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PII: S0044-8486(11)00561-8
DOI: doi: 10.1016/j.aquaculture.2011.06.050
Reference: AQUA 629778

To appear in: *Aquaculture*

Received date: 21 February 2011
Revised date: 27 June 2011
Accepted date: 30 June 2011

Please cite this article as: Young, Tim, Alfaro, Andrea C., Robertson, John, Effect of neuroactive compounds on the settlement of mussel (*Perna canaliculus*) larvae, *Aquaculture* (2011), doi: 10.1016/j.aquaculture.2011.06.050

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Running Head: Effect of chemical cues on larval settlement

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Abstract

Herein, we present the first laboratory study on the effects of pharmacologically active compounds on larval settlement of the green-lipped mussel, *Perna canaliculus*. Competent hatchery-reared larvae were exposed to seawater containing excess K^+ in the form of KCl and K_2SO_4 and the neurotransmitters γ-aminobutyric acid (GABA) and acetylcholine. Both KCl and K_2SO_4 were identified as active inducers of larval settlement with maximum inductions occurring after exposures to 10 and 7.5 mM, respectively. Peak settlement response to KCl was higher (>64%) than that achieved with K_2SO_4 (>41%). GABA did not induce larval settlement and displayed toxic and settlement inhibitive effects at 10^{-4} and 10^{-3} M. Acetylcholine induced larval settlement (>49%) at 10^{-4} M with minimal acute toxic effects (LC < 10%). To gain insight into the class of acetylcholine receptors involved, atropine was used to block the muscarinic-type receptors. Atropine treatment alone did not inhibit settlement compared to control assays, indicating that muscarinic-type receptors are not involved in settlement behavior. Furthermore, results showed that atropine did not significantly decrease acetylcholine induced settlement responses, which suggests an active role of the nicotinic-type receptors in the biochemical pathways of mussel settlement. Results of this study provide new insights on the mechanism of settlement behavior in *P. canaliculus*, which may have direct application to the growing New Zealand aquaculture industry.

Keywords: Larval settlement, Green-lipped mussels, *Perna canaliculus*, Chemical cues, Potassium, GABA, Acetylcholine, Receptors, Muscarinic, Nicotinic.

Introduction

Marine invertebrates have complex life histories and diverse behaviors, which are mediated by environmental and biological stimuli (Harder et al. 2002; Wikstrom and Pavia 2004; Briffa and Williams 2006). Approximately 80% of marine invertebrates (90,000 species) produce microscopic larvae that develop in the plankton (Thorson 1964; Costello et al. 2010). Depending on the species, they remain in their larval phases for minutes to months (Hadfield and Paul 2001). During this period, larvae may be swept great distances along ocean currents before contacting a suitable substratum for settlement and metamorphosing into their adult forms (Pawlik 1992).

Settlement of marine invertebrates may be defined as movement of larvae from pelagic to benthic environments, and subsequent attachment to the substratum (Rodríguez et al. 1993). This transition is modulated mostly by chemical cues of various biological origins (McClintock and Baker 2001). Exogenous cues bind to an assortment of receptors in the neural tissues of the larvae, activating neuronal networks (Hay 2008). Such biochemical stimulations lead to behavioral and morphological transformations in the organism. Although there is great interest in identifying natural settlement cues, few compounds have been isolated and specifically characterised to date (Swanson et al. 2006).

Representatives from all seven marine invertebrate Phyla have demonstrated particular responses to larval settlement cues. Exogenous regulation of settlement may be mediated by surface-associated compounds and waterborne substances released from microbial biofilms (e.g. Ganesan et al. 2010), macroalgae (e.g. Alfaro et al. 2006; Yang et al. 2007), sediments (e.g. Thiyagarajan 2006), and conspecifics (e.g. Eldbourne and Claire 2010) among others. These naturally occurring compounds often tend to be genus-, species- and

even intraspecies-specific in their abilities to induce larval settlement (Rodríguez et al. 1993; Williams et al. 2008; Ritson-Williams et al. 2010). The endogenous biochemical processes which occur after cue detection are of much interest to researchers. At the molecular and cellular level, receptors for cue-binding and the metabolites involved in signal transduction mechanisms are proving to be related structurally, functionally and evolutionarily to those of higher organisms (Murthey et al. 2009). Depending on the species, settlement can be regulated by manipulation of catecholamine, morphogenetic, nitric oxide synthase, regulatory and second messenger pathways (Murthey et al. 2009). The involvements of these processes often are investigated using pharmacologically active compounds to induce or inhibit settlement behavior. Compounds include: those affecting ion-gated channels (e.g. Yu et al. 2008), amino acid and choline derivatives (e.g. Dobretsov and Qian 2003; García-Lavandeira et al. 2005), enzyme inhibitors (e.g. Mesías-Gansbiller 2008), and drugs mimicking or affecting endogenous levels of neurotransmitters (e.g. Faimali et al. 2003) and other metabolites (e.g. Clare et al. 1995; Dahlström 2000; Lind et al. 2010). Results from such studies frequently demonstrate that neuronal control of settlement is diverse across marine invertebrate taxa. While the nervous systems of invertebrates are considered simple compared with higher organisms, researchers are continually discovering a higher level of complexity in invertebrates. Although comparative neurodevelopment in bilaterian invertebrates reveals a high degree of conserved molecular architecture from a common ancestor (Arendt et al. 2008), many differences exist. It is clear that various animals across phylogeny rely on the same neurotransmitters and modulators for signalling. However, there are cases where the function of a neurotransmitter has switched entirely across evolution (reviewed by Marder 2007). Determining the diversity among taxa in neuronal control of invertebrate behaviors, such as larval settlement, is an important step to understand

evolutionary developmental biology. Pharmacological induction of larval settlement in the laboratory provides insight into the functional evolution of receptors and metabolites.

As with exogenous regulators of larval settlement, larval responses to neuroactive compounds often are unique to particular invertebrate taxa. For example, excess K^+, a key regulator of cell membrane potentials, induces settlement in the bryozoan *Bugula stolonifera* (Wendt and Woollacot 1995), the oyster *Pinctada fucata martensii* (Yu et al. 2008), and the Asian green mussel *Perna viridis* (Ke et al. 1998), but fails to induce settlement in the blue mussel *Mytilus edulis* (Dobretsov and Qian 2003). Potassium is a universal regulator of ion gradients across cell membranes, and is involved in depolarisation of neurons, causing formation of action potentials. Induction of larval settlement by K^+ has been suggested to act *via* depolarisation of chemoreceptors, epithelial-bound excitable cells in the peripheral nervous system (PNS) normally responsible for binding naturally-occurring inducers (Leitz and Clingman 1990). Alternatively, exogenously applied K^+ may act directly on the central nervous system (CNS), or some intermediary site in the PNS by crossing epithelial membranes (Carpizo-Ituarte and Hadfield 1998; Hadfield et al. 2000). While the exact mode of action is still unclear, excess K^+ in seawater has demonstrated an ability to induce settlement behaviour in various marine invertebrate taxa.

The neurotransmitter and amino acid γ-aminobutyric acid (GABA) induces settlement in the sea urchin *Echinometra mathaei* (Rahmani and Ueharai 2001), the Mediteranean mussel *Mytilus galloprovincialis* (Garcia-Lavandeira et al. 2005) and some *Haliotis spp*. (e.g. *H. rufescens* [Morse et al. 1979] and *H. asinina* [Gapasin and Polohan 2004]). However, GABA is ineffective at inducing settlement in *H. discus hannai* (reviewed by Fusetani 2004) and the blue mussel *M. edulis* (Dobretsov and Qian 2003). GABA, an amino acid and neurotransmitter, is produced by the decarboxylation of glutamic acid. In vertebrates, GABA acts most commonly in the nervous system as an inhibitory neurotransmitter, causing

hyperpolarization of post-synaptic membranes through increase in permeability to negatively charged chloride ions (Kuffler et al. 1984). However, in some cases GABA has the ability to activate a depolarizing efflux of chloride out of the cell, inducing nerve firing (Kuffler et al. 1984). GABA was the first molecular inducer of settlement found for the gastropod *Haliotis rufescens* (Morse et al. 1979), and it was soon discovered that the mode of action involved relaxation of cilia movement in the velum appendage used for swimming (Akashige et al. 1981; Barlow 1990). It has been suggested that induction of settlement by GABA would be mediated by GABA-sensitive chemoreceptors exposed to the external environment, and the mechanism responsible would have to be one of excitory depolarization (Baloun and Morse 1984).

The neurotransmitter acetylcholine also has shown to be a potent settlement inducer in some marine invertebrates, including the Pacific oyster *Crassostrea gigas* (Beiras and Widdows 1995), the pearl oyster *Pinctada maxima* (Zhao et al. 2003), and the blue mussel *M. edulis* (Dobretsov and Qian 2003). Conversely, while acetylcholine causes larvae of the bryozoan *Bugula neritina* to cease active swimming, it does not induce attachment to substrata (Yu et al. 2007). Acetylcholine, an ester of acetic acid and choline, is a neurotransmitter in the PNS and CNS of many organisms (Martinez-Murillo and Rodrigo 1994), and modulates a variety of processes. Acetylcholine receptors form two primary classes – muscarinic- (mAChRs) and nicotinic-type receptors (nAChRs). mAChRs are G protein-coupled receptors composed of a single protein, and are indirectly linked with ion channels through second messengers (Gomperts et al. 2009). nAChRs are pentameric ionotopic receptors that are permeable to potassium, sodium, calcium, and sometimes chloride ions (Cooper et al. 1991; Corringer et al. 2000; Bolsover et al. 2003). Activation of nAChRs produces a variety of physiological responses in animals. For example, presynaptic neuron activation can facilitate release of neurotransmitters, including dopamine, norepinephrine, serotonin, GABA, and glutamate (Decker et al. 1995; Lopez

et al. 2001). Such compounds have been implicated in modulating diverse molluscan functions, including ciliary activity (Braubach et al. 2006), cardiac contraction (Sukhanova et al. 2008), metamorphosis (Leise et al. 2001), and regulation of swimming behavior (Panchin et al. 1995). In the mammalian CNS, nAChR's located within presynaptic neuronal cell membranes predominately regulate presynaptic neurotransmitter release, and rarely are involved in fast direct synaptic transmission of nerve impulses (Clarke and Reuben 1996; Alkondon et al. 1998; Kaiser and Wonnacott 2000; Reuben and Clarke 2000; Wonnacott et al. 2000; Grady et al. 2001). However, in the molluscan CNS, nAChR's are more involved in fast synaptic cholinergic transmissions, binding to acetylcholine receptors on postsynaptic neurons (Kandel et al. 1969; Blankenship et al. 1971; Yeoman et al. 1993; Woodin et al. 2002). A unique characteristic of molluscs is that they possess, in addition to excitory sodium-selective nAChRs, inhibitory chloride-selective nAChR's (Vulfius et al. 1967; Chiarandini and Gerschenfield 1967; Chiarandini et al. 1967; Watchel and Kandel 1971; Chemeris et al. 1982). It has been suggested that nAChR subtypes in molluscs are comparable in structure to those found in vertebrates, although the former display a much higher functional complexity (Neirop et al. 2006). In the gastropod mollusc *Lymnaea stagnalis*, it was suggested that virtually all the neurons in the CNS respond to acetylcholine through nicotinic-type receptors (Zeimal and Vulfius 1967; Vulfius et al. 1967). This may highlight the importance of these receptors in molluscan neuronal transmissions. However, the extent to which nAChR's are involved in the neuroethologies of molluscs is unknown. While effects of acetylcholine on larval settlement have previously been investigated, little attempt has been made to distinguish which class of acetylcholine receptors are involved.

The endemic New Zealand mussel, *Perna canaliculus*, is a species of considerable commercial value. Although this mussel is extensively cultivated in New Zealand, little is known about regulatory factors that govern its larval behavior. Currently, a major bottle neck

in hatchery production of mussel spat (juveniles) to seed the farms is that larval settlement often is inefficient (< 30%) and unpredictable (e.g., high mortality and poor health). Thus, there is a need for a better understanding of the cues and mechanisms that induce settlement, which may be applied to aquaculture. After a brief planktonic stage, larvae settle onto specific substrates before undergoing metamorphosis (Alfaro and Jeffs 2002; Alfaro et al., 2004). Previous investigations have shown that morphological characteristics of the substrata, water flow and oxygenation are important for primary settlement site suitability (Alfaro and Jeffs 2002; Alfaro et al. 2004; Alfaro 2005, 2006). In addition, crude non-polar solvent extractions of macro-algae, with which the juvenile mussels are known to be associated, have shown a significant capacity for inducing larval settlement in this species (Alfaro et al. 2006). Although these previous studies have provided valuable information, the biochemistry of settlement behavior in *P. canaliculus* remains elusive. To gain insights into the biochemical mechanisms of larval settlement in *P. canaliculus*, this study investigated potassium ions, GABA, and acetylcholine as potential settlement inducing cues. Larvae also were treated with the mAChR blocker atropine to investigate involvement of nicotinic-type receptors in the settlement process.

Methods and Materials

Larval Culture

Veliger larvae of *Perna canaliculus* were obtained from the Sealords hatchery in Ruakaka, northern New Zealand, where adult mussels were induced to spawn by thermal shock. Gametes were pooled and mixed from a number of individuals (ca. 200) to ensure genetic heterogeneity. Fertilised eggs were incubated in 1 µm filtered seawater (FSW) at

17±1ºC and 35 PSU salinity, until 50% or more had developed into the D-veliger developmental stage. Larvae were reared in 24,000 L tanks under static conditions with 48-hr water changes. Water was maintained at 20±1ºC and a mixed diet of *Chaetoceros calcitrans*, *Isochrysis galbana*, and *Pavlova lutheri* was introduced in a controlled ration allowing *ad libitum* access to food. Eyed pediveliger larvae were apparent at around 18–23 days post-fertilisation and considered competent for settlement. Competent larvae (ca. 40,000) were screened by filtering through 175 µm nylon mesh, corresponding to a shell diameter of > 215 µm. Larvae were placed in small polyethylene tubes (50 mm outside diameter) with breathable membranes fitted on both ends. Samples were packed in polystyrene containers filled with wet moss and containing an ice pack to keep them cool and damp during transport to the Auckland University of Technology (AUT) laboratory, Auckland, New Zealand.

Treatment Solutions

To test the effect of excess K^+ on larval settlement, two potassium-containing compounds were selected with different anionic compositions (KCl and K_2SO_4). This ensured that any attribution of inductive effects could be made with increased confidence to the cationic component of the compounds. The two potassium salts, along with GABA, acetylcholine chloride and atropine sulfate were each dissolved in 0.45 µm FSW. Stock solutions (10^{-2} M) of each treatment were prepared immediately prior to all settlement assays. Following serial dilution in FSW, treatment solutions were prepared as 10X concentrates. In order to maintain the same ion concentration, the final KCl concentration was double that of K_2SO_4. Final exposure concentrations were prepared by dilution in FSW (Table 1).

Settlement Assays

Several experiments were conducted to test the various compounds on larval settlement. Thus, different larval batches were tested in the different experiments. To standardize the data across experiments, a new set of controls was used for each batch cohort. Upon arrival to AUT, the larvae were transferred into a 2 L beaker with 1 L of 0.45 µm FSW. The larvae were left to stand for 30–60 min to separate healthy swimming veligers at the correct developmental stage from bottom dwellers that were either dead or had already shed their vela. The water was decanted into another beaker, leaving the undesired larvae behind. With constant stirring, the volume of FSW was increased to adjust the larval concentration until 20–30 larvae ml^{-1} could be drawn from the solution. Settlement assays were performed in sterile polystyrene Petri plates (60 mm in diameter, and 14 mm in depth) over 48 hours. All experiments were conducted with ten replicates per treatment at 17±1ºC under ambient light conditions. Treatment assays consisted of 8 ml FSW, 1 ml larval solution and 1 ml concentrated (10X) treatment solution. The only exception to this was the preparation of the combined acetylcholine and atropine treatment which consisted of 7 ml FSW, 1 ml larval solution and 1 ml each of concentrated (10X) acetylcholine chloride and atropine sulfate. Control assays consisted of 9 ml FSW and 1 ml larval solution. After 48 hrs, settlement was determined. Under a dissecting microscope at 20–45X magnification, a 200 µl pipette was depressed and brought within close proximity (0.5–1.5 mm) to each larva, and gentle suction was applied. Individuals that maintained firm attachment to the substratum were considered settled, and those moving freely with no resistance were considered unsettled. In many cases, settlement could be detected visually by the presence of thin transparent mucous-like threads, but settlement always was verified with suction. Percent settlement was calculated as the proportion of settled larvae from the number initially placed in each plate.

Mortality Assays

Mortality was recorded to determine acute toxicity effects. In most cases, mortality checks were conducted on the same individuals as those used in settlement assays. Exceptions to this were treatments with K_2SO_4, which were performed on a different batch cohort to those used for the settlement assay due to time limitations for mortality detections. Also, where selected treatment concentrations for mortality assays were different from those employed in the settlement assays, different individuals were used, but they always came from the same batch cohort as those used in the settlement assays. Since atropine sulfate was not tested as a potential settlement inducer, it was omitted from the mortality experiments. Mortality was identified under a stereo microscope at 20–45X magnification. Larvae that showed signs of movement of the velum, foot, or gut were considered alive. Since live larvae often were inanimate for periods of more than 15 min, the neutral red vital stain was used to corroborate mortality detection (see Platter-Rieger and Frank 1987; Jacobson et al. 1993). A 120 ppm solution of neutral red was prepared in FSW and diluted in the experimental medium to give a final stain concentration of 20 ppm. After 30 min, larvae were again viewed at 20X magnification under a stereo microscope. Larvae that did not incorporate the stain into their tissues were considered dead. Percent mortality was calculated based on the initial number of larvae within each plate.

Statistics

Percent settlement data were arcsin transformed prior to parametric statistical analyses. In replicates where zero larvae settled, the proportion was given a value of $1/4n$ before the transformation was computed, where n = number of larvae in a single treatment. Data that satisfied homogeneity of variance (Bartlett's test) and normality (Anderson-Darling test) were

analyzed using one-way ANOVAs to test for significant differences among treatment groups, followed by Tukey's *post-hoc* tests to determine pair-wise comparisons. Cases where some treatment levels resulted in complete inhibition of settlement (0%) across all replicates were omitted, and the remaining data were analyzed with a two-sample t-test. The level of significance chosen was 0.05 for all statistical tests. Data were analyzed using the Minitab version 15 statistical software package.

Lethal dose concentrations of treatments were estimated to provide acute toxicity effects of selected compounds. Statistical software developed by the US Environmental Protection Agency was used to analyze these data (EPA Probit Analysis Program Used for Calculating LC/EC Values Version 1.5). Raw mortality data for each treatment level were pooled across replicates to yield the total number of larvae responding to the chemicals.

Results

Larval Settlement

Excess potassium ions

Both of the K^+ containing salts tested (KCl and K_2SO_4) induced a significant proportion of larvae to settle (Fig. 1A–B). Settlement responses after exposure to KCl revealed a typical dose response curve (Fig. 1A) with significant difference detected among concentrations (ANOVA; $F_{4,45} = 13.17$; $p < 0.001$). Among the concentrations, excess K^+ at 5, 10, and 15 mM increased larval settlement compared to control assays (Tukey test; $p < 0.01$). A settlement peak maximum of 64.5% occurred after treatment with 10 mM KCl, showing an almost 380% increase in mean settlement response compared to the control. Induction of settlement with excess K^+ from K_2SO_4 revealed a similar dose response curve,

but overall lower settlement values compared to those with KCl (Fig. 1B). Significant differences were detected among concentrations (ANOVA; $F_{4,45} = 4.65$; $p < 0.01$), with 5 and 7.5 mM K_2SO_4 showing significant increases over the control. A 41.6% maximum settlement peak occurred at a K^+ concentration of 15 mM.

Neurotransmitters

Treatment with GABA did not induce larval settlement at any of the concentrations assayed in this study (Fig. 1C). Total settlement inhibition was detected after exposure to GABA at 10^{-4} and 10^{-3} M, and only a GABA concentration of 10^{-5} M produced a similar settlement response as the control (Tukey test; $p > 0.05$). Conversely, acetylcholine revealed a high capacity for inducing larval settlement, with a similar dose response curve as those generated after exposure to KCl and K_2SO_4 (Fig. 1D). Significant differences in settlement among different acetylcholine concentrations were detected (ANOVA; $F_{3,36} = 23.06$; $p < 0.001$), and all the concentrations were significantly different to the control (Tukey test; $p < 0.01$). A maximum settlement response of 49.6% was achieved after exposure of larvae to 10^{-4} M acetylcholine, which equated to over 450% settlement increase over the control.

Acetylcholine receptor blocker

Results from the acetylcholine with and without atropine experiment indicated significant differences among treatments and the control (ANOVA; $F_{3,35} = 26.41$; $p < 0.001$) (Fig. 1E). Exposure of larvae to 10^{-5} M atropine resulted in an increase in settlement compared to the negative FSW control (Tukey test; $p < 0.05$), but was lower than the settlement with both acetylcholine alone and acetylcholine with atropine (Tukey test; $p < 0.001$). The combined treatment of acetylcholine and atropine showed that the settlement

inductive ability of acetylcholine was not diminished by blocking the mAChR's. In addition, there were no significant differences in settlement responses between acetylcholine alone and acetylcholine with the blocker atropine (Tukey test; $p > 0.05$).

Mortality

Excess potassium ions

Results from the larval mortality assays indicate a difference in acute toxic effects between the two potassium salts tested (Figure 2A & B). From the mortality graphs, it appears that KCl is more acutely toxic than K_2SO_4 at lower concentrations, since exposure of larvae to 20 mM solutions resulted in approximately 36 and 11% mortality, respectively. Treatment concentrations for mortality assays were chosen based on pilot studies to ensure that the data fitted the assumptions of statistical tests. Similarly, estimated lethal concentration values indicate that KCl is more toxic than K_2SO_4, yielding lower LC1, 5, 10, and 15 concentration values (Table 2). However, at higher concentrations, KCl is less acutely toxic than K_2SO_4, since exposure of larvae to 40 mM solutions of each compound resulted in approximately 51 and 96% mortality, respectively. Statistical analyses of the data also revealed that K_2SO_4 becomes more toxic than KCl at higher exposure concentrations, with estimated LC50 values of 28 and 32 mM, respectively.

Neurotransmitters

Exposure of larvae to 10^{-5} M solution of GABA resulted in mortality comparable to control assays (Figure 2C). However, exposure of larvae to 10^{-4} M GABA increased mortality, with over 40% of mussels succumbing to toxic effects. Estimated lethal concentration values show that the first signs of acute toxicity (LC1) are apparent at 5.0 X 10^{-}

^{6} M, the most toxic of all compounds tested in this study. Treatment of larvae with acetylcholine (Figure 2D) revealed no significant increase in mortality when mussels were exposed to 10^{-5} and 10^{-4} M solutions compared to control assays (Tukey test, $p < 0.05$). However, estimated lethal concentration values show that acute toxic effects are apparent after a 48-hour exposure of larvae to 10^{-4} M acetylcholine chloride, which is estimated to kill between 5 and 10% of the population (Table 2).

Discussion

Adding to the list of species, results of the present study show that excess K^+, from KCl and K_2SO_4, is an effective inducer of larval settlement in *Perna canaliculus*. Optimal concentrations of K^+, upon complete dissociation of the compounds, were 10 and 15 mM, respectively. It is uncertain why these differences occurred. However, the possibility exists that interferences from cationic components of the compounds occurred, or may simply reflect a difference in larval responses among hatchery batches. A curious bimodal pattern of settlement induction with KCl across various concentrations has been demonstrated for a number of marine invertebrates including bivalves, gastropods and polychaetes. For example, Yu et al. (2008) found that KCl induced larval settlement of *Pinctada fucata martensii* at 10, 20 and 50 mM, but not at 30 mM. However, such a pattern was not observed in the Asian green mussel *Perna viridis* (Ke et al. 1998), nor in *P. canaliculus* in the current study. Optimal inducing concentrations of K^+ were similar in *P. canaliculus* (10-15 mM) and *P. viridis* (12 mM). This may indicate similarities in the stimulatory action of K^+ and/or the receptors involved in signal transmissions of larval settlement in this genus. Interestingly, the blue mussel *Mytilus edulis* does not respond to K^+ induction at any concentration (Dobretsov and Qian 2003).

In the present study, GABA showed no ability to induce larval settlement of *P. canaliculus* at any of the concentrations assayed. Similar results has been observed in the Pacific oyster *Crassostrea gigas* (Coon et al. 1985), or the blue mussel *M. edulis* (Dobretsov & Qian 2003). Conversely, GABA has been found to induce settlement in various other molluscan species, including the clams *Venerupis pullastra* and *Ruditapes philipinarum* (Garcia-Lavandeira et al. 2005), the oysters *Pinctada margaritifera* (Doroudi and Southgate 2002) and *Ostrea edulis* (Garcia-Lavandeira et al. 2005), and the Mediterranean mussel *M. galloprovincialis* (Garcia-Lavandeira et al. 2005). In this study, GABA was highly toxic to larvae. Conversely, some species that are susceptible to GABA settlement induction do not display toxic responses after exposure of larvae to between 10^{-5} and 10^{-3} M GABA solutions (e.g. Yu et al. 2007, Yu et al. 2008). While the mechanism of settlement induction by GABA may be one of excitory depolarization (Baloun and Morse 1984), perhaps toxic response is caused by inhibitory hyperpolarization of neuronal membranes. Alternatively, GABA may alter levels of other metabolites resulting in death of the organism.

In the current study, acetylcholine was an effective settlement inducer of *P. canaliculus* with optimal induction at 10^{-4} M. These results are similar to those obtained for *Crassostrea gigas* (Bieras and Widdows 1995), *Pinctada maxima* (Zhao et al. 2003), and *M. edulis* (Dobretsov and Qian 2003). It is unlikely that acetylcholine stimulates externally-bound chemoreceptors, and choline derivatives have been suggested to act directly on endogenous receptors, or by increasing levels of other neurotransmitters (Hadfield and Hirata 1986; Hadfield and Pennington 1990; Bieras and Widdows 1995). The acetylcholine induced response in *P. canaliculus* was not significantly reduced by blocking mAChR's with atropine sulfate, suggesting nAChR's were responsible for acetylcholine induction. Furthermore, atropine sulfate treatment alone increased settlement compared to control assays. It is uncertain why settlement was induced by blocking mAChR's, but this may indicate that

antagonism of mAChR's causes an increase in available endogenous acetylcholine at nAChR junctions, leading to higher settlement rate. Further investigation must be carried out to determine such a role. Nonetheless, our results infer an active involvement of nAChR's in biochemical mechanisms of larval settlement for this species.

In conclusion, we have conducted the first study to determine effects of artificial inducers of larval settlement in *P. canaliculus*. Unearthing additional factors for settlement site selection and characterizing cellular regulatory processes of settlement behavior is underway. Identification of receptors involved in critical life processes, such as larval settlement, provides information that may be useful for determining physiological effects of environmental change. Comparative neuronal control of behavior among taxa also may offer new perspectives on evolutionary development. Furthermore, such information has direct implications for the aquaculture industry. For example, identifying factors that can enhance larval rearing and settlement techniques may improve production of hatchery-supplied seed, which may reduce the reliability on the unpredictable wild-caught spat. Based on results of this study, we are currently investigating the commercial suitability of using K^+ and acetylcholine for increasing settlement rates of *P. canalicuclus* larvae under hatchery conditions, and we are performing further work to determine the mode of K^+ and acetylcholine actions on the biochemistry of this organism.

Acknowledgements

We are thankful to Jonathan Bilton and the Sealords hatchery, Ruakaka, northern New Zealand, for providing mussel larvae for these experiments. We are grateful to the laboratory technicians in the School of Applied Sciences, Auckland University of Technology, for their ongoing assistance. We also thank Annapoorna M. Ganesan, John Brooks, Colleen Higgins, and the rest of the Aquaculture Biotechnology Group at AUT for many fruitful discussions.

This research was supported by a Foundation for Research Science and Technology Pre-Seed grant (FRST-PSAF UOWX0716) and an AUT Research Grant to A.C. Alfaro.

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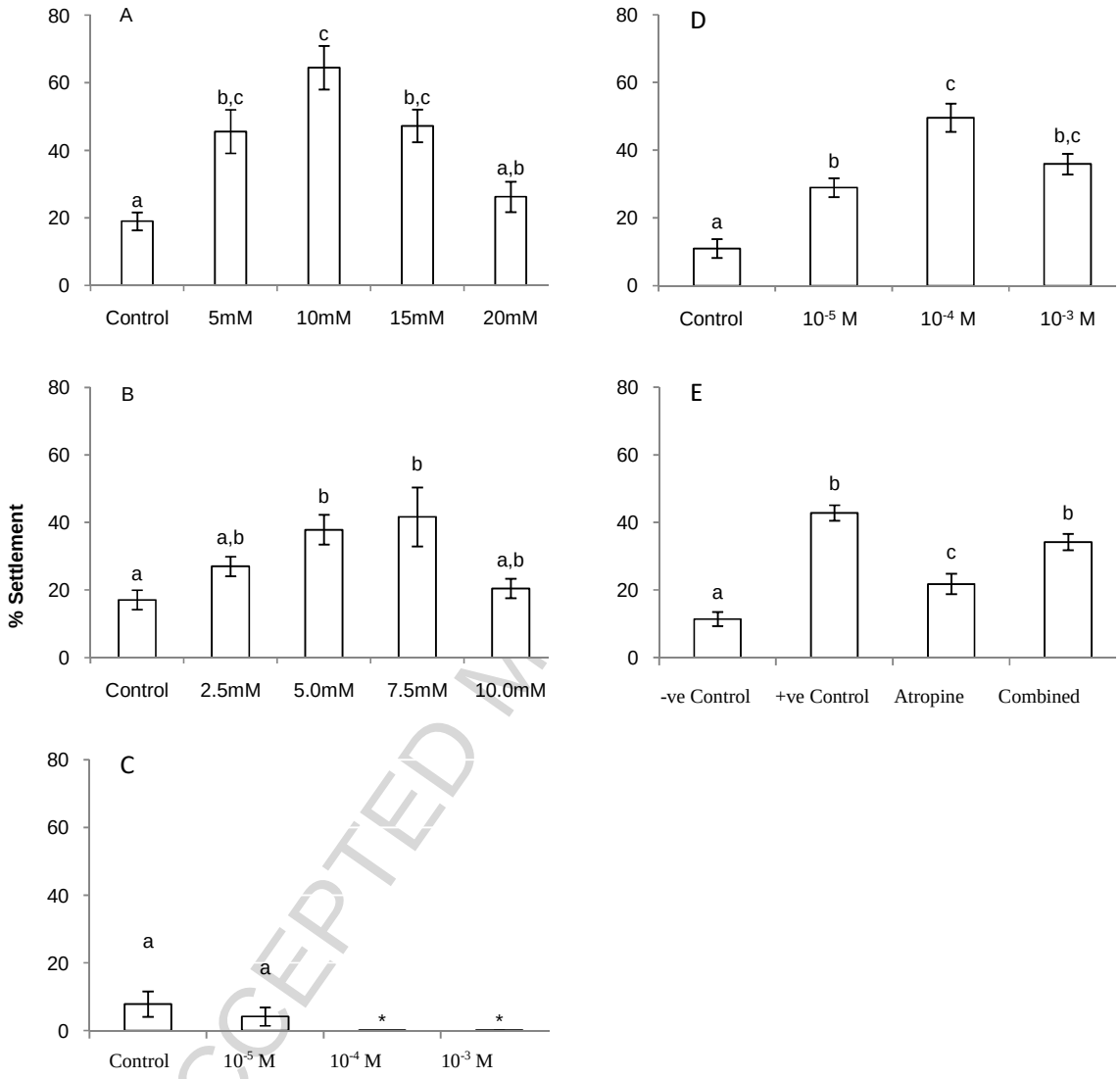

Figure 1. Settlement of *P. canaliculus* larvae exposed to treatment compounds. (A) KCl; (B) K_2SO_4; (C) GABA; (D) acetylcholine; (E) atropine and acetylcholine (-ve control = FSW, +ve control = $1x10^{-4}$ M acetylcholine chloride, atropine = $1x10^{-5}$ M atropine sulphate, combined = $1x10^{-4}$ M acetylcholine chloride + $1x10^{-5}$ M atropine sulphate). Data are mean ± SE of 10 replicates. Letters above error bars indicate pairwise differences (Tukey's HSD post hoc tests). Asterisks represent data omitted from statistical analyses due to 0% settlement being detected across replicates.

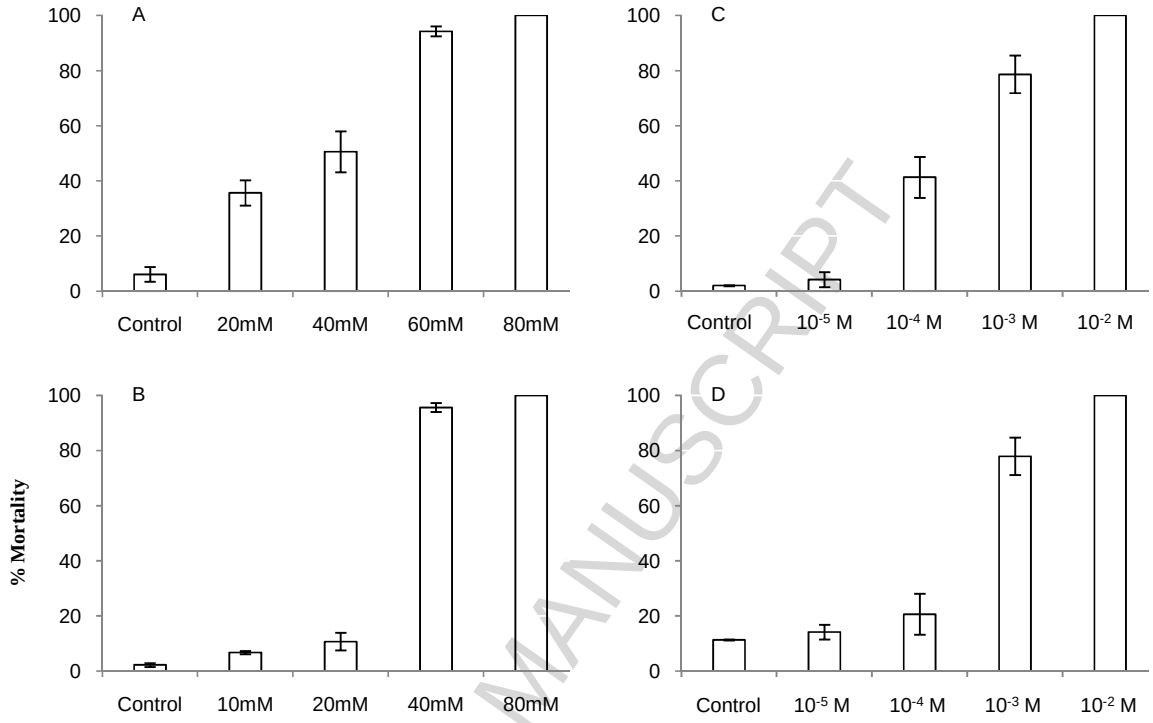

Figure 2. Mortality of *P. canaliculus* larvae exposed to treatment compounds. (A) KCl; (B) K_2SO_4; (C) GABA; (D) acetylcholine. Data are mean ± SE of 10 replicates.

Table 1. Final assay treatment concentrations of trialled settlement inducers

Treatment	Exposure concentration
KCl	5.0, 10.0, 15.0, 20.0 mmol L^{-1}
K_2SO_4	2.5, 5.0, 7.5, 10.0 mmol L^{-1}
GABA	10^{-5}, 10^{-4}, 10^{-3} mol L^{-1}
ACh	10^{-5}, 10^{-4}, 10^{-3} mol L^{-1}
Atropine	10^{-5} mol L^{-1}

Table 2. Estimated lethal concentration values of trialled settlement inducers.

Treatment	Lethal Concentration Values (mol L^{-1})				
	1%	5%	10%	15%	50%
KCl	$9.2x10^{-3}$	$1.3x10^{-2}$	$1.6x10^{-2}$	$1.8x10^{-2}$	$3.2x10^{-2}$
K_2SO_4	$1.7x10^{-2}$	$2.0x10^{-2}$	$2.1x10^{-2}$	$2.2x10^{-2}$	$2.8x10^{-2}$
GABA	$5.0x10^{-6}$	$1.4x10^{-5}$	$2.5x10^{-5}$	$3.6x10^{-5}$	$1.7x10^{-4}$
Acetylcholine	$3.7x10^{-5}$	$7.8x10^{-5}$	$1.2x10^{-4}$	$1.5x10^{-4}$	$4.7x10^{-4}$