Moreover, in the current study, epinephrine only displayed toxic effects in larvae in the highest concentration tested (10^{-3} M); and thus, treatments of 10^{-5} and 10^{-4} M (increasing settlement in approximately 40% and 80%, respectively) did not influence larval survival. Disagreements on settlement results between studies might be due to differences in some of the methodology procedures, such as larval density or age, light or temperature conditions. Regarding light conditions, it is known that epinephrine solutions deteriorate rapidly on exposure to light, and continuous exposure to adrenochrome, one of the epinephrine oxidation products, yielded no metamorphosis induction on *M. galloprovincialis* larvae (Yang et al., 2008). This observation reinforces the suitability of keeping trials based on epinephrine solutions out of light to avoid biased outcomes.

GABA is an amino acid derivative and acts as a neurotransmitter in vertebrate and invertebrate animals. The inductive effect of GABA on invertebrate larval metamorphosis and settlement was first demonstrated by Morse et al. (1979) for the gastropod *Haliotis rufescens*. Subsequent works have yielded highly variable results on the ability of GABA to induce settlement and metamorphosis across bivalve taxa. For example, GABA was ineffective with some oyster species (Beiras and Widdows, 1995 ; O'Connor *et al*., 2009), but enhanced settlement in some others (Yu *et al*., 2008 ; Zhao *et al*., 2003), as well as in the clam species *Venerupis pullastra* and *Ruditapes philippinarum* (García-Lavandeira et al., 2005). Regarding mussels, GABA did not induce settlement in *P. canaliculus* (Young et al., 2011) nor in *M. edulis* (Dobretsov and Qian, 2003), but was shown to be an active settlement promoter for *A.*

maoriana (Alfaro et al., 2011) and for *M. galloprovincialis*, as reported by García-Lavandeira et al. (2005) and by the present study. The mechanism by which GABA is able to induce settlement and metamorphosis in bivalves remains elusive. Yu et al. (2008) reviewed that GABA impairs ciliar activity in mollusk larvae; Alfaro et al. (2011) proposed an indirect mechanism of action by means of secretion of other substances. On the other hand, the current study reports for the first time to our knowledge the toxic effect displayed by GABA on *M. galloprovincialis* larvae: GABA enhanced mortality at all the concentrations tested, suggesting that this chemical would not act as a natural settlement promoter in larvae, which was also suggested byAlfaro et al. (2011) for *A. maoriana*.

To date, scarce studies have reported the effect of IBMX on marine invertebrate competent larvae. Although this compound was found to either inhibit settlement and metamorphosis or to be toxic for the oyster *Crassostrea madrasensis* (Sriyutha Murthy et al., 1999), it has been reported by contrast to induce metamorphosis in polichaetes (Bryan *et al*., 1997; Holm *et al*., 1998 ; Pechenik and Qian, 1998), barnacles (Clare *et al*., 1995 ; Holm *et al*., 2000), and in the bivalves *Chlamys varia* (Mesías-Gansbiller et al., 2008) and *M. edulis* (Dobretsov and Qian, 2003). To our knowledge, the present study yields the first outcomes on the effect of IBMX on larval settlement of *M. galloprovincialis*. Our results revealed that both settlement and mortality were enhanced with an increasing dose of IBMX, with peak values of 79.2 and 40.5%, respectively, at 10^{-3} M. It has been suggested that IBMX enhances intracellular levels of the secondary messenger 3',5'-cyclic adenosine monophosphate (cAMP) by inhibiting phosphodiesterases, the enzymes causing the cAMP degradation, which in turn would trigger the response of cAMP pathway, with a probable intervention of a protein kinase A (Bryan *et al*., 1997; Dobretsov and Qian, 2003; Li *et al*., 2008 ; Pechenik and Qian, 1998). However, the relationship between the elevated intracellular cAMP levels and larval settlement and metamorphosis response needs to be far further examined.

Potassium acts by depolarizing externally accessible, excitable cells involved in the perception of inductive stimuli or directly activating the nervous system (Yool *et al*., 1986; Yu *et al*., 2008 ; Zhao *et al*., 2003). The role of K^+ in settlement has been reported for a wide variety of marine invertebrate larvae including bryozoans (e.g. *Bugula neritina)*, polichaetes (e.g. *Phragmatopoma californica*, *Hydroides elegans*) and gastropod molluscs (e.g. *Haliotis asinia*, *Haliotis diversicolor*, *Astraea undosa*, *Babylonia* sp.) (Bryan and Qian, 1998; Bryan *et al*., 1997; Gapasin and Polohan, 2004; Ke *et al*., 2000; Yool *et al*., 1986; Yu *et al*., 2007 ; Yu *et al*., 2010). Regarding bivalves, the effectiveness of K^+ as settlement inducer has been proved for the oysters *Pinctada fucata martensii* (Yu et al., 2008) and *Pinctada maxima* (Zhao et al., 2003), and for the mussels *P. canaliculus* (Young et al., 2011) and *A. maoriana* (Alfaro et al., 2011), although, unexpectedly, it had no significant effect on the mussel *M. edulis* larval settlement (Dobretsov and Qian, 2003). The study by Yang et al. (2008) was the first to include *M. galloprovincialis* in the list of species metamorphosing in response to KCl, and our results confirm that excess K^+ has an inductive effect not only on the metamorphosis, but also on the settlement behavior of larvae. However, in the current study, the optimal concentration of K^+ varied depending on the anionic component of the compound assayed. Actually, K_2SO_4 was slightly more effective and toxic than KCl at the lowest concentration of K^+ tested (10 mM upon dissociation of the compound), and however, K_2SO_4 peak values for settlement and mortality did not overpass 70% and 40%, respectively. In contrast, KCl at exposures of 20 mM and higher induced nearly 100% larvae to settle, and led a maximum mortality rate of 80%. It is uncertain why these differences occurred. Similar results were reported in a recent work with *P. canaliculus* (Young et al., 2011), in which authors suggested that the effect of the anionic components of the compounds might have interfered in the signal transduction pathways leading to settlement in larvae. This coincidence among studies provides confidence to the hypothesis by Young et al. (2011), and suggests indeed that similarities may occur in the signal transmission of these anionic components for both mussel species.

Under the experimental conditions followed in the current study, the smaller LC50 value ($9.4 * 10^{-6}$ M) corresponded to GABA, which was consequently the most toxic among the chemicals assayed, enhancing larval mortality at all the exposure concentrations. GABA was also acutely toxic for *P. canaliculus*, with an LC50 of $1.7 * 10^{-4}$ M (Young et al., 2011) and for *A. maoriana* (Alfaro et al., 2011), although LC50 values were not provided for this species. Based on its highly acute toxic effect, Alfaro et al. (2011) inferred that GABA would be unlikely to be a natural settlement cue, and our results reinforce this hypothesis. LC50 value for epinephrine ($4.3 * 10^{-4}$ M) was also fairly high, however mortality was enhanced only in the higher concentration tested. KCl displayed the lowest toxicity among the substances assayed, and its LC50 value ($3.1 * 10^{-2}$ M) coincided with this of *P. canaliculus* ($3.2 * 10^{-2}$ M) reported by Young et al. (2011).

When comparing our results with those yielded by previous pharmacological studies with different mussel species, we have found unexpected conflicts and coincidences. Actually, *M. galloprovincialis* and *M. edulis*, which are phylogenetically closed, differed in the settlement and metamorphosis response of larvae when exposed to GABA and potassium ions (Dobretsov and Qian, 2003). In contrast with that, we found similarities between our results with *M. galloprovincialis* and those of species taxonomically and geographically remote; for example, the larval settlement and mortality response of *A. maoriana* to GABA (Alfaro et al., 2011), and of *P. canaliculus* to potassium ions (Young et al., 2011). As our knowledge on the molecular mechanisms that regulate larval settlement and metamorphosis is still rather limited, further studies will be needed to identify the signal-transduction pathways controlling these processes, and to understand why these intriguing differences and similarities might occur.

Finally, the present study yields promising results for the *M. galloprovincialis* aquaculture industry, as some of the chemicals assayed significantly induced settlement without a short-term effect on mortality. Specifically, the most convenient agreements between performance in promoting settlement and low toxicities were found with the epinephrine 10^{-4} and 10^{-5} M and the KCl 20 mM trials. These treatments are thus suitable to be tested in future long-term and bigger scale experiments, with the aim of providing the industry inexpensive and reliable techniques for the synchronous settlement of larvae in hatcheries.

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