Pharmacological induction of larval settlement in the New Zealand mussel *Perna canaliculus*

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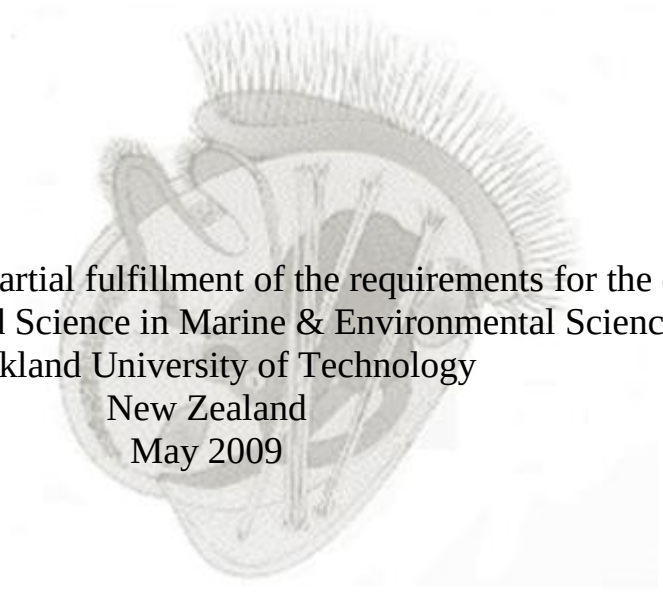

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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ABSTRACT

The endemic New Zealand green-lipped mussel (*Perna canaliculus*) is a commercially important species. Although this mussel is extensively cultivated in New Zealand, very little is known about its early larval behaviour. After a brief planktonic stage, the young mussels settle onto specific substrates before undergoing metamorphosis into their adult form. However, the timing of this settlement, and preference for substrata, is poorly understood. Chemical stimuli of biological origin that modulate neuronal signalling are thought to mediate this process. Exposure of marine invertebrate larvae to neurotransmitters, their precursors, or similar molecules that have the ability to induce larval settlement, have been used in numerous studies to give important insights into the biochemical mechanisms underlying settlement behaviour.

In the present study, 16 pharmacologically active compounds were assessed for their effectiveness at inducing larval settlement in *P. canaliculus*. In the laboratory, mussel larvae were exposed to the chemicals for 48 hours. Among the chemicals tested, potassium chloride, acetylcholine, atropine, epinephrine, L-DOPA, hydrogen peroxide, and cyclic adenosine monophosphate induced larvae to settle with minimal acute toxic effects. Exposure of larvae to potassium metabisulphite, sodium metabisulphite, ascorbic acid, caffeine, L-Tryptophan, L-Phenylalanine, and L-Tyrosine induced high levels of larval settlement, but were acutely toxic to the mussels. Exposure of larvae to gamma aminobutyric acid had inhibitive effects on the settlement process. These results suggest that such compounds, many of which are present endogenously, are naturally involved in the biochemical signalling pathways of larval settlement. An exploratory meta-analysis was performed on larval settlement responses to various inductive compounds among nine marine invertebrate species, clustering the organisms by similarity of response. This investigation may have identified a novel approach to species clustering; illuminating currently concealed relationships based on the biochemical mechanisms involved in larval settlement behaviours. There also may be

considerable applications for using these compounds in aquaculture to provide routine, inexpensive, and effective techniques for inducing synchronous settlement of hatchery-reared larvae.

A major concern for the green-lipped mussel aquaculture industry in New Zealand is the limited supply of larvae or juvenile mussel spat for cultivation. To stock farms, the primary source of juvenile mussels comes from 'beach cast spat', attached to drifting macroalgae washed up on the foreshore. Considerable effort also is made to catch planktonic larvae on 'spat catching ropes' suspended in the ocean. In the present study, weakly surface-bound acetylcholine was tested as a spat attractant to determine if the compound has commercial applications in areas other than larval settlement. Results showed no apparent capacity for acetylcholine to modulate chemotaxis in *P. canaliculus*. The effect of substrate surface charge on the ability of spat to attach showed that mussels have a significant preference for positively charged surfaces. Charged surfaces in the marine environment may modulate the colonisations of particular substrata, and may have commercial applications in promoting or inhibiting biofouling. Another problem suffered in the mussel aquaculture industry is poor retention of mussels on growing ropes. Acetylcholine showed no ability to enhance spat retention in a practical capacity, and neither did alterations of surface charge.

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

In this chapter, a brief summary of the relevant literature is given to provide background information on the topic and scope of this thesis. This literature review is not extensive since each of the following chapters contains their own introductions, and each chapter is discussed, in detail, in the context of the literature. In this chapter, the thesis aims and objectives also are provided.

1.1 Settlement of benthic marine invertebrates

Marine invertebrates have complex life histories and diverse behaviours, mediated by environmental and biological stimuli (Harder & Qian 2000; Engel et al. 2002; Harder et al. 2002; Wikstrom & Pavia, 2004; Briffa & Williams, 2006). Approximately 80% of marine invertebrates (roughly 90,000 species) produce microscopic larvae that develop in the plankton (Thorson 1964). These larvae, which have morphologies completely unlike those of their parents, may remain in their larval phases for minutes to months, depending on the species (McClintock & Baker 2001). During this time, larvae may drift great distances in the water column, being swept along by ocean currents, before contacting a suitable substratum and metamorphosing into their adult form (Pawlik 1992). The evolutionary advantages for having a dispersed larval stage includes avoidance of competition for resources with adults, reduction of benthic mortality while in the plankton, increased genetic heterogeneity among populations (i.e. decreased likelihood of inbreeding in the next generation), and increased chances of withstanding local extinctions (Pechenik 1999). At the end of the early planktonic stage, the swimming larvae settle out of the water column onto particular substrata, with the preference for these substratums often being species-specific (Pawlik 1992). Settlement of marine invertebrate larvae is defined as the movement of the organism from a pelagic environment to a benthic one, and subsequent attachment to the substrata (Rodriguez et

al. 1993). This transition is modulated by the chemoreception of chemical cues of various biological origins. These exogenous cues bind to an assortment of receptors in the neural tissues of the larvae (Mackie & Mitchell 1981; Hay 2008). In all but a few species, the identities and locations of these receptors are currently unknown (Zimmer & Butman 2000). However, the binding of these cues results in the activation, or deactivation, of specific neuronal networks by way of endogenous neurotransmitter chemicals within the organism (i.e signal transduction of the external stimulus which triggers a raft of endogenous metabolic pathways) (Baloun & Morse 1984; Rodriguez et al. 1993). These biochemical stimulations lead to behavioural and morphological transformations in the organism, such as settlement and metamorphosis respectively. Although there is great interest in determining what these natural chemical cues are, in over forty years of research few compounds have been isolated and identified (Rodriguez et al. 1993).

Chemical settlement cues originate from a variety of sources in the marine environment. These cues may be waterbourne or surface-bound, and associated with conspecific individuals, specific prey, locally-habituated microalga, or the surfaces of substrata (Rodriguez et al. 1993). Microbial biofilms which coat most marine surfaces have long been recognised as important for the settlement of some invertebrate larvae (e.g. Morse et al. 1984; Bonar et al. 1990). In many cases, particular species of bacteria have been demonstrated to facilitate settlement, and compounds released from bacteria may function as settlement inducers (Qian et al. 2007). There are numerous reasons why so few naturally occurring chemical inducers of larval settlement have been identified to date, including: (1) the low concentrations in which they are present in the environment; (2) the unstable nature of the compounds; and (3) many compounds in the marine environment are tightly complexed with other molecules. These factors make purifications and molecular characterisations extremely difficult (Zimmer & Butman 2000; McClintock & Baker 2001). Another major issue stems from limited collaborations between larval biologists and natural products chemists investigating larval settlement and natural triggering cues (McClintock & Baker 2001).

1.2 The study of larval settlement

1.2.1 Neuroethological approach

Since the elucidation of naturally-occurring chemical cues has routinely been demonstrated to be problematic, scientists often apply neuroethological techniques to investigate larval settlement processes. Neuroethology is the biological approach to the study of natural behaviors (often called intrinsic behaviors or innate behaviors) and its fundamental mechanistic control by the nervous system (Ewart 1980; Camhi 1984; Hoyle 1984; Wyeth & Willows 2006; ISN 2008). Natural behaviors may be thought of as those behaviors generated through means of natural selection (i.e. finding mates, navigation, locomotion, predator avoidance), including larval settlement (Watson et al. 2001; Sisneros & Tricos 2002; Wyeth & Willows 2006; Feary et al. 2007). Some of the areas that neuroethological inquiry attempts to address are summarised by Ewert (1982), these include: (1) how stimuli are detected by organisms; (2) how environmental stimuli in the external world are represented in the nervous system; (3) how information about a stimulus is acquired, stored and recalled by the nervous system; (4) how behavioural patterns are encoded by neural networks; (5) how behaviour is coordinated and controlled by the nervous system; (6) how the ontogenetic development of behaviour can be related to neuronal mechanisms. The neural approaches used within the field are diverse. For example, some neuroethologists solely rely on behavioural observation to suggest organisations of underlying neural circuits (e.g. Medeiros et al. 1998). Some neuroethologists use intracellular recording techniques, probing individual neuronal cells, to determine the involvement of neural circuits in particular behaviours (e.g. Faure & Hoy 2000). Other neuroethologists use pharmacologically active drugs to stimulate specific components of neural circuits and biochemical pathways in order to elicit, or inhibit, particular behaviours of interest (e.g. Winberg & Nilsson 1992; Elliot & Susswein 2002). This last technique tends to be most commonly used by scientists for investigating the internal biochemical mechanisms which control marine invertebrate larval settlements.

1.2.2 Inductions of larval settlement

To gain insight into the endogenous biochemical signaling pathways of larval settlement and metamorphosis in the natural environment, neuropharmacological agents are commonly applied in the laboratory to induce such early life-stage processes (Pawlik 1990). The study of non-naturally occurring chemical cues that induce larval settlement permits a better understanding of the mechanisms involved in the larval response (Rodriguez et al. 1993). Neurotransmitters, their precursors, or similar molecules have been reported to mimic the effect of naturally-occurring chemical cues, and are known to trigger larval settlement and metamorphosis in a variety of marine invertebrates. For example, neurotransmitters and their derivatives that affect larval settlement responses include acetylcholine (bivalves: Beiras & Widdows 1995; Dobretsov & Qian 2003; Zhao et al. 2003; Fusetani 2004; Urrutia et al. 2004; Yu et al. 2008; arthropods: Faimali et al. 2003; ascidians Coniglio et al. 1998), serotonin (bivalves: Fusetani 2004; gastropods: Leise et al. 2001; hydroids: Walther et al. 1996; McCauley 1997; Zega et al. 2007; arthropods: Yanamoto et al. 1996), L-DOPA and the catacholamines dopamine, norepinephrine, and epinephrine (bivalves: Coon et al. 1985; McAnally-Salas et al. 1989; Garcia-Lavandeira 2005; Satuito et al. 2005; Mesias-Gansbiller et al. 2008; Yang et al. 2008; gastropods: Pires & Hadfield 1991; polychaetes: Jensen & Morse 1990; Pawlik 1990), gamma amino-butyric acid (gastropods: Morse et al. 1979; Bryan & Qian 1998; Gapasin & Polohan 2004; echinoderms: Pearce & Scheibling 1990; Rahman & Uehara 2001). Surface-bound neurotransmitter mimetic compounds have been suggested as natural inducers of settlement in a variety of marine invertebrate larvae (e.g. Morse et al. 1985). However, criticisms of this theory have been advanced (Pawlik 1990). In the few cases where naturally occurring settlement inducers have been isolated and identified, the compounds are unrelated to neurotransmitters (Pawlik 1992). Some neurotransmitters such as L-DOPA and gamma amino-butyric acid are small water-soluble amino acids. It is well established that dissolved amino acids can be actively transported from seawater into the bodies of marine invertebrate larvae (e.g. Jaeckle & Manahan 1989). Therefore, it is most likely that these pharmacologically active compounds stimulate larval settlement behavior by influencing the nervous system internally, rather than acting on external epidermally-bound chemoreceptors

(Hirata & Hadfield 1986; Pawlik 1992). Other compounds which are known to induce larval settlements include the secondary messenger cyclic adenosine monophosphate (bivalves: Dobretsov & Qian 2003; Tao et al. 2003; Zhao et al. 2003; Yu et al. 2008; gastropods: Hadfield 1984; Trapido-Rosenthal & Morse 1986b; arthropods: Rittschof et al. 1986; Clare et al. 1995; Clare 1996; Holm et al. 2000; Li 2007; polychaetes: Jensen & Morse 1990), the neurotransmitter precursor choline (gastropods: Morse et al. 1979; Levantine & Bonar 1986; polychaetes: Pawlik 1990; nudibranchs: Hadfield 1984; Hirata & Hadfield 1986; Todd et al. 1991), and compounds which affect the movement of ions across cell membranes such as picrotoxin (arthropods: Rittschof et al. 1986), and potassium ions (bivalves: Ke et al. 1998; Yu et al. 2008 gastropods: Li et al. 2006; bryozoans: Wendt & Wollacot 2005; ascidians: Denegan et al. 1997). The responses of larvae to these pharmacologically active drugs have been used to formulate complex intracellular signal transduction mechanisms controlling settlement or metamorphic activations (e.g. Baxter & Morse 1987). However, variations in the applications of the drugs, and the assessments of the larval responses, may lead to erroneous conclusions (Pawlik 1990). Nevertheless, the induction of larval settlement with bioactive compounds remains to be a relatively simple and effective means of gaining insight into the biochemical pathways underlying marine invertebrate larval settlement behaviours.

Understanding the environmental factors that have the ability to influence the settlement behaviours of marine invertebrate larvae is critically important (McClintock & Baker 2001). Gaining an understanding of this process by investigating the neuroethologies of marine invertebrate larvae will enhance knowledge in diverse areas such as developmental biology, marine benthic community ecology, biofouling, and aquaculture. In this thesis the process of larval settlement in the native New Zealand green-lipped mussel is investigated by the exogenous application of neurotransmitters, precursors, or other pharmacologically active compounds which have been shown to induce larval settlement in other marine invertebrate species.

1.3 The green-lipped mussel

The endemic New Zealand green-lipped mussel, *Perna canaliculus*, is a filter feeding mollusc with distinctively coloured green shell margins (Figure 1). This mussel can grow up to 260 mm in shell length (anterior-posterior axis) (FAO 2008). It is distributed throughout New Zealand, but is most commonly found in warmer central and northern parts of the country, where it frequently forms dense mussel beds of up to 100 m^2 (FAO 2008; Ministry of Fisheries 2007). This species is a typical bivalve of the intertidal zone and open coast with a preference for moderately exposed habitats and full salinity (FAO 2008). *P. canaliculus* tends to be found from the mid-littoral down to depths of around 50 m. This mussel is a dioecious (separate sexes) broadcast spawner and generally spawns in late spring to early autumn (Ministry of Fisheries 2007). However, spawnings may occur throughout the year (Alfaro et al. 2003).

Figure 1. Green-lipped mussels, *Perna canaliculus*.

The life cycle of *P. canaliculus* is complex compared to many other marine invertebrates. During the early stages, the species undergoes two separate settlement events (Figure 2). After initial fertilisation, larvae develop and remain planktonic for periods of up to six weeks (Jeffs et al. 1999; Alfaro et al. 2004). During this planktonic stage the larvae are able to swim only vertically but may be transported great distances by ocean currents. When they are around 220–350 µm in size, the larvae settle out from the water column to lead a temporary benthic life (FAO 2008). Primary settlement is most intense from late winter to early summer, although settlement is highly variable, both spatially and temporally (Alfaro & Jeffs 2003; Alfaro 2006b; FAO 2008).

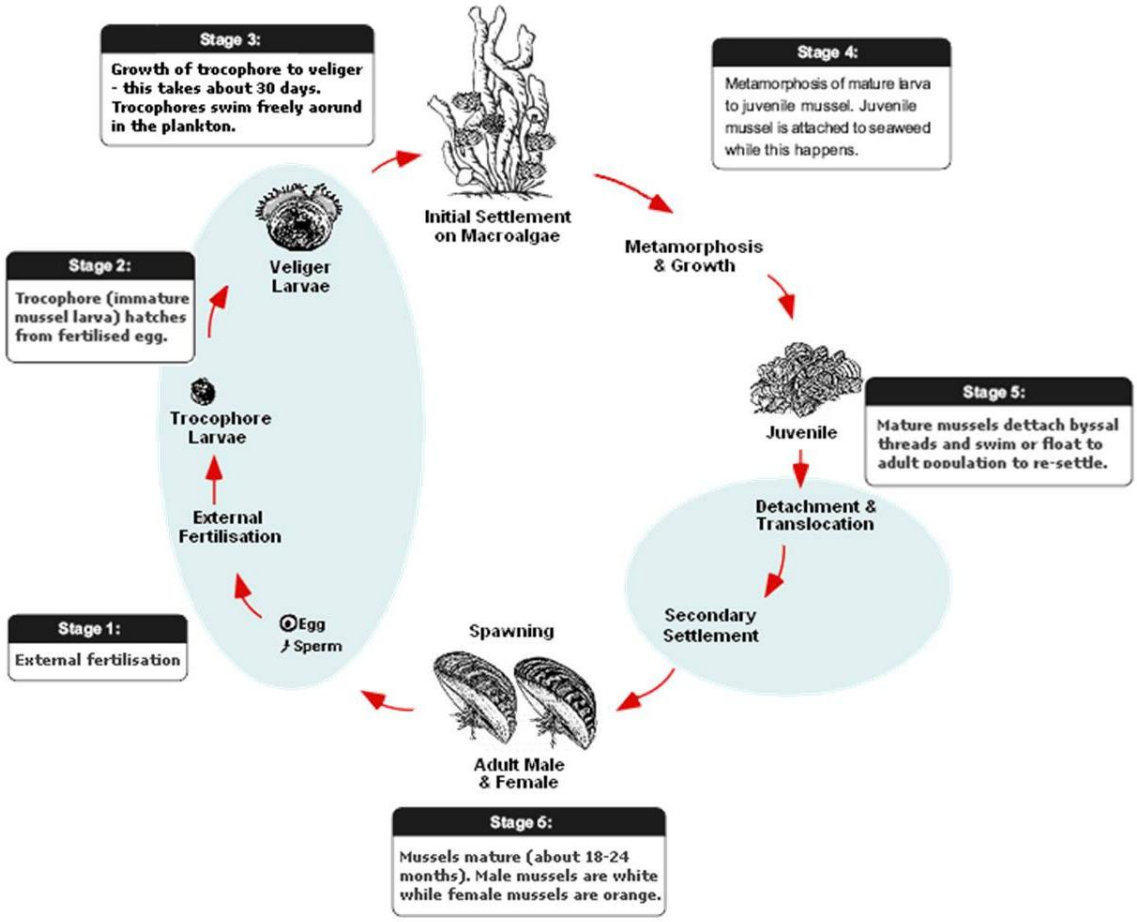

Figure 2. Life cycle of *Perna canaliculus*. Lightly shaded blue areas represent pelagic stages.

Larvae tend to settle over a wide range of depths and prefer filamentous substrata such as hydroids, bryozoans, and filamentous red macroalgae (Alfaro & Jeffs 2003; Alfaro et al. 2004, 2006; FAO 2008). Primary settlement rarely occurs onto adult mussel beds, although may take place on surrounding seaweeds (Alfaro 2006). When larvae come into contact with a suitable substrate they produce adhesive mucoid exudates which are used to attach to the substrate. Settlement is completed with the attachment of fine mucoid threads (resembling primitive byssus threads). Once this process has occurred the young mussels undergo metamorphosis, turning into their adult forms (FAO 2008). After primary settlement and metamorphosis, juvenile mussels may undergo a secondary settlement event and re-attach to other substrata (Alfaro et al., 2003). In such cases juvenile mussels secrete mucoid filaments and detatch themselves from their primary settlement subsratum, being transported by ocean currents (byssopelagic migration or mucous drifting) (FAO 2008). Mussels may relocate numerous times during this early juvenile stage and it is thought that the drifting ability is lost once spat reach about 6 mm in length (FAO 2008).

Little is known about the natural larval settlement process in *P. canaliculus* compared to other mussel species, such as *Mytilus edulis*. Some factors which may mediate primary settlement include: temperature, light conditions, nutrient availability, water flow, chemical cues, and physical cues of substrata. In many marine invertebrate species, induction of larval settlement is associated with physical characteristics of substrata (Folino-Rorem et al. 2006), and similarly so for *P. canaliculus* (Alfaro & Jeffs 2002). Primary settlement of green-lipped mussel larvae also has been found to be associated with chemical cues from filamentous red macroalgae. Alfaro et al. (2006) showed that crude organic extracts of red macroalgae induced larval settlement in the laboratory. However, the identity of the specific inducing molecules remains unknown. Although the effects of L-DOPA have been investigated on the swimming ability of larvae (Buchanan 1999), no attempts have been made to induce larval settlement in this species using pharmacologically active compounds. Nor have there been any studies which have previously investigated the neuroethology, or general biochemistry, of larval settlement behaviour in *P. canaliculus*.

1.4 Aquaculture

1.4.1 Commercial value and industry growth

The green-lipped mussel is a commercially important species for New Zealand. In 2008, over 33,000 tonnes of farmed mussels were exported providing export-only market revenue of over $200 million. The mussel industry is New Zealand's biggest aquaculture sector, and by value, the largest single species of seafood currently exported (NZGovt. 2009). As of 2005, there were almost 900 mussel farms in New Zealand, covering an area of approximately 6535 hectares (Ministry of Fisheries 2007). The global demand for green-lipped mussels currently exceeds supply, and there is significant scope for growth. Interest has been mounting in the future management of this economically lucrative and sustainable resource. In a combined effort, the NZ Aquaculture Council, NZ Seafood Industry Council and the Ministry of Economic Development have recently launched *The New Zealand Aquaculture Strategy*. The strategy aims to increase the NZ aquaculture sector sales to $1 billion per annum by 2025 (NZGovt 2007). Some main features of the development plan include strengthening stakeholder partnerships, securing and promoting investment in aquaculture, developing the market for NZ aquaculture support, and maximising opportunities for innovation. As a response, the government has recently published *Our Blue Horizon - The Government's Commitment to Aquaculture* to facilitate the sector strategy in areas where the government has leverage, such as building the confidence to invest, promoting Maori success, capitalising on research and innovation, and increasing market revenues (NZGovt 2007). Therefore, a significant opportunity exists to develop new technologies which may aid in the development and expansion of the New Zealand mussel aquaculture industry.

1.4.2 Mussel farming practices

Mussel farming in New Zealand was first trialed in the mid 1960's, using pontoon-style (Spanish-Raft) cultivation techniques (Bartley 2005). In the mid 1970's, traditional Japanese long-line technology was introduced. Since then, long-line technology in New Zealand has been adapted to allow for mechanised farm management practices, providing high cost-effectiveness. A typical mussel farm contains approximately 10 long-lines. Each long-line is composed of two backbone ropes (over 100 m in length) and supported by 30–40 large floats (Butler 2003). Dropper lines, approximately 3500 m in length, are attached to the backbone ropes, providing a large vertical surface area for growing mussels (Figure 3).

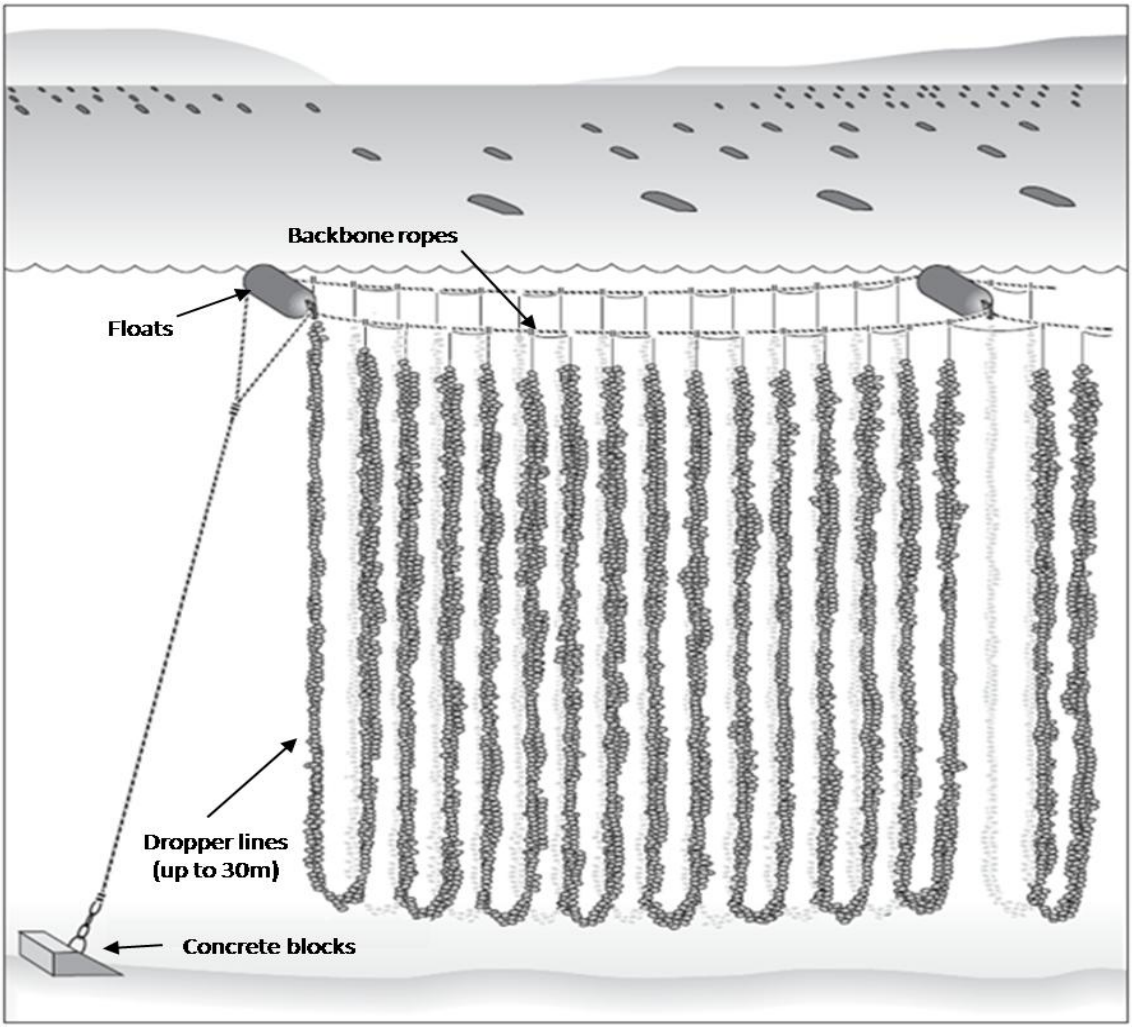

Figure 3. Long-line suspended culture technique for farming *P. canaliculus*. Adapted from Lloyd 2003.

The mussel aquaculture industry in New Zealand relies heavily on obtaining juvenile spat from natural stocks and re-settling them onto ropes for on-growing (Alfaro & Jeffs 2002). A natural phenomenon which occurs in the region of Kaitaia, northern New Zealand, provides a significant source of the juvenile mussels. Large quantities of newly settled spat, found densely attached to drifting macroalgae, are washed up on the foreshore at irregular times throughout the year (Alfaro & Jeffs 2002; Alfaro et al. 2004). The timing of this event often correlates with strong winds and high swell conditions (Alfaro 2001). Over 160 tonnes per annum of this 'beach cast spat' are distributed to mussel farms, accounting for approximately 80% of the industry's requirements (Alfaro et al. 2001). This spat source, being dependent on local environmental conditions, is often unreliable and unpredictable in timing and volume (Hay & Grant 2004). This makes it difficult for mussel farmers to manage their operations (Hay & Grant 2004). The other 20% of the industry's requirements for mussel spat is caught directly from the water column on 'spat catching ropes'. Farmers suspend spat catching lines in areas that are known to produce high quantities of planktonic larvae that are ready to settle (NZMIC 2005). Juvenile mussels caught from the water column tend to be of high quality. However, the results of such practices also are variable, and the technique is costly (Hayden 2002; NZMIC 2005).

After collection of beach cast spat, the seaweed is carefully, and quickly, transported to mussel growers in other parts of the country (NZGovt 2008). Once the juvenile spat arrive at the mussel farms, they are re-settled onto nursery lines. The seaweeds, with the attached spat, are positioned along the length of nursery ropes and covered by a continuous tubular biodegradable socking, firmly holding the mussels in place (NZMIC 2005). This procedure is performed automatically by machine (Figure 4). During this process the spat are seeded onto the ropes at a density of 1000 to 5000 spat per meter of rope (NZMIC 2005).

Figure 4. Automatic machine seeding of 'beach cast spat' onto nursery lines. Mussels and seaweed are covered with continuous biodegradable cotton socking to hold spat in place.

After initial seeding of 'beach cast spat', the nursery lines are suspended in the ocean where the seaweed and socking degrade within weeks (Kaspar 2005). After 3–6 months, the nursery lines are lifted and the high density young mussels (now approximately 10–30 mm in length) are removed from the ropes. The mussels are then re-seeded onto thicker growing ropes at a density of around 150–200 mussels per meter (NZMIC 2005). To secure the mussels, they are once again covered with biodegradable cotton socking to keep them in place while they establish a firm attachment to the ropes. The growing ropes are then attached in loops (dropper lines) to backbone ropes, and suspended in the ocean for final on-growing (Figure 5).

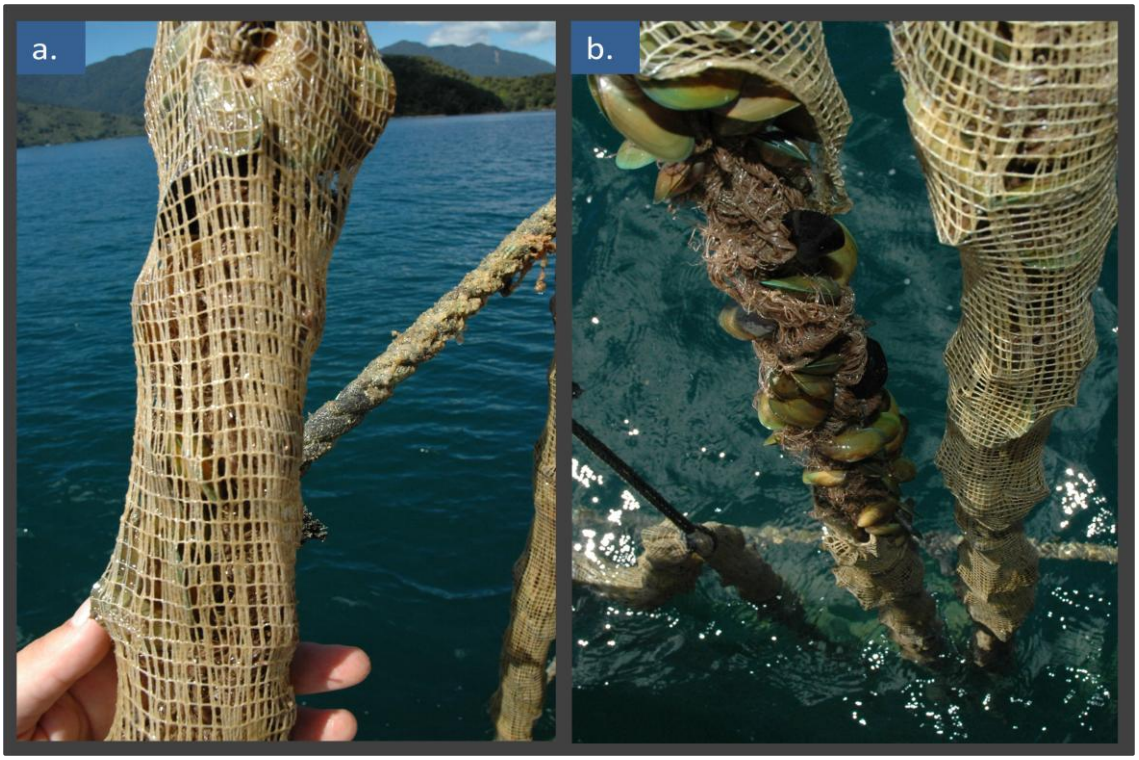

Figure 5. Biodegradable cotton socking for retaining juvenile mussels on nursery lines (a); partially degraded cotton socking (b).

The duration of the complete culture cycle from seed to harvest varies from site to site, and depends on the densities of mussels attached the ropes. However, this cycle generally takes 12–18 months, with mussels growing to 90–120 mm in length (NZMIC 2005). An overview of the green-lipped mussel culturing process is illustrated in Figure 6.

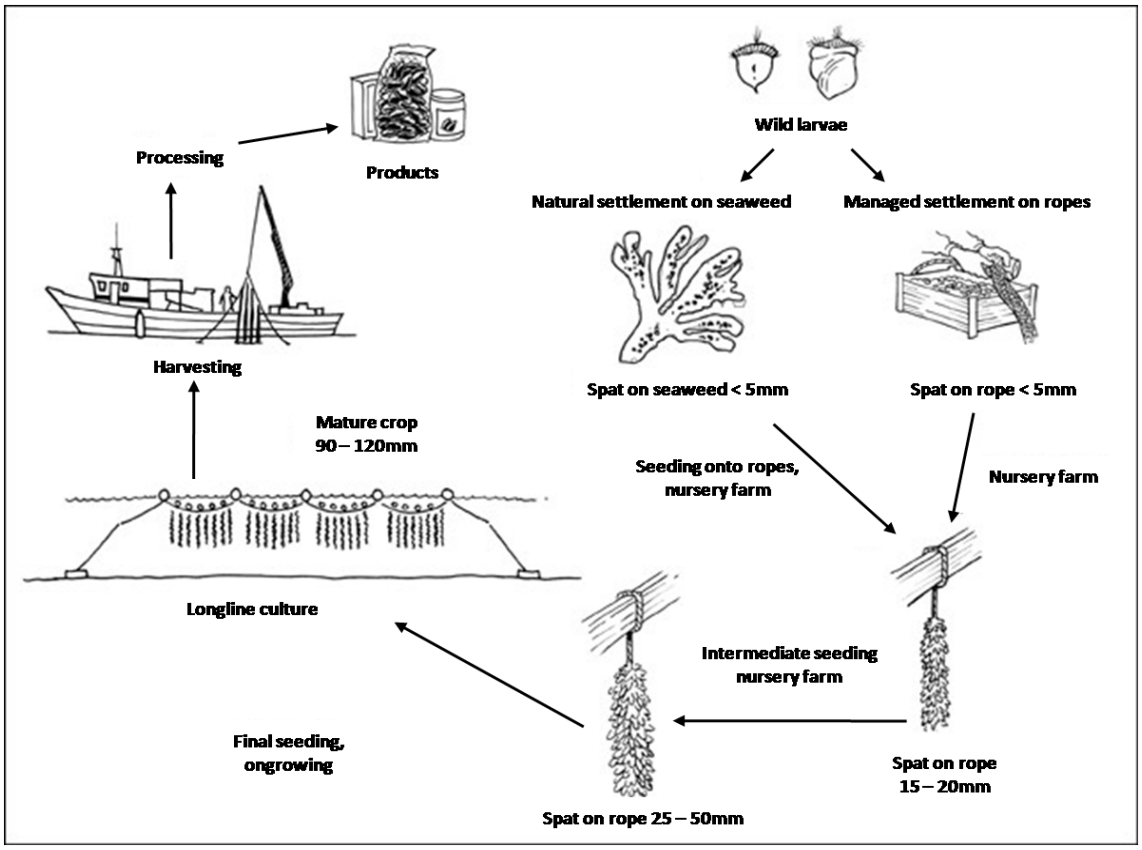

Figure 6. Overview of the green-lipped mussel industry in New Zealand.

1.4.3 Concerns within the industry

A major concern for the mussel industry relates to the unreliability of sourcing juvenile mussels to stock mussel farms with. Because of this, there has been considerable interest in the development of land-based mussel hatchery rearing facilities. In New Zealand there are two commercial hatcheries in operation, Sealord Ltd. in Ruakaka, North Island, and the Cawthron Institute in Nelson, South Island. At these facilities, adult broodstock mussels are spawned, and the gametes fertilised to produce larvae. The swimming veliger larvae are reared in tanks until they become competent to settle. This rearing process usually takes around 24–28 days, and is dependent on the development stage of the larvae (J. Bilton, hatchery manager, Sealord Ltd, personal communication,

23 April, 2008). Some factors which may contribute towards larval development in a particular production batch include broodstock source, diet, temperature, light conditions, and water quality - the timing of settlement competency tends to vary from batch to batch. Once the larvae are ready to settle, they are transferred into 'setting tanks' where they are settled onto nursery ropes. The settlement process may take up to 10 days to complete (J. Bilton, hatchery manager, Sealord Ltd, personal communication, 23 April, 2008). Afterwards, the attached mussels are distributed to mussel farms for on-growing. Because of the high costs involved in hatchery production of mussel larvae, and the unpredictability in batch cycle times (i.e. caused by variations in larval development and setting periods), this method of providing juvenile mussels also has its concerns. Compared to other juvenile mussel sources (i.e. beach cast spat and wild caught larvae), these hatcheries do not supply the aquaculture industry with significant quantities of mussels.

Another major concern for the green-lipped mussel industry in New Zealand relates to poor spat retention on growing ropes. Losses of up to 100% have been reported in the past, with spat losses commonly exceeding 70% (NIWA 2006). Once spat are seeded onto growing ropes, poor spat retention often occurs over the following months, after the cotton socking has degraded. Although some of this loss is suspected to occur due to poor initial spat attachment during seeding, predation by fish, high water currents, bad water quality, limited food supply, poor weather conditions, disease, and biofouling, very little is known about the actual causes of poor spat retention in *P. canaliculus* (NIWA 2006).

1.4.4 Opportunities for development

There are three main areas within the mussel aquaculture industry that could be improved to increase the production and financial returns of this commercially important species.

1. **Enhancing the volume of juvenile mussels obtained from natural stocks.**

   By enhancing wild spat collection techniques there is the potential to significantly increase the supply of juvenile mussels to stock farm. By identifying compounds which have the ability to induce larval settlement, there may be an opportunity to incorporate such compounds into spat catching ropes to increase the number of larvae caught from the water column. Such technology may improve the temporal distribution of juvenile mussels to local farms by smoothing seasonal variations in spat supply. The enhancement of spat catching techniques would decrease the financial overheads involved, making this mussel source more economical and sustainable than it currently is. Furthermore, it has been estimated that technology which has the capacity to increase larval settlement on catching ropes could displace the beach cast spat by up to 25% (C. Hensely, director, Kaitaia Spat Ltd, personal communication, 26 October 2008). This would improve accessibility and reliability of the mussel spat resource.

2. **Enhancing hatchery production techniques.**

   One of the costs associated with hatchery production of larvae is caused by the duration of time between production batches. The larvae are reared in a rotational batch process and any improvement which may decrease the time between cycles would be beneficial. There are usually variations in the development of larvae during a production cycle, with some larvae becoming competent to settle before others. This non-synchrony means that batches often have to be left for longer than others, to allow the ‘slow’ larvae time to develop. Although this may only be a matter of a few days, the costs involved in

prolonging a cycle are substantial. Furthermore, the quality of some larvae may be reduced under such circumstances. Once larvae are determined to be at a suitable development stage for settlement, they are relocated into 'setting tanks' where they settle onto nursery ropes over 10 days or so. By identifying compounds which can quickly induce larval settlement and provide synchronous attachments of the larvae to substrata, the duration between production batches may be reduced considerably. This would not only increase the economic viability of rearing larvae in hatchery facilities, but also would make the management of such processes easier with outcomes being more predictable.

3. Enhancing the retention of mussels on nursery and growing ropes.

Because the retention of spat on ropes often is poor, the development of new techniques to enhance retention would be greatly beneficial. Much of the beach cast spat which is seeded onto growing ropes is ultimately wasted, increasing the overheads for marine farmers. Furthermore, larvae caught on larval/spat catching ropes to stock mussel farms also are wasted due to poor spat retention – this may result in unnessecary pressure being placed on wild mussel populations. The incorporation of compounds into growing ropes which increase spat retention may provide an urgently needed solution to this problem. Alternatively, the identification of substrates with particular physical properties that enhance spat retention also may provide a remedy.

1.5 Thesis outline & structure

1.5.1 Aims & Objectives

This thesis aims to increase the understanding of the larval settlement process in *P. canaliculus* by identifying compounds which have the ability to induce settlement. In doing so, insights into the biochemistry of this organism may be gained. Based on these results, this thesis also aims to provide comprehensive discussions on the biochemistry of settlement inductions in order to identify areas of future research, not only for *P. canaliculus*, but also for other marine invertebrate species.

There are three reasons why *P. canaliculus* was chosen as the organism in this thesis to investigate the larval settlement process. (1) the green-lipped mussel is an endemic species to New Zealand, therefore providing a unique opportunity for study. (2) Very little is known about the larval settlement processes of this species, and the biochemical signalling pathways which regulate such processes. (3) *P. canaliculus* provides a source of revenue for New Zealand through aquaculture, and there are opportunities for developing new technologies which may enhance the production of this species, such as the inducement of larval settlement.

The primary objective of this thesis was to identify pharmacologically active compounds which have the ability to induce larval settlement in *P. canaliculus*. Based on previous studies investigating the effects of various compounds on larval settlements in numerous other species, a range of compounds was chosen to be trialled. In the order in which these compounds are investigated in this thesis, this initial selection comprised of: gamma aminobutyric acid (an amino acid and neurotransmitter), L-Tryptophan

(an amino acid and precursor to the neurotransmitter serotonin), the potassium ion as a chloride salt (an ion transport effector), acetylcholine (a neurotransmitter), epinephrine (a neurotransmitter and hormone), L-DOPA (an amino acid and precursor to epinephrine), L-Tyrosine (an amino acid and precursor to L-DOPA), L-Phenylalanine (an amino acid and precursor to L-Tyrosine), cyclic adenosine monophosphate (a secondary messenger of signal transduction mechanisms), and caffeine (a phosphodiesterase inhibitor and stimulator of endogenous levels of cyclic adenosine monophosphate). Due to the results of these investigations, a further range of compounds was selected to expand upon previous results. These compounds were: the potassium ion as sulphate and metabisulphite salts, atropine (an antagonist of a specific class of acetylcholine receptors), hydrogen peroxide (an oxidation breakdown product of epinephrine and L-DOPA), ascorbic acid (an antioxidant), and sodium metabisulphite (also an antioxidant). The justifications for selecting these compounds are given in following chapters.

During the progression of this thesis, two secondary objectives also were developed. The first of these objectives involved a meta-analysis of the inductive larval responses determined in this thesis with results gained from other studies in various marine invertebrate species. This investigation explored the use of clustering techniques to group species based on settlement responses to pharmacologically active compounds. The aim was to elucidate currently unknown relationships among the species based on the settlement neuroethologies of the organisms analysed. The second objective involved a study in an attempt to identify technologies which may enhance areas within the mussel aquaculture industry not already covered by the identification of larval settlement inducing compounds. Two main areas were investigated for enhancement: catchment of wild larvae/spat (through an attraction study using surface bound acetylcholine, and an attachment study using charged surfaces), and retention of spat on growing ropes (through the use of charged surfaces, and exogenous application of acetylcholine).

1.5.2 Layout and hypotheses

This thesis is composed of twelve chapters. Chapter 2 involves an investigation into the suitability of producing larvae onsite at the AUT aquaculture facility – to provide organisms for later settlement assays. Chapter 3 involves an investigation into factors which may influence the results of later settlement assays. Chapters 4–9 detail the investigations of settlement induction assays and are divided based on chemical groupings of pharmacological compounds, or to provide a logical sequence of events. Chapter 10 involves a cluster and meta- analysis of larval responses to inductive compounds, Chapter 11 details investigations into mussel spat attraction, attachment, and retention, and Chapter 12 provides a brief general discussion and recommendations for future research. Each of the ten middle chapters include their own introductions and literature reviews, and also provide extensive discussions of the results in the context of the literature.

1.5.3 Hypotheses

Chapters 3–11 all have at least one hypothesis. Chapter 2 does not since it was based around investigatory pilot work with no expected outcome. The reasoning behind these hypotheses mostly are described in the individual chapter introductions. These hypotheses are:

Chapter 3:

- Larval age (18–23 days old) has no effect on settlement rate.
- Hatchery production batch has no effect on settlement rate.
- Hatchery protocol has no effect on settlement rate.
- Settlement rate is dependent on incubation time.

Chapter 4:

- Exogenous application of gamma amino butyric acid induces larval settlement.
- Exogenous application of L-Tryptophan induces larval settlement.

Chapter 5:

- Exogenous application of potassium chloride induces larval settlement.
- Exogenous application of potassium sulphate induces larval settlement.
- Exogenous application of potassium metabisulphite induces larval settlement.

Chapter 6:

- Exogenous application of acetylcholine induces larval settlement.
- Exogenous application of atropine has no effect on larval settlement
- Exogenous application of atropine has no effect on acetylcholine induced settlement.
- The nicotinic class of acetylcholine receptors are responsible for settlement induction with acetylcholine.

Chapter 7:

- Exogenous application of epinephrine induces larval settlement.
- Exogenous application of L-DOPA induces larval settlement.
- Exogenous application of L-Tyrosine induces larval settlement.
- Exogenous application of L-Phenylalanine induces larval settlement.

Chapter 8:

- Exogenous application of hydrogen peroxide induces larval settlement.
- Exogenous application of ascorbic acid does not induce larval settlement.
- Exogenous application of sodium metabisulphite does not induce larval settlement.
- Exogenous application of sodium metabisulphite causes high mortality.
- Ascorbic acid is a suitable antioxidant for preventing oxidation of catecholamine treatment solutions.

Chapter 9:

- Exogenous application of cyclic adenosine monophosphate induces larval settlement.
- Exogenous application of caffeine induces larval settlement.

Chapter 10:

- A cluster analysis of settlement responses to pharmacologically active compounds will group species in a similar manner to morphological and genetic taxonomic groupings.

Chapter 11:

- Surface-bound acetylcholine will attract juvenile mussel spat.
- Substrates with a positive surface charge will enhance spat attachments to substrata.
- Substrates with a negative surface charge will inhibit spat attachments to substrata.
- Substrates with a positive surface charge will retain mussels longer on substrata than negatively charged surfaces.
- Exogenously applied acetylcholine will enhance spat retention on glass substrates and on coir substrates.

1.5.4 Scope

The investigations undertaken in this thesis, regardless of outcome, will provide important information about the developmental biology, biochemistry, pharmacology, and neuroethology of the endemic green-lipped mussel. This information will supply a baseline for future research. By determining the larval responses of *P. canaliculus* to pharmacologically active compounds, further insight into comparative biologies among marine invertebrate species will be gained. This will provide greater understanding of

the diversity and complexity of life, substantiating the foundations of human effort to secure a sustainable environmental future.

Due to the considerable interest in New Zealand mussel aquaculture, it is necessary to have an in depth knowledge of the biology and behaviours of the comercially important green-lipped mussel. Gaining an understanding of the various natural processes, such as larval settlement, is vital for the management of fishery stocks, the development of future aquaculture methods, and construction of environmental impact assessments. The results gained in this thesis have potential to provide fundamental information for the New Zealand aquaculture industry to increase productivity, thus improving economic viability. This information could be of value for the development of efficient new methods in juvenile mussel sourcing, hatchery production of mussel larvae, and the enhancement of mussel spat retention.

CHAPTER 2

LARVAL PRODUCTION: SPAWNING & FERTILISATION TRIALS

1. INTRODUCTION

To obtain larvae for this investigation, the spawning of adult mussels was performed in the laboratory. It is well known that fertilisation success rates of gametes spawned in hatchery facilities vary between bivalve species (Helm et al. 2004). Under ideal conditions of maximum gamete viability, fertilisation success of ova is primarily dependent on gametes making contact. However, numerous factors contribute to this viability, such as, gamete concentration and ratios, gamete age at time of mixing, and the temperature and density at which gametes are stored prior to mixing (Sprung & Bayne 1984; Levitan 1995). In the case of *P. canaliculus*, Tong and Redfearn (1985) suggest that a large proportion of gametes are not viable. However, Buchanan (1998) found that under set optimal parameters for broodstock selection and conditioning (i.e. methods of spawning induction, temperature and density and period of gamete storage, and gamete ratio prior to mixing) a fertilisation success rate of 98% could be consistently achieved.

Buchanan (1998) suggested that *P. canaliculus* broodstock individuals should contain gonads with high follicle and gamete density. These conditions may be maximised by sampling at specific times of the year, based on temporal variations in the reproductive cycle of this species. Helm et al. (2004) suggested that broodstock tanks used during the conditioning period should have a continuous flow of water exceeding 25 ml per minute per adult. He also indicated that a maximum of 5 kg live weight biomass of stock should be contained in tanks no less than 120 L. Broodstock individuals should be conditioned for ten weeks if the gonads are under-developed, 4–9 weeks if gametogenesis is well underway, and 7–12 days if individuals with high indices of gonad development have been obtained (Helm et al. 2004). Water temperature during the conditioning period should be equivalent to that observed in the natural environment at the specific times of the year at which normal gonad maturity is reached (Helm et al. 2004). For *P. canaliculus*, a temperature of

12°C is suggested by Buchanan (1998). However, mean sea-surface temperatures during these peak gonad maturity periods are variable depending on the geographic location of the population from which the broodstock individuals are obtained.

A number of methods are available to induce spawning in bivalves, including gamete stripping, thermal cycling (temperature shock), and treatment with various chemical compounds. Spawning induction methods for *P. canaliculus* should not incorporate the gamete stripping technique since eggs undergo a maturation process during their traverse down the oviducts, which is required for successful fertilisation (Helm et al. 2004). Instead, the thermal cycling technique has been successfully used for this species (Buchanan 1998). Chemical induction should only be employed as a last resort, if the success of thermal cycling is limited. The selection of chemical compounds for this mode of induction is also important. For example, serotonin induction is ineffective for *P. canaliculus* (Buchanan 1998). Conversely, for the related mussel species, *Perna viridis*, treatment with hydrogen peroxide at low concentrations (0.3%) is effective (Sahavacharin et al. 1984, 1985, 1988). *P. canaliculus* is a dioecious bivalve, and like other species of this type, the males are invariably the first to release gametes during spawning induction. The sperm age more quickly than the eggs, and if left for more than 1 hour may cause a substantial reduction in the rate of fertilisation. It is common practice to wait until the females spawn before inducing the males (Helm et al. 2004).

Once gametes are obtained, it is suggested that they be stored in cool filtered seawater (10–12°C) for a maximum of 30 minutes before mixing, and only if necessary (Buchanan 1998). However, Hay and Hooker (1994) suggested that gamete storage of 30 minutes prior to mixing results in a 25% reduction in larval yield, although the storage temperature was not revealed in this study. If storage is necessary, spermatozoa should be stored at concentrations of $>1x10^6$ sperm/ml to maximise their lifespan (Buchanan 1998). Before mixing of gametes, egg suspensions should be gently filtered (90µm aperture) to remove contaminating faecal pellets produced by

the adults, reducing subsequent proliferation of bacteria, which may become problematic later on in the culture process (Helm et al. 2004). For *P. canaliculus*, Buchanan (1998) advocated that 50-100,000 sperm/ml should be combined with egg concentrations below 1000/ml to maximise fertilisation success.

2. METHODS

2.1 Broodstock

Adult mussels, approximately 18 months old, were obtained in July 2007 from Westpac Mussels Ltd, Wharekawa, Firth of Thames. Mussels were collected from the farm and kept cool and moist for 2 hours while transporting to the AUT aquaculture facility in Auckland. Shells were thoroughly scrubbed and rinsed to remove epifaunal organisms and sediment. To check for sexual maturity, ten randomly selected individuals were dissected and observed. The visual gonad grading system, developed by Buchanan (1998) for rapid assessment of gonad reproductive status, was used to predict the spawning activity of female specimens (Table 1).

Table 1. Description of the visual grading system for determining reproductive status of adult male and female mussel specimens (modified from Buchanan 1998).

Male VG	Female VG	Description
1	5	Undifferentiated gonad, clear or smooth white appearance. No or minor visible follicles. Females may have pale colouration being of >30% cover.
2	6	Light orange colour for females. Follicles sparse and small, cover being of <50%. Gonad may appear patchy with colour and development. Gonad ducts small and difficult to see.
3	7	Follicles cover 50-75% of the gonad with colour becoming darker orange for females. Ducts becoming more visible.
4	8	Follicle cover at the gonad surface is >75%. Colour a bright orange to pink for females and a creamy white for males. Follicles large and densely packed, gonad appears 'granular' and textured. Ducts are clear and easy to see between follicle covered areas. Gonad often distended with mature gametes.

Of the ten individuals, six were females containing large orange/pink gonads with a granulated appearance (VG = 7). These individuals were identified as being ripe enough for the spawning trials (e.g. Figure 7b). All male specimens observed contained large developed gonads with a VG = 4 (e.g. Figure 7d).

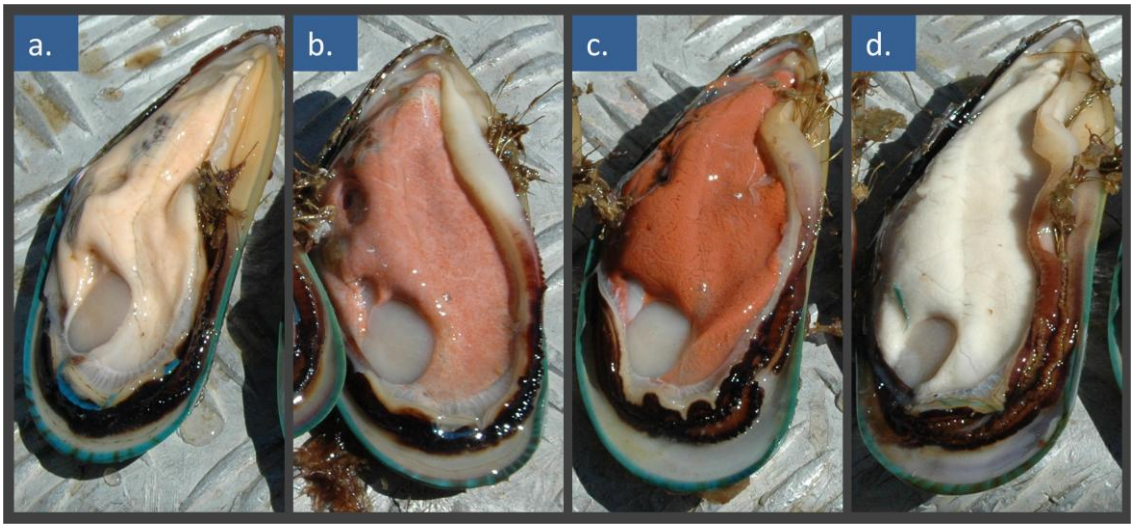

Figure 7. Sexual maturity in the gonad tissues of adult mussels. Immature female (a) showing slight yellow/orange colouration (VG = 6). Female reaching sexual maturity (b) showing first signs of granulation, an increase in gonad volume, and change in colouration (VG = 7). Very sexually mature female (c) containing full volume gonad tissue, very dark orange/red colouration and heavy granulation (VG = 8). Mature male individual ready to spawn (d) showing large gonad volume and a dense milky white appearance (VG = 4).

2.2 Conditioning

The remainder of the mussels were placed in an 80 L broodstock tank with fresh seawater maintained at 17±1°C for five days prior to spawning induction. The seawater was not filtered since the diversity of food species present in unfiltered seawater is beneficial in the conditioning process. During the five day period, mussels were fed a microalgal mixed diet consisting of *Tetraselmis suecica*, *Isochrysis galbana*, and

Chaetoceros calcitrans. The seawater was replaced every 24 hours to remove waste material. Adult individuals were fed approximately 10 mg organic weight (ash-free dry weight) of each microalgal culture per mussel daily during the conditioning period. The food rations provided for broodstock conditioning were calculated using the following equation as prescribed by Helme et al. (2004):

Ration (g) per adult per day = 3 x mean dry tissue weight (g) / 100

Algal stock cultures were diluted accordingly to provide the required ration using known cell mass values (Table 2) for each microalgal species in the conditioning diet. Cell counts were determined using a Neubauer haemocytometer.

Table 2. The cell volume, organic weight and nutritional content of the three microalgal species used for feeding broodstock mussels during the conditioning period (modified from Lavens and Sorgeloos 1996; Helm et al. 2004). Note: Protein, carbohydrate, and lipid compositions are expressed as percent organic weight.

Species	Median cell volume (μm^3)	Organic weight (µg per $1x10^6$ cells)	Protein %	Carbohydrate %	Lipid %
Dinoflagellates:					
Tetraselmis suecica	300	200	26	10	8
Isochrysis galbana	45	22	42	19	31
Diatom:					
Chaetoceros calcitrans	35	7	55	10	27

2.3 Microalgae cultures

Microalgae were obtained as pure monocultures from the National Institute of Water and Atmospheric Research Ltd (NIWA), and cultured at AUT in 500 mL Erlenmeyer flasks in UV sterilised 0.45 µm filtered seawater (FSW) at 22–23°C. The diatoms *Isochrysis galbana* and *Chaetoceros calcitrans* were cultured in standard Guillards F/2 enrichment media (Guillard & Ryther 1962, Guillard 1975) prepared as described in Table 3. The dinoflagellate species *Tetraselmis suecica* has no requirement for silica due to the lack of silicic acid composed frustules, a primary differentiating feature between diatoms and dinoflagellates. Hence, this species was cultured in standard Guillards F/2 enrichment media without the addition of sodium silicate (Na_2SiO_3) - also known as F/2-Si medium.

Table 3. Compositions of microalgal culture media.

F/2 Medium			Trace Metal Solution (TMS)		Vitamin Solution (VS)	
Compound	**Stock Conc.**	**Stock/L media**	**Compound**	**Stock Conc.**	**Compound**	**Stock Conc.**
$NaNO_3$	75.0 gL^{-1}	1.0 ml	$FeCl_3.6H_2O$	3.2 gL^{-1}	Vitamin B_{12}	1.0 mgL^{-1}
$NaH_2PO_4.H_2O$	5.0 gL^{-1}	1.0 ml	$Na_2EDTA._2H_2O$	4.4gL^{-1}	Biotin	1.0 mgL^{-1}
$Na_2SiO_3.9H_2O$	30.0 gL^{-1}	1.0 ml	$CuSO_4.5H_2O$	9.8 gL^{-1}	Thiamine HCl	200.0 mgL^{-1}
TMS	-	1.0 ml	$Na_2MoO_4._2H_2O$	6.3 gL^{-1}		
VS	-	0.5 ml	$ZnSO_4.7H_2O$	22.0 gL^{-1}		
			$CoCl_2.6H_2O$	10.0 gL^{-1}		
			$MnCl_2.4H_2O$	180.0 gL^{-1}		

2.4 Spawning

Two standard methods of inducing spawning were tested; thermal cycling and induction with dilute hydrogen peroxide. Three trials were conducted with different cohort populations. In the first trial only the thermal cycling regime was employed. The second and third trials involved thermal cycling and hydrogen peroxide induction. Although thermal cycling is the preferred method (hydrogen peroxide is slightly toxic at 0.3%), individuals which were not induced to spawn via temperature shock treatment were later exposed to dilute hydrogen peroxide to increase the chances of obtaining enough gametes for successful fertilisations and to provide sufficient larvae for future settlement assays.

2.4.1 Thermal Cycling

After five days of conditioning, 40 individuals were separated based on sex (visual grading system) and placed into two 20 L tanks containing standpipe drains and fresh 1μm filtered seawater at 12±1°C. Two header tanks were constructed; one with a submerged heater and thermostat (Jager 50W) which maintained fresh seawater at 28±1°C and the other with chilled seawater containing sealed ice bags. Water was carefully drained from the broodstock tank and simultaneously replaced with water from the heated header tank until the broodstock tank water reached 22°C. The warm water was left for 30 minutes before being drained and replaced with the cooler seawater until a temperature of 12°C was achieved and left for a further 30 minutes. The complete thermal cycling regime was repeated eight times. Individuals which revealed signs of spawning were immediately placed into separate 1 L beakers containing fresh filtered seawater at 12±1°C to complete the spawning process. Females were induced first with males being induced at the onset of female spawning.

2.4.2 Hydrogen Peroxide

Conditioned individuals which did not spawn during the thermal cycling regime were kept separated based on sex in the original 20 L tanks. Mussels were given 24 hours equilibration time in fresh seawater at 17±1°C and fed the previously described mixed algal diet. 400 ml of 15% hydrogen peroxide (H_2O_2) were gradually added to each tank with slow stirring to give a final concentration of 0.3%. Individuals which revealed signs of spawning were immediately placed in separate 1 L beakers containing 0.3% H_2O_2 in fresh filtered seawater at 17±1°C to complete the spawning process. Females were induced first with males being induced at the onset of female spawning.

2.5 Fertilisation

Within 20 minutes of male spawning the combined gametes from all individuals for each treatment were collected via pipette and placed separately, based on sex, into two 1 L beakers containing FSW at 12±1°C. Released gametes from the two induction methods employed were kept separate in case of adverse treatment effects. Using a compound microscope and Neubauer haemocytometer, the water in each beaker was carefully adjusted to give gamete concentrations of approximately 50,000 sperm per ml and 100 eggs per ml. The gametes from the respective treatment method which induced spawning, were mixed together and 1ml samples were taken every 15 minutes for 6 hours. Samples were viewed under a microscope at 400X magnification to observe the quality of gametes and determine fertilisation success.

3. RESULTS

The results of the three spawning induction and subsequent fertilisation trials are shown in Table 4. Males were induced to release gametes in every trial, whereas females were induced in trials two and three only. In total, the cumulative percentage of males induced by both treatments was greater than that of females (36.7% *vs*. 21.9%). In every occasion where induction by temperature shock was followed by hydrogen peroxide treatment, individuals from both sexes which had failed to release gametes in the first treatment were induced to spawn. From successful spawning trials, collection and mixing of gametes at the optimum concentrations prescribed by Buchanan (1998) failed to produce fertilised eggs in any significant quantities.

Table 4. Spawning induction and fertilisation success – results of three trials using two methods for inducing gamete release.

Cohort population	Spawning method	Males (n)	Females (n)	Male spawning success	Female spawning success	Fertilisation success
1	Thermal cycling	(16)	(24)	12.5%	0.0%	-
2	Thermal cycling	(18)	(22)	22.2%	0.0%	-
2	Hydrogen peroxide	(14)	(22)	28.6%	19.0%	Failure
3	Thermal cycling	(26)	(14)	30.8%	35.7%	Failure
3	Hydrogen peroxide	(18)	(9)	16.7%	11.1%	Failure

Initial observations showed spermatozoa to be healthy, with cells forming a milky suspension and exhibiting a high degree of motility when observed under the microscope. In every trial, eggs tended to be liberated in clumps, showing signs of

reduced viability. However, 15 minutes after contact with the seawater, approximately 80-90% of eggs had hydrated and assumed a spherical shape with the remaining cells being pear-shaped. During mixing of gametes, 20–40 sperm cells were observed attempting fertilisation by surrounding each egg cell (e.g. Figure 8a). After 45 minutes, approximately one in every 100-150 eggs had been penetrated by the spermatozoa with extrusions of the first polar body being evident (e.g. Figure 8b). Within 1–2 hours these cells had divided and developed into the two-cell stage with the appearance of the second polar body (e.g. Figure 8c). This two-cell stage produced a pear shaped structure through unequal cleavage. 3–4 hours after initial mixing of gametes, egg cells had reached the four-cell stage (e.g. Figure 8d) and within 4–6 hours many of those cells which had been fertilised had developed into the eight-cell stage (e.g. Figure 8e). Although fertilisation was observed, the percent fertilisation never exceeded 0.5–1.0% of the total number of eggs.

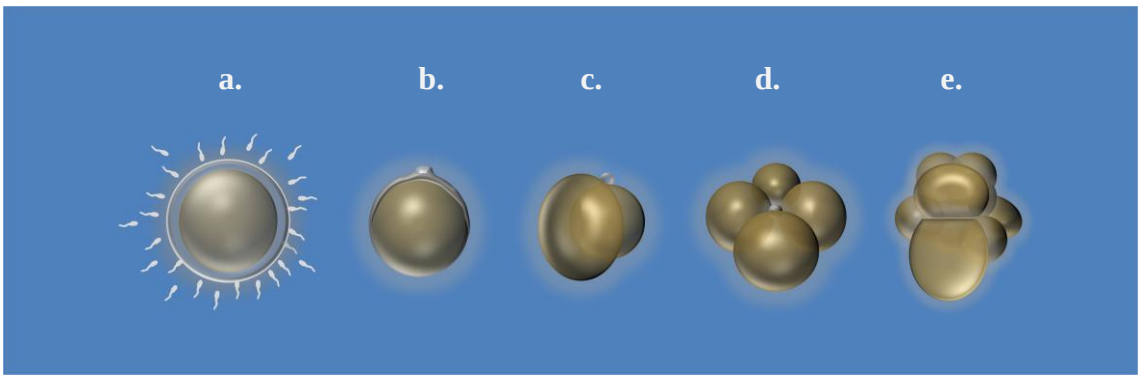

Figure 8. Illustrated early stages of egg development observed in fertilisation trials; (a) spermatozoa swimming and attempting fertilisation; (b) extrusion of the first polar body after fertilisation; (c) development of the two-cell stage showing unequal cleavage; (d) four-cell stage; (e) eight-cell stage.

4. DISCUSSION

Although spawning induction was achieved, the poor fertilisation success rate was too inefficient to provide larvae in the quantities required for subsequent settlement assays. Three possible explanations for this are discussed: conditioning and fertilisation parameters, spatio-temporal variations, and weather anomalies. Any of these factors may have individually caused the poor fertilisation success rate observed. However, it is likely that a combination of all three influences were responsible.

4.1 Conditioning and fertilisation parameters

The conditioning parameters used for the larval production trials incorporated those suggested from the literature, where possible. However, due to equipment and time constraints, three of these parameters were altered. Although female broodstock individuals had a VG value of 6, the conditioning time was limited to only five days. This period was decided on since the trials were initially designed as a pilot study, and due to problems with the circulatory system and lack of space. At the time, five days was considered manageable. The water temperature during conditioning was maintained at 17±1°C, controlled by the air temperature inside the aquaculture lab. This compromise also was due to availability of the circulatory system incorporating a chiller unit. Although this temperature is higher than the 12°C recommended by Buchanan (1998), it may not have had a significant impact. Buchanan's (1998) study focused on mussel populations from southern New Zealand where winter sea-surface temperatures are considerably lower than those observed in Firth of Thames, 11–12°C *vs*. 15–16°C respectively. Since 17°C is only slightly higher than natural conditions during expected normal peak gonad conditions, the negative effect of increased temperature in the laboratory is likely to be reduced for broodstock individuals from the Wharekawa area. Conditioning of broodstock was performed under static conditions with regular water changes, as opposed to the recommended flow-through system.

However, since the flow-through system recommended by Helm et al. (2004) was for large numbers of broodstock when conditioning periods of up to 10 weeks are employed, it is suggested that the low broodstock numbers in the present study, and the daily water changes performed, was sufficient enough to remove waste materials and maintain optimal water conditions. Also to support this argument, the water volume to stock biomass ratio, suggested by Helm et al. (2004), was kept well over the minimum volume advocated.

4.2 Spatio-temporal variations

There is evidence suggesting that spatio-temporal variations in the reproductive cycle of *P. canaliculus* exist throughout New Zealand (Flaws 1975; Hickman et al. 1991; Buchanan 1998; Alfaro et al. 2001; Alfaro et al. 2003). At locations in northern New Zealand, unique conditions are thought to be responsible for the gonad development disparities observed between these populations and those in southern New Zealand (Alfaro et al. 2001). Differences in the reproductive cycle also have been observed between intertidal and subtidal populations with indications that the latter may have one prolonged trickle spawning season (Alfaro et al. 2003).

The seasonal reproductive trends in *P. canaliculus* determined by Buchanan (1998) relied solely on samples taken from a natural intertidal population in the French Pass, Marlborough Sounds, and north of the South Island New Zealand. In his study, Buchanan suggests that follicle and gamete density in both sexes peak primarily in July and spawning follows in early spring, with a second late summer spawning (Figure 9). These findings largely agree with those summarised by Hickman et al. (1991). However, it is the general consensus among authors of comprehensive reproductive studies, including in the far north of New Zealand, that one gametogenesis stage in both sexes is initiated around April/May, providing mature gonads early-mid winter.

Based on this information, the broodstock specimens obtained in the present study were sampled during July. Due to availability, broodstock individuals were obtained from the western side of the Coromandel peninsula in the Firth of Thames, located just north of central New Zealand.

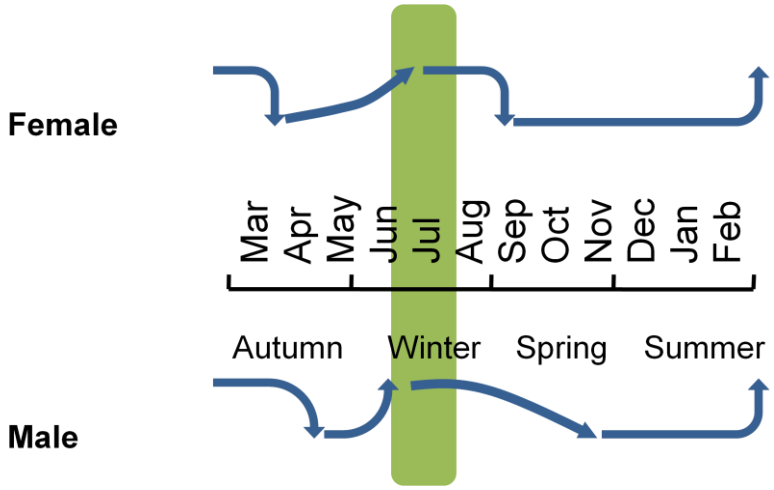

Figure 9. Illustration of temporal variation in the reproductive cycle of *Perna canaliculus* as suggested by both Buchanan (1998) and Hickman et al. (1991). Blue arrows show levels of gonad maturity in females and males. Inclines represent gametogenesis periods and declines represent spawning periods. Sampling time of broodstock individuals for the current study is indicated (green box).

It is possible that the spatio-temporal variations in the reproductive cycle of *P. canaliculus* observed in previous studies may be responsible, in part, for the poor fertilisation success of the larval production trials in the present study. It was expected that the gonads of female individuals during July would contain high follicle and gamete density, corresponding to a VG value of 8 using Buchanan's visual gonad grading system. However, female broodstock individuals were identified as having a VG value of 6 – slightly less mature than ideal. Male gonads had maximal follicle and gamete density. Perhaps the long gametogenesis period in the few months prior to

winter, observed by Buchanan (1998) and Alfaro et al. (2003), is delayed or prolonged in the case of mussels from Wharekawa. This would in effect cause a shift to the right in the female reproductive cycle represented in Figure 9. If this was the case, the gonads of individuals in July would not be as developed as previously expected, leading to the subsequent induced spawning of underdeveloped eggs in the larval production trials. Because ova were observed to clump together immediately after liberation, this suggests that eggs were not fully developed. This supports the hypothesis of a gametogenesis lag in subtidal zones of the Firth of Thames compared with the reproductive cycle of *P. canaliculus* in other New Zealand locations.

Although not a primary interest of this study, observations of the reproductive status of *P. canaliculus* at Wharekawa during July 2007 may provide pointers for future areas of research. The differences observed in the gonad development stages between females and males may reflect less reproductive synchronicity between the sexes in this area than has been shown for other locations. Alfaro et al. (2003) provided evidence that subtidal populations, albeit from naturally occurring mussel beds, have consistently higher gonad indices and more reproductive synchronicity than their intertidal counterparts. Although the number of individuals sampled in the current study was very small, and comparisons were not made between subtidal and intertidal populations, the different gonad stages identified between sexes may reflect possible spatial variations, on a wider geographic scale, in the spatio-temporal variations between different depths at singular geographic locations. This could be worth investigating in the future.

4.3 Weather anomalies

In July 2007, weather conditions throughout New Zealand were described as being extreme and contrasting. In the weeks prior to sampling, the Coromandel Peninsula experienced high temperatures, high rainfall producing flooding in the area, thunderstorms, and extreme winds with several tornado events (NIWA, 2007). Combined, these conditions are likely to have caused increased wave action and

mechanical stress, increased turbidity, alterations in salinity and seawater chemistry, and potential adverse effects on food sources. In addition, 2007 was an El Niño year. It is possible that gametogenesis and gonad development during this time were delayed or prolonged due to reserved energy supplies being utilised for survival rather than reproduction. Although these ideas are speculative, such weather conditions may have contributed to the poor fertilisation success observed from the gametes of the broodstock individuals. It may be these conditions which are the cause of the hypothesised lag in gonad development outlined in the previous discussion on spatio-temporal variations between the Wharekawa site and those locations described in the literature.

To eliminate possible complications affecting the poor fertilisation success observed in the larval production trials, a mitigation strategy was conceived. Sampling of broodstock individuals to obtain specimens with fully mature gonads was planned during autumn 2008, when another spawning event occurs at specified locations (e.g. Hickman et al. 1991; Buchanan 1998). Also, plans to improve the broodstock conditioning parameters were devised i.e. decreasing the conditioning temperature, increasing the conditioning period, and designing a system with water flow-through conditioning tanks. However, before these proposed alterations to the larval production parameters could be implemented, an opportunity to obtain regular batches of healthy larvae at the correct development stage immediately prior to settlement competency was presented. Two commercial hatcheries with well-refined processes for culturing *P. canaliculus* larvae, Sealord Ltd and the Cawthron Institute, kindly offered to supply veliger larvae for this study. Due to this proposal, and since the larval production trials were only initiated to provide specimens for subsequent settlement assays, the suggested alterations to the parameters were never applied.

CHAPTER 3

LARVAL SETTLEMENT: AGE, BATCH, HATCHERY, & INCUBATION EFFECTS

1. INTRODUCTION

When performing settlement inducing assays, the age at which subjects are selected is very important. It is essential that larvae are sufficiently competent to settle yet not competent enough that high settlement percentages result in control assays. This chapter describes an assessment of the effective post-fertilisation age of larvae required for assays under treatment conditions in later chapters. The effects of larval production batch and hatchery protocols on larval settlement rates were examined to determine bias. Also, the incubation/exposure time required for settlement assays was investigated.

The pelagic or free swimming stage of most marine invertebrate larvae is an inherent part of their biology designed to maximise larval dispersal. Larvae are adapted to be retained in the water column for various periods of time depending on the species and the environment in which they live (Abelson & Denny 1997). For most of these organisms, the settlement process onto suitable substrates is a stage of normal development which must occur before metamorphosis can take place: it is vital for their survival. Age effects on settlement rate can be variable within species. For example, Bayne (1965) suggested that larvae of *Mytilus* sp. usually settle after a 3–4 week pelagic period but may delay settlement for up to ten weeks. Age effects are also variable between marine invertebrate species. For example, Satuito et al. (1997) suggested that cyprid larvae of the barnacle *Balanus amphitrite* may settle 12–72 hours post-fertilisation, this is considerably quicker than *Mytilus* sp.

Selecting the correct larval age for settlement studies for a particular species is important because high settlement rates in control assays is undesirable. If high rates do occur in control assays, it may be difficult to identify marked increases caused by the treatments administered. Also, if a high settlement rate

naturally occurs in the control, there is the possibility that the larvae may have lost their ability to detect or respond to inducers/inhibitors (e.g. Gribben et al. 2006). If larvae are too young, biological processes (i.e. in the case of mussels, the ability to produce mucous adhesives or fine byssal filaments) may not yet be developed enough for settlement to occur. In this situation, under treatment conditions, no detectable improvement in settlement rates may be observed. Therefore, ideal conditions for settlement assays require that the age range selected is old enough that settlement can occur, but young enough that a low percent settles under control conditions with limited variability. Based on pilot work, it was decided that the selected age range of larvae suitable for settlement assays must result in at least 10% settlement (initial stages of competency) with an absolute maximum settlement rate of 30% occurring under control conditions.

Since the opportunity arose to obtain consistently healthy larvae from two commercial hatcheries, some important factors had to be considered. At these facilities, larvae are reared in batch processes with 3–6 week cycle times. Because larvae provided for settlement assays were to come from separate batches, the following question was raised: What is the effect of different batches on larval settlement rates? Although the production parameters at both hatcheries have been finely tuned over the periods each have been culturing *P. canaliculus* larvae, single batch production techniques inherently produce larger variations than semi-continuous, or rotational batch, practices. Variation in larval quality is however a major concern for both techniques. The Cawthron Institute employs a single batch process due to their smaller scale and relatively low larval demand, whereas Sealord Ltd. employs a hybrid production with a rotational batch process on a much larger scale. The set rearing conditions applied at each hatchery are constantly monitored and adjusted according to strict quality control procedures. Although these parameters are rigorously controlled, differences in larval development among batches have been observed. For example, the stage at which larvae obtain eye-spots, a primary indication of developmental stage, can vary among batches by 2–3 days. Also, the growth rates of larvae may differ slightly between batches. It is possible that these factors are determined by inconsistencies in

production conditions or by genetic variations of broodstock populations. Therefore, since production batch effects may cause differences in settlement rates between independent assays it was decided to determine the magnitude, if any, of this effect within each hatchery.

Since larvae were to be sourced from two hatcheries with different production parameters, it was determined that the effect of these larval rearing conditions should be investigated between hatcheries. The broodstock used at each facility come from very different populations with observable differences in their external morphology and colouration, indicating a degree of genetic variability. Although an effort is made at each hatchery to ensure genetic heterogeneity by using multiple broodstock individuals, the numbers of adult broodstock used are significantly different between the two facilities. Both hatcheries employ similar spawning induction techniques of broodstock individuals. The volumes of larval rearing tanks are markedly different, as is the incubation and culture media between the two facilities. Cawthron Institute uses sterilised seawater and Sealord Ltd. uses non-sterilised seawater. The incubation temperature at both facilities is the same until larvae reach the D-veliger stage, at which point alterations are made at both hatcheries resulting in different final culturing temperatures. Finally, the compositions of microalgal diets provided are also different between the two hatcheries. Details of all production parameters are described in the methods section.

The exposure time allowed for settlement studies may influence the results and subsequent conclusions made, especially if insufficient time is given for larvae to settle. Since settlement rates differ between and within species, it is important to determine the time required for the settlement process to occur, with limited variability, for the particular species under investigation. Even if settlement occurs quickly once larvae become competent, some considerations must be taken into account. Any stresses induced immediately prior to the settlement assays may affect settlement rates over short time periods. For example, the processes of sampling,

storage, transportation, and any handling of larvae involved in experimental set-up may be traumatic for the organisms. Also, changes between the environmental parameters employed during the larval rearing period at the hatchery and those applied during settlement assays may cause undue stress (i.e. adjustments in seawater chemistry, temperature, light conditions, oxygenation, water flow, larval densities, and food supply). It is likely that these effects may partially inhibit or promote initial larval settlement and cause high levels of variation over shorter time periods. Due to the possibility of age and production batch effects, allowing longer exposure periods so that the larvae have time to equilibrate to their new surroundings may reduce unwanted variation between experiments.

2. METHODS

2.1 Organisms

Larvae were obtained from two commercial hatcheries, Cawthron Institute in Nelson (South Island, New Zealand), and Sealord Ltd at the Bream Bay Aquaculture Park in Ruakaka (northern New Zealand). At these facilities, adult mussels were induced to spawn by thermal shock. Gametes were pooled and mixed from a number of individuals to ensure genetic heterogeneity (≥8). Fertilised eggs were incubated in sterile seawater (Cawthron Institute) or non-sterile seawater (Sealord Ltd.) at 17°C and 35 ppt salinity until 50% or more larvae had metamorphosised into the D-veliger developmental stage.

At the Cawthron Institute, veliger larvae were transferred into bullet-shaped 2.5 L polycarbonate tanks, as described by King et al. (2005). This system was supplied with aerated 1 µm filtered seawater (18±1°C, 35 ppt). Water inflow was maintained at 80 ml/min (non-circulatory) and dosed with two species of microalgae; *Chaetoceros calcitrans* and *Isochrysis galbana* (T-Iso clone).

At the Sealord Ltd hatchery, with its large scale operation, larvae were reared in 24,000 L tanks under static conditions with regular water changes every 48 hours. Water was maintained at 20±1°C (1 µm filtered), and a mixed diet of *Chaetoceros calcitrans*, *Isochrysis galbana*, and *Pavlova lutheri* was introduced in an increasing controlled ration, based on batch volume and growth rates, allowing *ad libitum* access to food. Table 5 outlines the key differentiating production parameters employed by each hatchery.

Table 5. Hatchery production parameters for larval rearing. Key differences between Sealord Ltd. and Cawthron Institute.

Hatchery	Broodstock (n)	Incubation & culture medium	Incubation & culture system	Culture volume	Final culture temperature	Diet[a]
Cawthron Institute	8	Sterile seawater (1µm filtered)	Flow through system (non-circulatory)	2.5L	18±1°C	Cc, Ig
Sealord Ltd.	200	Non-sterile seawater (1µm filtered)	Static, 48hr water change	24,000L	20±1°C	Cc, Ig, Pl

[a] *Chaetoceros calcitrans* (Cc); *Isochrysis galbana* (Ig); *Pavlova lutheri* (Pl)

At both facilities, most larvae had metamorphosised into pediveligers after approximately 18–21 days post fertilisation. At this stage, with the development of a foot and appearance of eye spots, individuals were considered capable of settlement. Approximately 40,000 competent swimming veliger larvae were screened by filtering through a 175 µm nylon mesh sieve, corresponding to a shell diameter of >215 µm. Larvae were placed in a small polyethylene tube with breathable membranes fitted on both ends. Samples were packed in polystyrene containers filled with moss and containing an ice pack to keep them cool and damp during transport to the aquaculture facility at AUT in Auckland.

2.2 Settlement Assays

The effects of post-fertilisation age, incubation period, hatchery production batch, and hatchery protocol on larval settlement was investigated. An outline of the sampling and experimental parameters, and the comparisons, to determine these effects are shown in Table 6.

Table 6. Experimental design to determine effects of age, incubation period, hatchery production batch, and hatchery protocol on larval settlement.

Effect investigated	Hatchery protocol	Larval age (days)	Batches sampled (n)	Incubation time (hrs)	Comparisons made
Larval age and incubation time	Sealord Ltd.	18, 21, & 23	1	24 & 48	Between ages for each incubation time
Larval batch and incubation time	Sealord Ltd.	21	3	24 & 48	Between production batches for each incubation time
	Cawthron Institute	19	3	48	Between production batches
Hatchery protocol	Sealord Ltd.	19	2	48	Between hatchery protocols
	Cawthron Institute	19	4	48	

Upon arrival of larvae to AUT, organisms were immediately transferred into a 2 L beaker and approximately 1 L FSW was added. The larvae were left to stand for 30–60 minutes to separate healthy swimming veligers at the correct development stage from bottom dwellers that were either dead or had already lost their velums. The water was carefully decanted into another beaker leaving the undesired larvae behind. With constant stirring, using a magnetic stirrer bar, the volume of FSW was increased until a constant 20–30 larvae/ml could be drawn from the solution. Using a dissecting

microscope at 4X magnification at least ten 1ml replicate samples were viewed and adjusted until a constant density was obtained. 1ml aliquots of this larval solution was pipetted into 60x14 mm sterile polystyrene Petri plates (Biolab Ltd.) containing 9 ml FSW, providing 20–30 larvae per plate. All assays were conducted at 17±1°C with ten replicates. Larvae were incubated for 24 and/or 48 hours depending on the experiment being conducted.

After incubation, settlement was determined by recording the percent of larvae firmly attached to the bottom of the Petri plates. Four methods of detecting settlement were trialled; decantation, agitation, discharge and suction. Decantating of the experimental medium, slow circular agitation, and water discharge by pipette proved ineffective. Since larvae were often weakly attached to the Petri plates, movement of the medium by decantation and agitation routinely resulted in mechanical detachment of settled larvae. In addition, the vigour of this movement was difficult to control thereby introducing an unnecessary factor. Close proximity water discharge by pipette to each individual was also ineffective since the consistency in the force applied was difficult to maintain and overshoot of the water jet often dislodged larvae in other areas of the Petri plate before detection of settlement was achieved. However, suction using a pipette was found very effective as a means of detecting settlement. This method has been successfully used by Ruppert and Rice (1983) for detecting attachment of marine worm free swimming larvae (Sipuncula: *Golfingia misakiana*).

Under a dissecting microscope at 20–45X magnification a 200 µl pipette was depressed and brought within close proximity (2–4 mm) to each larva and suction was applied. Individuals which moved freely with no resistance were considered unsettled and those which maintained a clear attachment to the substratum were considered settled. In many cases settlement could be detected visually by the presence of thin transparent mucous-like threads, but settlement was routinely verified with suction. Once counted, the settled and non-settled individuals were removed from the plates to avoid double counts.

2.3 Statistics

2.3.1 Larval Settlement

Percent settlement data were arcsin transformed. This is necessary since proportion distributions cannot be symmetrical due to the left-hand tail of the distribution being truncated by the theoretical minimum of zero. Furthermore, in proportion distributions both tails are truncated because all values must lie on a scale with absolute limits of 0 and 1. When observations are grouped at one end of the scale (i.e. proportions close to 0.1 or 0.9) errors which might arise are greatest, and least when in the middle (i.e. a proportion of 0.5). For statistical testing of percent data, the variance for the binomial distribution of the observed proportion is a function of that proportion. Since the variance of *p* depends on its particular value, this violates the homogeneity of variance assumption across subjects, which is required for the computation of parametric statistical tests.

$$VAR(p) = (p)\frac{(1-p)}{(n-1)}$$

The arcsine transformation is a mathematical function of *p* whose variance is essentially free of the value of *p* and moves very low or very high values towards the centre, giving them more theoretical freedom to vary. Sometimes called an *angular transformation*, the arcsine transformation equals the inverse sine of the square root of the proportion.

$$p_i' = arsin\sqrt{p_i}$$

To improve the arcsin transformation, when n < 50 and $p = 0$ the proportion was calculated as 1/(4n) and a proportion of 1 was calculated as 100-(1/4n) before the transformation was computed.

All data were analysed using the statistical computer software Minitab version 15.0. Data which satisfied homogeneity of variance (Bartlett's test) and normality (Anderson-Darling test) were analysed using parametric 1-way Analysis of Variance (ANOVA), with Tukey's post-hoc test to determine all pair-wise comparisons. For rare cases where only two independent samples were compared, a 2-sample t-test was employed. Data which failed to satisfy homogeneity of variance and/or normality were analysed using the non-parametric Kruskal-Wallis test. To assess multiple comparisons using non-parametric data analysis methods, a specialised macro, Minitab Dunns Macro test for testing the variance of medians (Orlic 2004), was sourced from the Minitab online library database. The level of significance chosen was 0.05 for all statistical tests. Reference to significance within the text is denoted as: p </> 0.05 and, for cases where a very high level of significance was found; p </> 0.01. Actual p-values for every test can be found in Appendix I, p277.

3. RESULTS

3.1 Age and incubation time effects

Eyed veliger larvae obtained from Sealord Ltd., which were almost ready to settle, were assayed to determine the post-fertilisation age and exposure time for all subsequent settlement experiments under treatment conditions. Larvae from a cohort population were sampled on days 18, 21 and 23 after fertilisation. Settlement was detected after 24 and 48 hour incubation periods. The percent of *P. canaliculus* larvae settled within each age class (18, 21, & 23 days old) is shown in Figure 10. After 24 hours incubation there was a significant difference in settlement among the three age classes (ANOVA; $F_{2,27}$=3.74; p<0.05). After 48 hours incubation percent settlement differences between age classes were not significant (ANOVA; $F_{2,27}$=1.25; p>0.05).

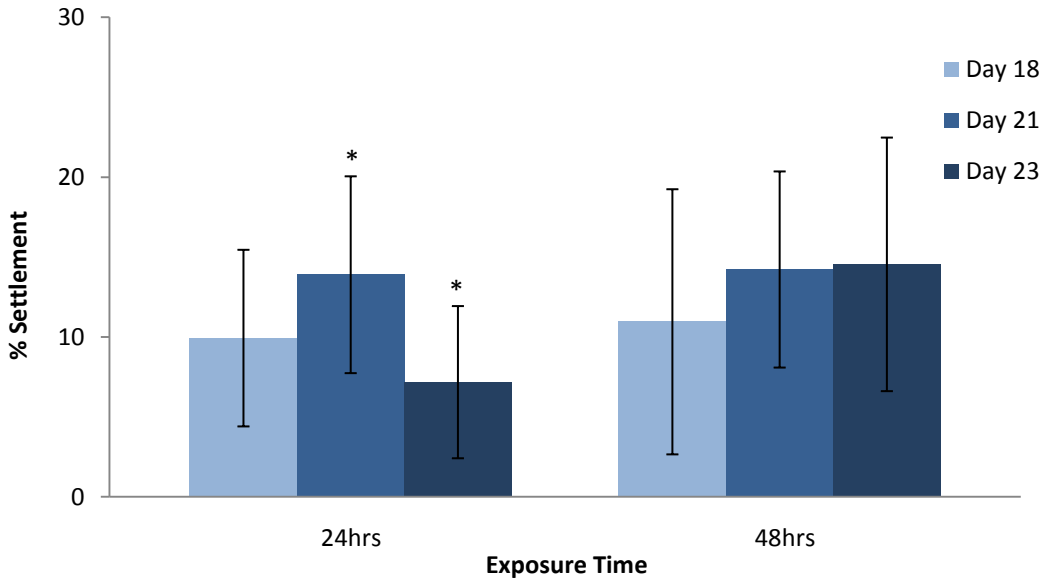

Figure 10. Effect of post-fertilisation age on percent larval settlement of *Perna canaliculus* after 24 and 48 hour incubations. The three age groups assayed came from a single hatchery bred batch population. Data plotted are mean ± standard deviation of 10 replicates. Asterisks above bars represent significant difference (Tukey test).

The effect of larval age from specimens obtained from the Cawthron Institute was not assessed because the post-fertilisation age of larvae provided by this hatchery facility only ever came from cohort populations which were 19 days old.

3.2 Batch and incubation time effects

To investigate the variation among production batches of hatchery-reared larvae from Sealord Ltd., and incubation time allowed, percent larval settlement of 21 day old post-fertilisation larvae were determined. Larvae were sampled from three batches over three consecutive months, and settlement was detected after 24 and 48 hour incubations for each batch. The percentage of *P. canaliculus* larvae that settled in each production batch is shown in Figure 11. After 24 hours incubation, there was a significant difference in settlement among the three batches (ANOVA; $F_{2,27}$=4.76; p<0.05). After 48 hours incubation, similar settlement were observed with no significant differences between production batches (ANOVA; $F_{2,27}$=1.06; p>0.05).

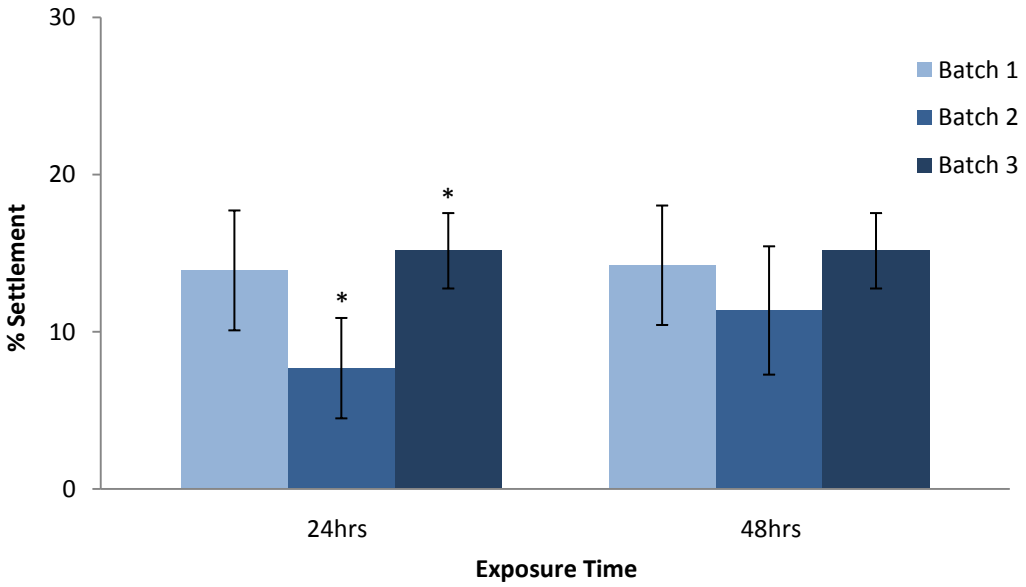

Figure 11. Effect of Sealord Ltd. production batch on percent larval settlement after 24 and 48 hour incubations. The three batches assayed were all conducted with 21-day old post-fertilisation larvae. Data plotted are mean ± standard deviation of ten replicates. Asterisks above bars represent significant difference (Tukey test).

The effect of production batch *vs*. larval settlement was also determined for larvae sourced from the Cawthron Institute (Figure 12). Because larvae from this hatchery facility were only ever provided at 19 days post-fertilisation, this was the age at which production batch effects were assayed. Also, since incubation times were previously shown to influence the consistency of larval settlement rates between batches (Figure 10 & Figure 11), assays were performed over 48 hour incubations only. Between the three batches sampled, no significant differences in percent larval settlement was detected (ANOVA; $F_{2,27}$=1.64; p>0.05). These results agree with those determined from the Sealord Ltd. data after a 48 hour incubation period.

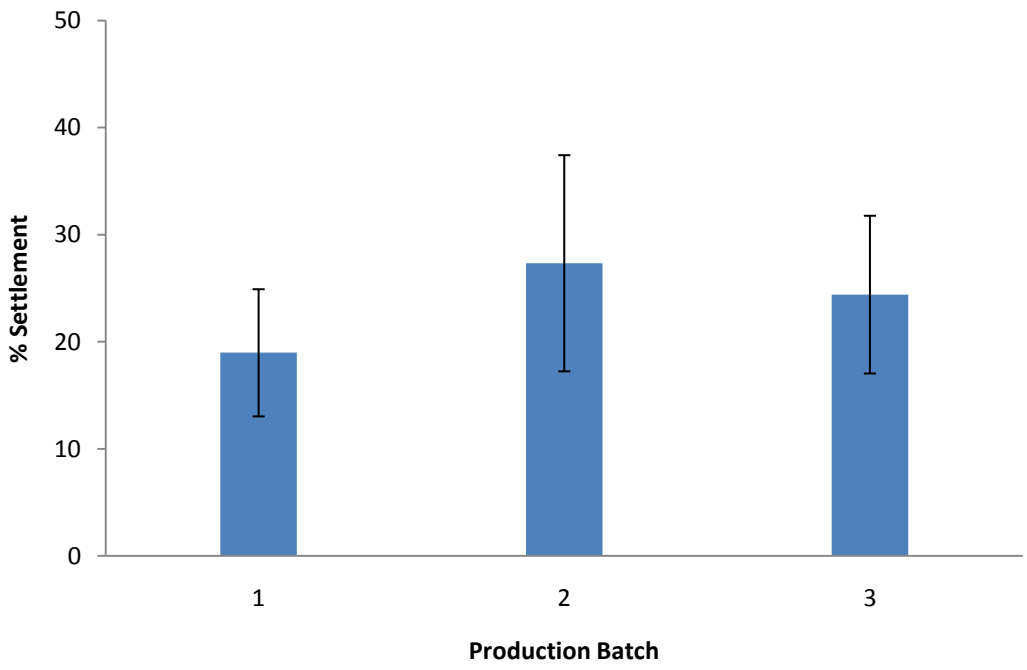

Figure 12. Effect of Cawthron Institute production batch on percent larval settlement after 48 hours incubation. The three batches assayed were all conducted with 19-day old post-fertilisation larvae. Data plotted are mean ± standard deviation of ten replicates.

3.3 Hatchery effects

The effect of hatchery production facility on percent larval settlement was determined to investigate whether contrasting production parameters may cause differences in settlement rates (Figure 13). Due to larval availability and time constraints, the number of batches sampled from each hatchery was different (Sealord Ltd., n = 2; Cawthron Institute, n = 4). All data came from populations 19 days post-fertilisation. Since batch effects at each facility were deemed negligible after 48 hour incubations (Figure 11 & Figure 12), the batch data from each hatchery were pooled and analysed. Larvae sourced from the Cawthron Institute had greater variation and generally higher settlement rates than those obtained from Sealord Ltd (2-sample t-test; $p<0.05$).

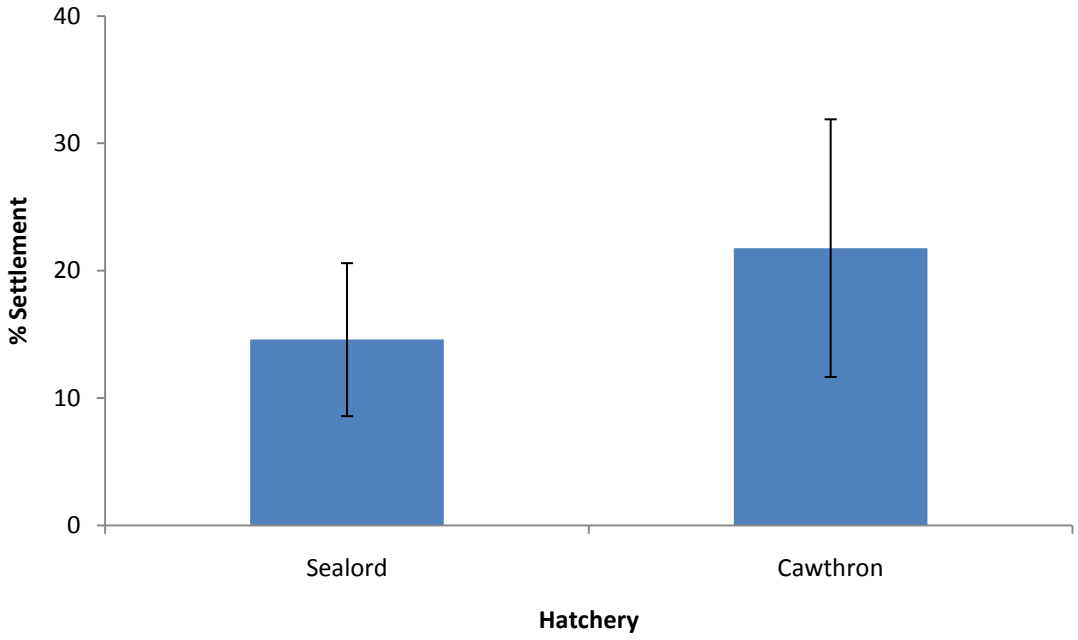

Figure 13. Effect of hatchery facility on percent larval settlement after 48 hours incubation. Individuals were sourced from 19-day old post-fertilisation larval batches. Data plotted are mean ± standard deviation of ten replicates.

4. DISCUSSION

4.1 Age and incubation time effects

To determine the post-fertilisation age range which could be used for subsequent settlement assays under treatment conditions, larvae between 18–23 days old were collected from a single batch cohort population reared at the Sealord Ltd. hatchery. This age range was selected after discussion and advice from the hatchery manager. Depending on the incubation period, the effect of post-fertilisation age on percent larval settlement revealed dissimilarities. It was expected that between an age range of six days, a gradual increase in settlement rates would occur regardless of incubation period. After 24 hours a significant difference was observed between 18, 21, and 23 day old larvae however a general increasing trend was not observed. Settlement rates after 24 hours incubation were also often below the decided 10% competency threshold. Over 48 hour incubations a very slight age dependant incline in mean values were detected with the differences between age classes becoming non-significant. Therefore, it was determined that larvae between 18–23 days old could be used successfully for subsequent settlement assays. In addition, to maintain consistency between experiments, an incubation period of 48 hours was selected, and is recommended for future studies investigating inductions larval settlement in *P. canaliculus*.

Although it was determined that larvae between the ages of 18–23 days old could be used for future settlement assays with limited variation, it was decided that only larvae between 19–22 days post-fertilisation would be used to ensure differences would not occur. Industrial batch processes are commonly associated with the potential ‘shifting-of-mean’ effect over time (i.e. increased variation caused by the manual correction of parameters when expected results are not achieved often leading to further deviations in expected results). Therefore, this narrower age range (i.e. 19–22 days old)

was selected to minimise any deviations in larval quality and competency which may occur due to the possibility of less rigorous quality control measures being employed at the hatchery.

Perhaps a reason why settlement rates were variable after 24 hour incubations was the adverse effects caused by stresses involved in the sampling, storage, transportation, and subsequent alterations in the environmental parameters used at the AUT aquaculture facility. Larvae were kept cool and moist during transport. However, they were not stored in seawater. The duration of transport was also slightly different between the three age classes sampled (8–12 hours). At the AUT laboratory larvae were placed in FSW (taken from the Sealord Ltd. hatchery). However, the temperature was maintained at 17±1°C, 3°C lower than what the larvae had been reared at. After being stored in the dark during transport, larvae were subjected to bright light while setting up experiments, then placed in diffuse light for the duration of the settlement assays. Also, to initially provide a larval solution calibrated at 20–30 larvae/ml, larvae were placed in a 2 L beaker with FSW and continuously mixed with a magnetic stirrer bar. This process created high water velocities and turbulence which may have caused undue stress for the organisms. It is possible that a combination of these conditions were responsible for the variations observed in settlement rates after 24 hours incubation. The decrease in variation after 48 hours incubation suggests that larvae had equilibrated to their environment and any stress induced by transportation, and the processes involved when initiating the settlement assays, had dissipated.

Although larvae were only sampled between 18–23 days post-fertilisation to determine an acceptable age range for subsequent settlement assays, perhaps younger or older larvae could have been suitable. It may be expected that rates of larval settlement increase in an exponential fashion from a particular developmental stage, approaching 100% settlement shortly thereafter. No literature for *P. canaliculus* has been generated on this topic, and little information for other mussel species could be sourced, especially detailing age effects under non-treatment conditions. Based on anecdotal evidence

supplied by the hatchery managers at both facilities, it was suggested that hatchery-reared larvae first become competent to settle from around 16–18 days old. Larvae may be placed into settling tanks around day 21–24 depending on the development of that particular batch. The settling period allowed can be around 10 days, again depending on batch. After this process, most larvae have settled and are approximately 30–35 days old. Jeffs et al. (1999) estimated that the larval phase of *P. canaliculus* in the natural environment is between 4–6 weeks. Taking into consideration these suggestions for the hatchery reared larvae, and incorporating the averages of the repeated measures data collected for the three age classes, a hypothetical age dependant settlement curve can be postulated (Figure 14). Due to possible initial effects of stress caused by transport and temperature equilibration, only data generated from the 48 hour incubations were incorporated with final larval age being plotted on the x-axis at the time of settlement detection.

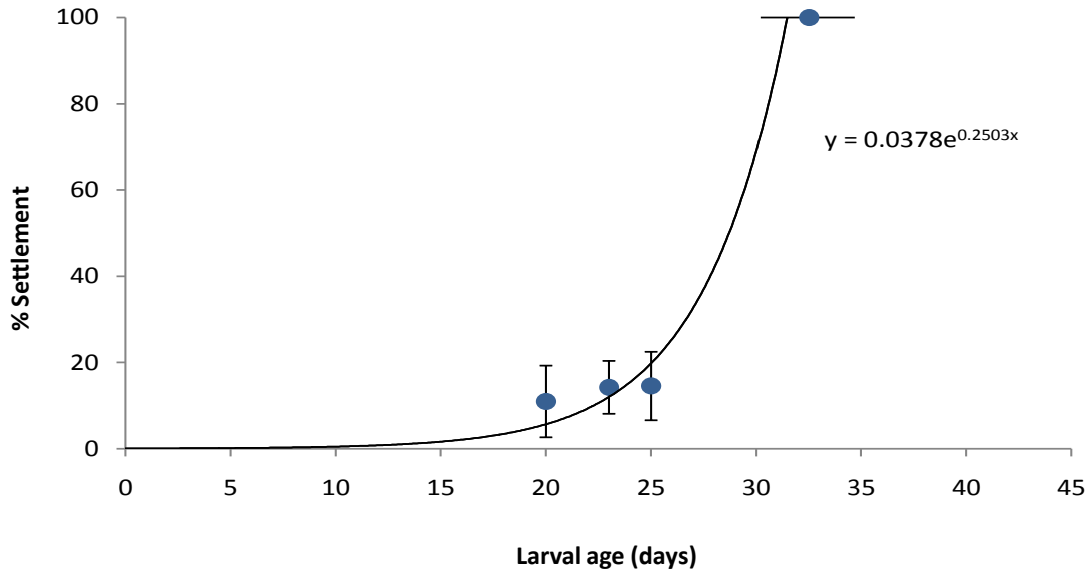

Figure 14. Hypothetical age dependant settlement curve based on experimental data and anecdotal evidence. Mean settlement is plotted with error bars representing standard deviation. Age at which larvae reach 100% settlement has a horizontal error bar and represents the age range suggested. The fitted line and related equation is derived from a best-fit exponential curve.

It is true that such a regression model should be performed as a logistic function due to the limiting 100% settlement factor. However, such a model could not be computed with missing data. An exponential curve fitted the data well and is used for subsequent interpretation. From the exponential settlement curve, some predictions may be hypothesised. It must clearly be stated here that since anecdotal evidence was incorporated, there is no statistical foundations for making such predictions.

Larvae over 23 days old are likely to be unsuitable for settlememt assays. Individuals older than this begin to reach the upper 30% settlement control limit. Also, from the variance in the data generated for each age class tested, larvae older than 23 days old may show significant mean differences in settlement rates compared to younger 18 day old larvae. It is possible that selection of younger larvae, 16–17 days post-fertilisation, may have the potential to be effectively used without variation problems. However, larvae at this age may result in settlement rates below the lower 10% control limit decided upon to ensure larval settlement competency. These predictions formulated from the hypothetical settlement curve in

Figure 14 suggest that the post-fertilisation age range selected for subsequent settlement assays in this thesis may contain the lower and upper ages suitable for *P. canaliculus* larvae. However, to corroborate this prediction and provide evidence for the age range which is acceptable, it would be imperative to perform a detailed study of percentage settlement *vs*. age of hatchery-reared larvae.

4.2 Batch and exposure time effects

The effect of production batch on percent larval settlement was determined to investigate whether different batches of larvae have the potential to influence results in future settlement assays. After analysing settlement data from each hatchery facility, it

was revealed that differences may occur depending on incubation time allowed. After 24 hour incubations there were significant differences. However, these inconsistencies are just as likely to have occurred due to the possibility of the early induced stresses outlined in the above discussion. After 48 hours incubation, production batch effects are minimal with no detectable differences occurring among Sealord Ltd. batches or among batches produced at the Cawthron Institute. Considering this, these results support the notion that both hatcheries have excellent production processes in place for larval rearing, and that the regulation of production parameters are strictly controlled. The effect of incubation period in this investigation provides further evidence that 24 hours may not be long enough to ensure settlement variation is minimised. It is therefore suggested that larvae can be used from any production batch in future settlement assays providing 48 hour incubations are maintained, and any data generated from shorter incubation periods should be regarded with caution.

4.3 Hatchery effects

Due to the different culture techniques and conditions employed at Sealord Ltd. and the Cawthron Institute, the effect of hatchery facility on percent larval settlement was investigated. Even after 48 hour incubations, a significant difference was detected between the two facilities. Some distinct morphological differences also were observed between larvae obtained from each hatchery. Larvae from the Cawthron Institute were generally much smaller at 19 days post-fertilisation than those obtained from Sealord Ltd. A number of possible explanations for this observation may be drawn including effects of differing production parameters. Larvae from the Cawthron Institute are reared in water that is 18ºC, lower than the 20ºC employed at Sealord Ltd. This cooler temperature may cause slower growth rates. It has been shown that for the larvae of the marine mussel *Mytilus galloprovincialis*, growth rates are significantly greater if reared at 20 ºC rather than at 16 ºC (Ruiz et al. 2008). This temperature effect also is seen in the larvae of the tiger prawn *Penaeus semisulcatus*, with slightly lower temperatures causing a significant reduction in growth rate (Kumlu et al. 2000). The diets provided

by each facility also are different. At Sealord Ltd. larvae are fed with three microalgal species; *Chaetoceros calcitrans*, *Isochrysis galbana*, and *Pavlova lutheri*, whereas at the Cawthron Institute only the first two species are used. These nutritional differences also are a likely cause for some of the disparities in growth rates observed.

The broodstock individuals used may also lead to differences in growth and development rates. Sealord Ltd. uses approximately 200 individuals to provide the large quantities of eggs required, but also to maintain genetic heterogeneity. Conversely, the Cawthron Institute only uses around eight individuals. Depending on the source of these broodstock, some inherent genetic variations may be present. Sealord Ltd. uses broodstock individuals from Kaitaia (upper north, New Zealand) and also from the Marlborough Sounds (South Island, New Zealand) to ensure a successful larval rearing cycle is maintained throughout the year. The Cawthron Institute often relies on wild broodstock obtained from Golden Bay in the Marlborough Sounds, but sometimes uses cultured mussels produced by their selective breeding program. It has been suggested that mussel populations throughout New Zealand contain significant genetic differences indicating minimal gene flow between geographically isolated populations (Gardner et al. 1996). These differences are theorised to result from localised hydrographic conditions affecting larval dispersal. Conversely, a later study by Apte and Gardner (2001) suggests genetic subdivision in *P. canaliculus* is lacking and the overall species population maintains genetic homogeneity accompanied by high genetic variations within localised populations. Although these two studies are contradictory, there still remains the possibility that distinct genetic divisions are present – especially in adult mussels obtained from a selective breeding program. Therefore, the sources of broodstock used by each hatchery facility may play a role in the growth and settlement rate disparities observed. In summary, although a significant difference exists between each hatchery, rates of larval settlement are within the control limits set (10–30%) and therefore larvae sourced from either facility can be used for future assays. However, perhaps comparisons made between assays involving a different source of larvae should be cautioned against.

4.4 Additional observations

During this investigation into the effects of age, batch, hatchery facility, and incubation period on larval settlement, some additional observations were made. It was noted that larvae have the ability to settle very quickly – within 5 minutes in some cases. It also was observed that larvae may attach and translocate numerous times before finally forming a permanent attachment. Even when larvae were firmly attached an ability to release their hold on the substrate was obvious. The method larvae seemed to employ to detach from the substrate appeared to involve mechanical torsion stress of the small mucous filaments - larvae tended to swim in circles numerous times until the attachment was broken. This is interesting since it infers that the detachment process is much simpler than using biochemical enzymatic techniques to break the mucoid adhesive. Also, over the 48 hour incubations, most attached larvae retained their vela. In many cases they remained extended and although sometimes withdrawn it appeared that the potential for swimming was maintained. Could this be an evolutionary advantage to act as a safety mechanism in case the initial substrate chosen is poorly selected and translocation from adverse conditions is required?

During the settlement assays there were three places in which larvae can settle; on the bottom horizontal surface of the Petri plates, on the vertical sides, or in the corners of the Petri plates. The selection of settlement location was variable. It was noted that approximately 25% of the time larvae settled in the corners of the Petri plates, but those individuals almost always orientated into a vertical position with the attached filaments on the sides of the Petri plates. Approximately 15% of larvae also settled higher up directly onto the vertical portion. When the overall vertically attached proportion of larvae is taken into consideration along with the surface areas of the two substrate planes available, a substantial difference in settlement rate between the planes are presented. It was determined that the approximate percentages of larvae attached per cm^2 to the bottom and sides of the Petri plates were 2.1 and 4.2% respectively.

Furthermore, bearing in mind larvae would have to swim much further to reach the vertical sides than the horizontal bottom surface of the Petri plates, this apparent preference for a vertical substrate becomes more apparent. It is well known that the physical structure of substrates affect settlement rates of many marine invertebrate larvae (e.g. Alfaro & Jeffs 2002; Railkin 2004; Yang et al. 2007), however little research has been conducted into the selection of substrate plane.

Larvae also have some sort of attraction to one another. On numerous occasions it was observed that larvae attach in pairs or even in larger groups of 5–8 individuals. Sometimes these larvae were firmly attached to each other but not attached to the substrate. However, within these clusters, it was generally more common for at least one larva to be firmly attached to the substrate. Although any of these circumstances arose well under 5% of the time, it does lead to some interesting questions about larval communities, communication, and evolutionary implications. The details of these questions and possible answers are not discussed since they are outside the scope of this thesis. However, these observations may lead to areas of future research.

It was noted that during homogenous mixing of larvae in preparation for setting up experiments, larvae were induced to secrete large quantities of mucoid material under high water flows. It has been shown that barnacle cyprids are induced to settle by turbulation and water flow (Crisp 1955, 1974; Jonsson et al. 2004). For these organisms low local flow rates at the boundary layer of a substrate induce settlement whereas high flow rates are inhibitive (Crisp 1955). Larvae of the marine mussel *Mytilus edulis* also are induced to settle by increased water velocity and turbulence to a certain degree (Pernet et al. 2003). In addition, Alfaro (2005) suggested larvae of *P. canaliculus* are induced to settle by moderate water flow. The observations made in this thesis may provide evidence to support Alfaro's suggestion. Since *P. canaliculus* larvae produce mucous threads in the laboratory under high water flow conditions, what is the purpose? Is it due to the organisms sensing a turbulent dangerous environment, therefore producing mucous filaments in order to attain an attachment onto a firm substrate – in

order to maximise survival of the individual? This reasoning supports the view that settlement is induced by water flow. However, what if the larvae are sensing high flow rates and are producing mucous threads in order to 'sail' through the water column thereby maximising the opportunity to increase rates of larval dispersal? The purpose of this may be to maximise population connectivity and genetic heterogeneity within localised populations, hence ensuring survival of the whole species. This is an interesting question and one which should be thoroughly investigated. Unfortunately, settlement studies investigating flow dynamics are always performed in small enclosed areas relative to the natural environment – for larvae to be quantified, they must be retained. An explanation for the detected settlement observed in the literature may simply be due to the larvae having vastly greater chances of making contact with a substrate than they would in the ocean. It would be interesting to determine if larvae produce mucous threads relative to increasing water velocities when they are pre-competent to settle. If this was the case, perhaps larval dispersal is the primary biological objective, and the increased settlement rates observed in the laboratory only result due to the maximised contact of larvae, possessing 'sticky' mucoid exudates, with a substrate (container walls/sides). It also is possible that the perception of water flow by the larvae and subsequent induced behavioural responses change over time during larval development - thereby giving the ability to respond to various water velocities a double purpose. The implications of these questions are likely controversial. However, investigating some of the points outlined may provide further evidence to support common theories, or they may lead to new discoveries and increase knowledge within the field of marine invertebrate larval settlement.

CHAPTER 4

EFFECTS OF GABA & L-TRYPTOPHAN ON LARVAL SETTLEMENT

1. INTRODUCTION

This chapter details the investigation of the effects of the amino acids γ-aminobutyric acid (GABA), and L-tryptophan on larval settlement in *P. canaliculus*.

The amino acid and neurotransmitter γ-aminobutyric acid (GABA) is produced by the decarboxylation of glutamic acid. In vertebrates, GABA acts most commonly in the nervous system as an inhibitory neurotransmitter (Kuffler et al. 1984). In this case, GABA causes hyperpolarisation of post-synaptic membranes through the increase in permeability to negatively charged chloride ions. This induces an influx of chloride ions into the neuronal cells, resulting in a greater charge difference across the membrane. When this occurs, depolarisation is difficult or impossible to achieve. Therefore, nerve impulses are inhibited and the 'messages' normally sent for various biological functions are not transmitted. However, in some cases GABA has the ability to activate a depolarising 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. 1979a). 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). Other species of *Haliotis* are also induced to settle by GABA e.g. *H. iris* (Moss & Tong 1992; Roberts & Nicholson 1997), *H. discus hannai* (Akashige et al. 1981; Yang & Wu 1995), *H. virginea* (Roberts & Nicholson 1997), *H. australis* (Moss 1999), *H. asinina* (Gapasin & Polohan 2004; Laimek et al. 2008), and *H. diversicolor* (Bryan & Qian 1998). The effective doses for these species vary between 0.5–10 μM implying species specificity. GABA also has been found to induce various other molluscan species including the clams *Venerupis pullastra* and *Ruditapes philipinarum* (Garcia-Lavandeira et al. 2005), the

oysters *Pinctada margaritifera* (Doroudi & Southgate 2002) and *Ostrea edulis* (Garcia-Lavandeira et al. 2005), the black chiton *Katharina tunicate* (Rumrill & Cameron 1983), and the sea urchins *Strongylocentrotus droebachiensis* (Pearce & Scheibling 1990) and *Echinometra* spp. (Rahman & Uehara 2001). GABA also induces settlement in larvae of the marine mussel *Mytilus galloprovincialis* (Garcia-Lavandeira et al. 2005). Conversely, no significant effect has been observed in the cnidarian *Alcyonium siderium* (Sebens 1983), the polychaete *Phragmatopoma lapidosa californica* (Pawlik 1990), the arthropod *Balanus amphitrite* (Mishra & Kitamura 2000), the echinoid *Dendraster excentricus* (Burke 1983), or the molluscs *Mytilus edulis* (Dobretsov & Qian 2003), *Crepidula fornicata* (Pechenik & Heyman 1987), *Phestilla sibogae* (Hadfield 1984) and *Crassostrea gigas* (Coon et al. 1985).

Exogenously applied GABA is effective at inducing settlement in *Haliotis* spp. It has been suggested that the activity of GABA is due to its close structural relationship with naturally occurring inducer molecules (Morse & Morse 1984a,b). The GABA isoforms γ-hydroxybutyric acid and δ-aminovaleric acid have been detected in crustose coralline algae which *Haliotis* spp. are associated with. These compounds have a demonstrated ability to induce larval settlement (Morse et al. 1979b, 1980, 1984; Morse & Morse 1984a,b; Morse 1991). The analogous molecules, muscimol, gabuline and baclofen, are also inducers of settlement and metamorphosis in *Haliotis* spp. (Trapido-Rosenthal & Morse 1986a,b). The apparent activity of related GABA molecules reflects the stereospecificity of larval receptors (Trapido-Rosenthal & Morse 1986a,b). It has been suggested that induction of settlement or metamorphosis 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 depolarisation (Baloun & Morse 1984). Although these concepts relate only to *Haliotis* spp., perhaps they also are appropriate for other marine invertebrate species that are induced to settle or undergo metamorphosis in the presence of exogenously applied GABA.

As previously mentioned, GABA was demonstrated to be an inducer of settlement in the marine mussel *M. galloprovincialis*. Since GABA or GABA mimetic compounds have been detected in crustose coralline algae which *P. canaliculus* larvae and spat are known to be associated with (Alfaro et al. 2004), it was hypothesised that GABA also would be partly responsible for induction of larval settlement in this mussel species. Therefore, GABA as a settlement inducer molecule was investigated in this thesis.

Another neurotransmitter, serotonin, is a known inducer of larval settlement in barnacles, and acts as a metamorphosis inducer in numerous marine invertebrate species. Serotonin is synthesised in the nervous system in serotonergenic neurons by the two-step conversion of the essential amino acid L-Tryptophan (Figure 15). L-Tryptophan is first oxidised to 5-hydroxytryptophan by the enzyme tryptophan 5-hydroxylase, and then decarboxylated to serotonin by the enzyme aromatic L-amino acid decarboxylase (Suni-Ichinose et al. 1992; Wang et al. 2002).

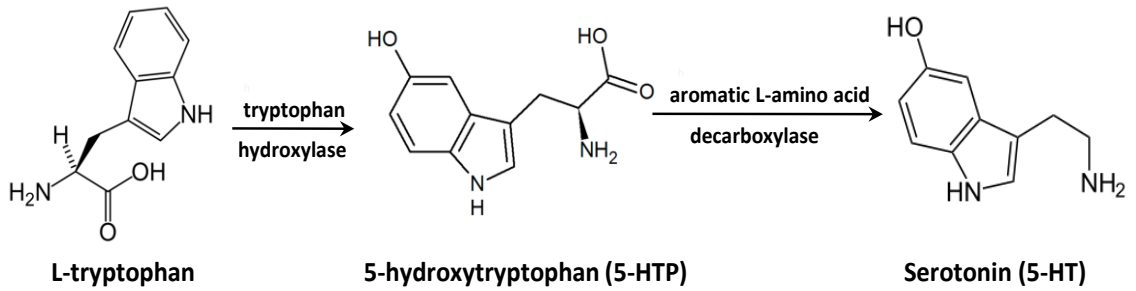

Figure 15. Serotonin biosynthesis pathway.

In the barnacle, *B. amphitrite*, it has been determined that treatment with the serotonin agonists, N,N,N,-trimethylserotonin, α–methylserotonin, and serotonin creatinine sulphate, promote larval attachment (Yanamoto et al. 2000). Evidence also shows that the serotonin antagonists, LY-53,857 maleate (6-methyl-1-(1-methylethyl)-ergoline-8b-carboxylic acid 2-hydroxy-1-methylpropyl ester maleate), LY-278,584 maleate (1-methyl-N-(8-azabicyclo-oct-3-yl)-1H-indazole-3-carboxamide maleate), cinanserin, cyproheptadine, ketanserin, and propranolol, significantly inhibit the attachment process (Yanamoto et al. 2000). Furthermore, in a separate study, a decrease in serotonin stimulated by the serotonin re-uptake blocker, amitriptyline, inhibited larval settlement of *B. amphitrite* (Yanamoto et al. 1996). Endogenous levels of serotonin also were detected in the cyprids of these organisms, and it is suggested that serotonin is a key regulator in the biochemical mechanisms of larval settlement for this organism (Yanamoto et al. 2000). When treated with p-chlorophenylalanine, an irreversible inhibitor of the enzyme responsible for the bioconversion of L-Tryptophan to serotonin (tryptophan 5-hydroxylase), a significant reduction in serotonin levels was observed in larvae of the echinoderm *Hemicentrotus pulcherrimus*. It also was demonstrated that the serotonin biosynthesis pathway is vital for the normal swimming behaviour of veliger larvae of *H. pulcherrimus* (Yaguchi & Katow 2003).

The presence of serotonin-containing cells has been detected very early in the larval development process, which reflects its importance as a neurotransmitter and regulator of metabolic processes in some marine invertebrate species. For example, in *H. pulcherrimus*, expression of the tryptophan 5-hydroxylase gene during neurogenesis is initiated with transcription of the enzyme occurring in the late gastrula stage in cells of the apical ganglion (Yaguchi & Katow 2003). Furthermore, neurogenesis in the marine mussel *Mytilus trossulus* also begins early, during the trocophore stage with the appearance of a serotonin-like immunoreactive (*lir*) sensory cell of the apical extreme. Shortly after, five serotonin-*lir* sensory cells can be distinguished forming an apical neuropil. These cells remain within the neuropil and do not project posteriorly towards the visceral ganglia until the late veliger stage (Voronezhskaya et al. 2008). In the case of *M. trossulus*, perhaps these serotonin-containing cells are important in biochemical

pathways involved in larval settlement and/or metamorphosis since they do not proliferate until veligers become competent to settle.

Induction of larval metamorphosis by treatment with exogenous serotonin has been identified for the clam *Ruditapes philippinarum* (Fusetani 2004), the marine snail *Ilyanassa obsolete* (Leise et al. 2001), and the hydroids *Eudendrium racemosum* (Zega et al. 2007), *Hydractinia echinata* (Walther et al. 1996), and *Phialidium gregarium* (McCauley 1997). Conversely, little metamorphic response is observed in the mussel *M. galloprovincialis* (Satuito et al. 1999), the oyster *C. gigas* (Beiras & Widdows 1995), and the scallop *Patinopecten yessoensis* (Kingzett et al. 1990). Induction of larval settlement by exogenous application of serotonin has been successfully shown for *B. amphitrite* (Yanamoto et al. 1996). In the sea star, *Patiriella regularis*, it has been demonstrated that as larvae develop, serotonin is redistributed within the nervous system, suggesting that activity is being redirected towards settlement (Chee & Byrne 1999). Considering the literature on larval responses to serotonin, the neurobiology of early development in marine invertebrate organisms, and the biosynthesis mechanism of serotonin production, it is evident that the serotonin precursor, L-Tryptophan, must be involved. Endogenous levels of L-Tryptophan likely regulate the serotonergic inductions of metamorphosis and settlement. It has been shown that exogenous treatment with L-Tryptophan has the ability to induce larval settlement in *B. amphitrite* (Delort et al. 2000; Mishra & Kitamura 2000), probably by conversion to serotonin. However, most studies focus on the metamorphosis induction effects of serotonin or related molecules with few investigating larval settlement. In this study, the role of L-Tryptophan as a possible larval settlement inducer was investigated for *P. canaliculus*.

2. METHODS

2.1 Organisms

Larvae for these assays were sourced from the Sealord Ltd. hatchery facility in Ruakaka (northern New Zealand) and from the Cawthron Institute (South Island, New Zealand). Organisms were reared according to the production parameters outlined in the methods section in Chapter 3, p60. Larvae were sampled between 18–22 days post-fertilisation and transported cool and moist to the AUT aquaculture laboratory. For details of larval source and age for each settlement assay performed, see Appendix II, p285.

2.2 Settlement Assays

Settlement assays were set-up and conducted using the same methods and environmental parameters outlined in Chapter 3, p62. Briefly, assays were performed at 17±1°C in sterile polystyrene Petri plates with 20-30 larvae per plate (from a larval solution pre-calibrated to 20–30 larvae/ml). Because some of the treatment compounds used in later chapters readily undergo oxidation, all assays were conducted under diffuse light conditions to limit chemical degradation of those compounds, and also to maintain consistency between assays. Ten replicates were performed for every control and level of treatment. Percent settlement was detected after 48 hour exposures to treatments using the suction-by-pipette technique. See Figure 16 for photo of settlement assay.

Figure 16. Settlement and mortality assays.

Individuals that died as a result of exposure to treatment compounds but showed clear evidence of mucoid thread attachment to the substrate, were considered settled. In such cases, it could be argued that settlement behaviour may have been a response to toxicity effects, and therefore counting dead individuals as settled would over estimate the inductive ability of those treatments. However, for such an argument to have foundation, a positive correlation between percent settlement and mortality would have to result in every case where treatments were found to be highly toxic. There was no consistent evidence to support such a relationship between settlements *vs*. toxicity. In some experiments, high percent settlement was detected after exposure to toxic treatments when mortality rates also were high (i.e. many dead individuals were firmly attached to the substrates). However, in some cases where high mortality was the result of exposure to toxic treatments, a decrease in settlement *vs*. treatment concentration was detected (i.e. dead individuals were not firmly attached to the substrate). This would infer that larval settlement is not a response to toxic effects of

treatments. Furthermore, from an evolutionary perspective, it seems unlikely that settlement would be a response to a toxic environment. It is logical that settled individuals must have been induced to settle before they died, and since a general trend in settlement *vs*. toxicity was not observed, attached dead individuals were considered settled. However, distinguishing features of dead larvae were taken into consideration to determine if settlement had actually occurred. Dead individuals which showed signs of exogenous tissue release were not considered settled due to the possibility of misinterpreting the mode of attachment. The exogenous release of tissues outside of the shell margin often provided a sticky substance which was observed to fix onto the bottom of the Petri plates. If not taken into account, this bonding of dead larvae to the substrate could lead to over estimation of larval settlement. Therefore, only those dead individuals which maintained their tissues within the margins of the shell, and had clear evidence of mucoid filamentous thread attachment, were considered settled.
It is possible that using this criterion, larval settlement was under estimated in this thesis since larvae may have attached prior to exogenous tissue release. The presence of exogenous tissue release is a feature which may possibly have the potential to obscure the results of settlement assays in the literature depending on the methods employed – especially when a microscope is not used and careful examination of every individual is not performed.

2.2.1 Treatments

The amino acids γ-amino butyric acid and L-Tryptophan were dissolved in 0.45 μm filtered seawater (FSW). Stock solutions of each treatment were prepared immediately prior to all settlement assays. Following serial dilution in FSW, treatment solutions were prepared as 10X concentrates. Controls consisted of 9ml FSW and 1 ml larval solution. Treatments consisted of 8 ml FSW, 1ml larval solution, and 1 ml treatment solution. Final exposure treatment concentrations for both GABA and L-Tryptophan were $1x10^{-5}$, $1x10^{-4}$, and $1x10^{-3}$ M.

2.3 Mortality assays

Mortality assays often were performed concurrently with settlement induction assays on the same larvae after percent settlement was determined. However, when time was a limiting factor, mortality assays were performed on different individuals, and in some cases, from different larval production batches. Experimental parameters used for mortality assays were the same as those employed for larval settlement.

Mortality was more difficult to quantify than settlement. Under a stereo microscope at 20–45X magnification, larvae which showed signs of movement of the velum, foot, or gut were considered alive. However, in many cases the larvae were observed to lie motionless with no movement for periods of more than 15 minutes while still alive. Therefore, relying on movement alone was not considered a viable method for detecting mortality of large numbers of larvae under experimental time constraints. In conjunction, a vital staining technique was used. The vital stain Neutral Red is a commonly used method of determining mortality in various small organisms, including larvae of the fresh water mussel, *Vilosa iris*, and *Anodonta grandis* (Jacobson et al. 1993) and the marine mussel *M. edulis* (Platter-Rieger and Frank 1987).

A solution of 120 ppm was prepared in FSW. After 48 hours incubation, 2ml of the stain was added to each Petri plate containing 10 ml of the experimental medium giving a final stain concentration of 20 ppm. Larvae were left for 30 minutes to absorb the stain before being viewed at 20X magnification under a stereo microscope. All dead individuals did not take up the stain. However, a small proportion of live larvae (detected by movement) also did not take up the stain. For determining mortality rates, the detection of movement technique and the vital stain methods have similar limitations since both may result in conservative mortality estimates. By using these techniques in conjunction, experimental design error was minimised and estimates of mortality were much closer to the real values.

To determine the toxicity of treatment compounds used for settlement induction experiments, lethal concentration values (LC_x) were calculated. Lethal concentration values are estimates, based on mortality data generated in real toxicity studies, to provide an indication of what concentration will kill a particular proportion of a population. For example, the LC_{50} value is the concentration of a compound/toxin in water that will kill 50% of the population of a particular aquatic species. Perhaps more useful is the LC_1 value (i.e. concentration that kills 1% of the population) since it gives an indication of the first observable acute toxic effect of a certain compound. These values may be useful in deciding if any of the compounds tested in this thesis have the potential to be used in commercial aquacultural activities. By increasing percent larval settlement in hatcheries or shortening the time it takes for settlement to occur, productivity, and hence economical viability of producing and rearing larvae, may be substantially improved. The LC values are significant since the concentrations of inducing compounds administered, would ideally be lower than the concentrations at which toxic effects are observed.

LC values calculated from toxicity studies are dependent on the conditions and experimental parameters employed. Common exposure times used for toxicity studies are: 12, 24, 48, 72, and 96 hours. Some other parameters which affect the outcome of these studies include: temperature, oxygenation, presence or absence of food, light conditions, and flow rate (water circulating tanks or static designs). There is no universal standard for toxicity studies, but all of these experimental conditions should be documented.

2.3.1 Treatments

Solutions of GABA and L-tryptophan were prepared as previously described for the settlement assays. The final treatment concentrations used for mortality assays were: GABA; $1x10^{-5}$, 1^{-4}, $1x10^{-3}$, $1x10^{-2}$, and $1x10^{-1}$ M, and L-tryptophan; $1x10^{-5}$, $1x10^{-4}$, and $1x10^{-3}$ M.

2.4 Statistics

2.4.1 Larval Settlement & mortality

Statistical methods used for settlement induction and mortality assays are the same as those outlined in the methods section in Chapter 3, p64. Briefly, all percent data were arcsin transformed and analysed using statistical software, Minitab v.15. Where data satisfied the assumptions of parametric analysis, 1-way ANOVA with Tukey's multiple comparisons test was used. Although the primary comparison of interest is between treatment level and control, the Tukey's test was performed to distinguish concentration effects. The Tukey's statistic also gives a more robust analysis as it is more conservative than the Dunett's test. However, Dunett's statistics can be found along with actual *p*-values for all comparisons in Appendix I, p277. Where data were not normally distributed, and/or homogeneity of variance failed between independent samples, the non-parametric Kruskal-Wallis test was employed with a specialised Dunn's macro enabled for multiple comparisons of medians.

2.4.2 Larval Toxicology

The following statistical methods were used to analyse mortality data and predict toxicological parameters for treatment compounds. These are the general methods employed for all future toxicology studies in later chapters. When calculating lethal concentration values, some conditions should be met to decrease sources of error in the estimated concentrations. For example, control assays should have low levels of mortality; there should be at least one level of treatment (preferably more) that produces mortality rates that are greater than in the control, and less than 100%; at least one level of treatment should result in 100% mortality. These conditions guarantee that LC_x estimations are not calculated from extrapolation of the data. They also ensure that the estimated values relate more closely to the actual toxicities of treatment concentrations that were not tested and that the slope of the mortality curve is more correct. Therefore, it was decided that lethal concentration values would only be calculated when: mortality in controls was ≤10%, at least one level of treatment resulted in ≤80% mortality but still greater than in control assays, and exposure to at least one concentration level resulted in 100% mortality. Since mortality was frequently observed in control treatments, and the regression model dose response curves varied among treatments (linear *vs*. cumulative), specialised statistical software and mathematical functions were implemented. The models utilised in the software incorporated their own data transformation techniques so the raw percent mortality data were analysed without arcsin transformation. Depending on the heterogeneity of the data (Chi-square) one of two statistical methods was used to analyse the data (probit analysis or Spearman-Karber analysis).

In cases where the variance was heterogeneous (the regression slope was different from 0, or linear), and the observed response proportions were monotonically non-decreasing, a probit analysis was performed. The probit method is based on regression of the probit of mortality on the log of concentration. Statistical software developed by the US Environmental Protection Agency was used to analyse these data (EPA Probit Analysis Program Used for Calculating LC/EC Values Version 1.5). This program was designed

for the analysis of mortality data from acute and short-term chronic toxicity tests with fish and other aquatic organisms to be performed with effluents, receiving waters, and reference toxicants by regulatory agencies under the US National Pollutant Discharge Elimination System, and was ideal for most of the data obtained in this study.

Where heterogeneity of variance was not successful and/or the regression slope was not different to 0, indicating that the data did not fit the probit model, the Spearman-Karber method was used and performed manually. The Spearman-Karber method is a non-parametric statistical procedure for estimating the LC_{50} and the associated 95% confidence interval (Finney, 1978). The Spearman-Karber method estimates the mean of the distribution of the $\log_{10}$ of the tolerance. If the log tolerance distribution is symmetric, this estimate of the mean is equivalent to an estimate of the median of the log tolerance distribution. In cases where the response proportions did not monotonically increase with increasing concentration, the data were smoothed using Abbott's procedure to adjust the concentration response proportions for mortality occurring in the control replicates. For the data to fit this model, the smoothed adjusted proportion mortality for the lowest concentration not including the control must be zero, and the smoothed adjusted proportion mortality for the highest concentration must be one.

The first step in the analysis requires that all concentrations are expressed as a percentage of the maximum concentration which resulted in 100% mortality. All concentrations were transformed into this format. The second step requires all response proportions are monotonically increasing i.e. $p_0 \leq p_{k1} \leq ... \leq p_{kx}$ where p_0, and $p_{k1}.....p_{kx}$ denote the observed response proportion mortalities for the control and k treatment concentrations, respectively. Where this was not true, the line was smoothed, obtaining p^s values, by replacing any adjacent pk's with their average. For example, if $p_k < p_{k-1}$ then:

$$p_{k-1}^{s} = p_{k}^{s} = \frac{(p_k + p_{k-1})}{2}$$

For cases where more than two consecutive concentrations were not monotonically increasing, averages of all those concentrations were determined.

To adjust for control mortalities, each smoothed observed proportion was adjusted using Abbott's formula (Finney 1971):

$$p_{kx}^{a} = \frac{(p_{kx}^{s} - p_{0}^{s})}{(1 - p_{0}^{s})}$$

Where: p_0^s = the smoothed observed proportion mortality for the control
p_{kx}^s = the smoothed observed proportion mortality for treatment concentration k

The following formula was employed to calculate the $\log_{10}$ of the estimated LC_{50}, m:

$$m = \sum_{k=1}^{t} -1\,(p_{k+1}^{a})\frac{(X_k + X_{k+1})}{2}$$

Where: p_k^a = the smoothed adjusted proportion mortality at concentration k
X_k = the log10 of concentration k
t = the number of treatment concentrations tested, not including the control

To calculate the variance of m, the following formula was applied:

$$V(m) = \sum_{k=2}^{t} -1 \frac{p_k^a(1-p_k^a)(X_{k+1}+X_{k-1})^2}{4(n_k-1)}$$

Where: X_k = the log10 of concentration k
n_k = the number of organisms tested at treatment concentration k
p_k^a = the smoothed adjusted proportion mortality at treatment concentration k
t = the number of treatment concentrations tested, not including the control

From the variance of m, V(m), 95% Confidence Intervals were calculated:

$$m \pm 2.0\sqrt{V(m)}$$

Since the treatment concentrations for the Spearman-Karber analysis requires that they are expressed as percentages of the minimum concentration which killed 100% of subjects, the estimated LC_{50} value and its associated 95% confidence intervals also are expressed in terms of that percentage. In addition, the estimated values from the analysis are presented as logarithmic percentages. Therefore, they needed to be converted back into original molar concentrations. This was done by taking the $base_{10}$ antilog of m and the 95% CI values, then converting those values into molar concentrations from the minimum molar concentration which killed 100% of the larvae.

3. RESULTS

3.1 Settlement

3.1.1 GABA

The amino acid and neurotransmitter GABA, showed no ability to induce larval settlement in *P. canaliculus* (Figure 17). With increasing GABA concentrations, a negative settlement trend was observed. At a concentration of $1x10^{-5}$ M, there was no significant difference between treatment and control (Tukey test; $p>0.05$). However, settlement inhibition was detected after exposure to GABA at concentrations of $1x10^{-4}$ M and $1x10^{-3}$ M, with 0% settlement being observed.

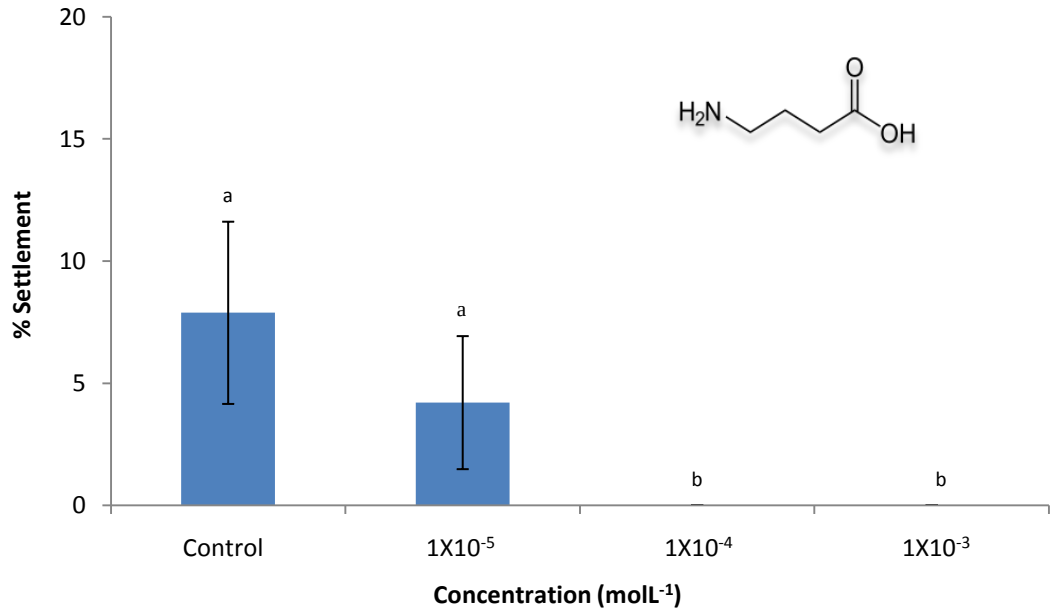

Figure 17. Effect of GABA on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.2 L-Tryptophan

Exposure to the amino acid L-Tryptophan (precursor molecule in the serotonin biosynthesis pathway) to mussel larvae resulted in high settlement (Figure 18). A One-way ANOVA test revealed significant differences in mussel larvae settlement among all treatments (ANOVA; $F_{3,36}>50$; $p<0.001$). At all concentrations assayed, significant induction was observed compared to the control (Tukey test; $p<0.001$ in each comparison). However, no differences were detected between each level of treatment (Tukey test; $p>0.05$ in each comparison).

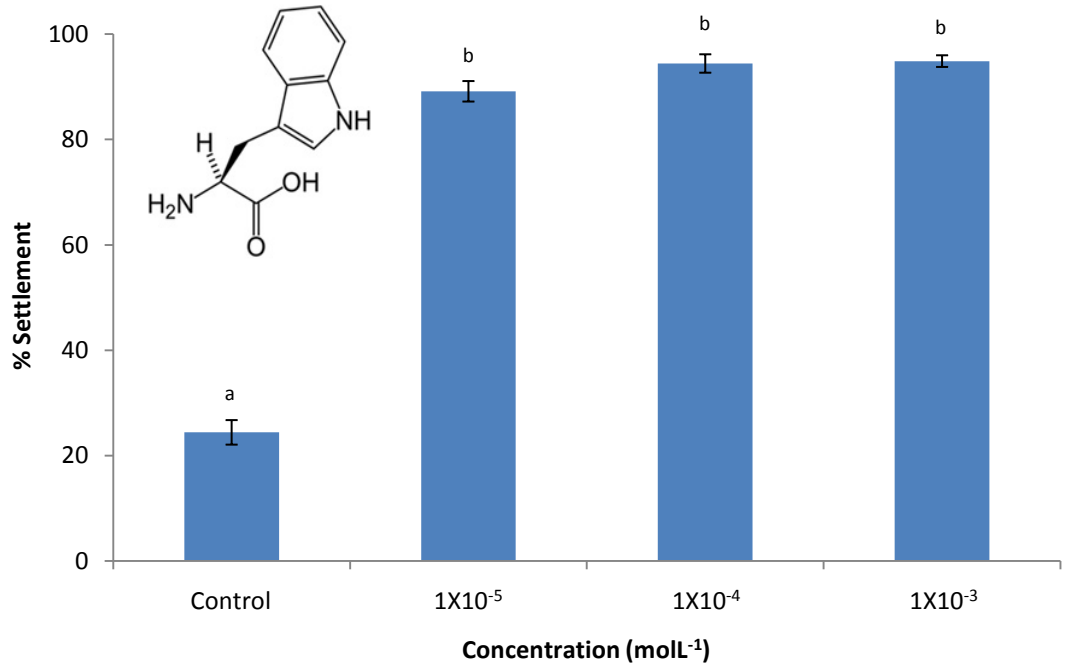

Figure 18. Effect of L-Tryptophan on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.2 Mortality & toxicology

3.2.1 GABA

Exposure to GABA for 48 hours at the five concentrations assayed revealed a typical toxicity dose response curve (Figure 19), with significant difference detected among treatments (ANOVA; $F_{5,54}>50$; $p<0.001$). No significant differences were detected between the control and $1x10^{-5}$ M GABA treatment solutions (Tukey test; $p>0.05$). Higher concentrations ($1x10^{-4}$ M, $1x10^{-3}$ M, $1x10^{-2}$ M, $1x10^{-1}$ M), were all significantly different from the control (Tukey test; $p<0.05$ in each comparison). Exposure to $1x10^{-2}$ M GABA solutions resulted in 100% mortality.

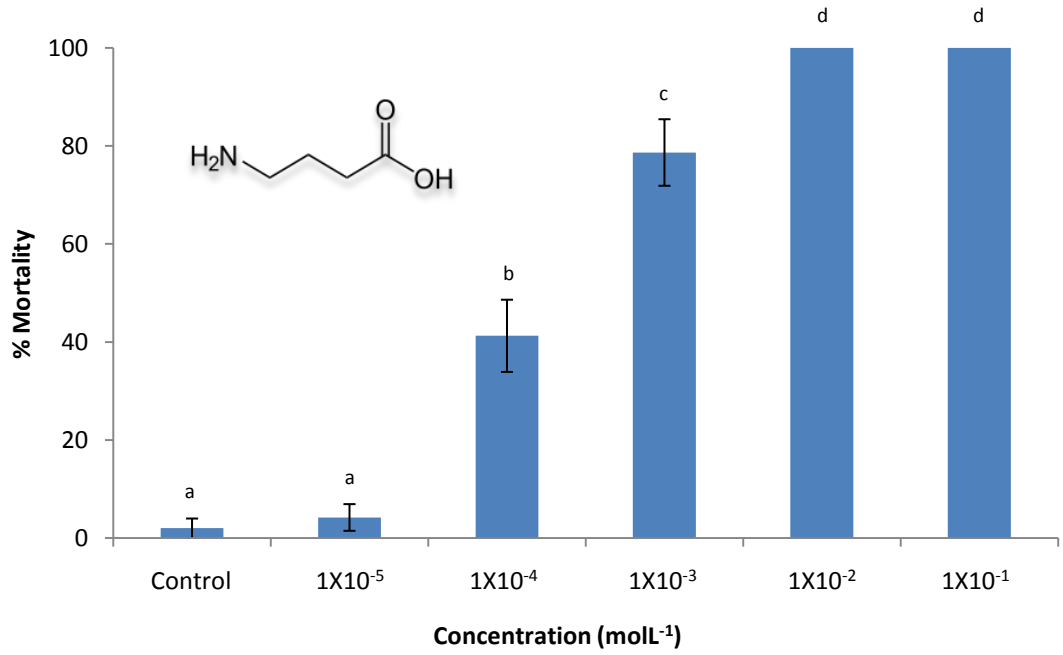

Figure 19. Effect of GABA on larval mortality after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Lethal concentration values were obtained from the raw mortality data by further advanced statistical analysis using the EPA Probit Analysis Program for Calculating LC/EC Values (v 1.5). The LC_1 (first observable effects), LC_{50}, and LC_{99} values and their associated 95% confidence intervals are shown in Table 7. Additional LC*x* data are presented in the computer outputs of the analysis in Appendix III, p287. The first signs of acute toxicity are estimated at a concentration of $5x10^{-6}$ M with a GABA solution of $1.7x10^{-4}$ M being estimated to kill 50% of the population.

Table 7. Estimated toxicological parameters for GABA: lethal concentration values and their associated 95% confidence intervals (italics).

Treatment	Treatment Levels (n)	Total larvae (n)	LC_1 (mol L^{-1})	LC_{50} (mol L^{-1})	LC_{99} (mol L^{-1})
GABA	5	1447	$5.0x10^{-6}$	$1.7x10^{-4}$	$5.8x10^{-3}$
			0.0 - $3.0x10^{-5}$	*$2.8x10^{-5}$ - $4.6x10^{-4}$*	*$1.6x10^{-3}$ - $5.0x10^{-2}$*

3.2.2 L-Tryptophan

Exposure to L-Tryptophan for 48 hours at three concentrations resulted in high levels of mortality for all treatment levels (Figure 20), with significant differences detected among treatments (ANOVA; $F_{3,36}>50$; $p<0.001$). Similar to GABA exposure, a dose dependant response curve was generated from mean percentage mortalities. However, this curve was not as distinct at the lower concentrations due to the high level of toxicity. Significant differences between the control and all treatment levels were detected (Tukey test; $p<0.05$ in each comparison). Almost 100% mortality was observed at $1x10^{-4}$ M.

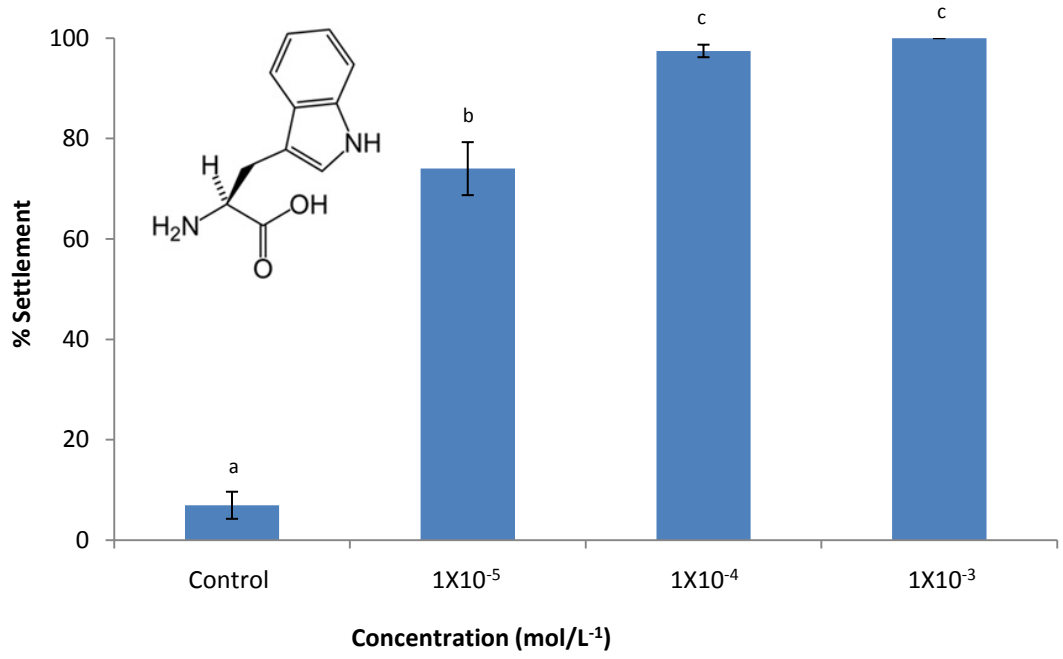

Figure 20. Effect of L-Tryptophan on larval mortality after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Toxicological calculations based on the mortality data from L-Tryptophan exposure were performed to determine estimated lethal concentration values (Table 8). From the mortality graph and the estimated LC_x values, L-Tryptophan is more toxic than GABA. First signs of acute toxicity are estimated to occur at a much lower concentration of $7x10^{-8}$ M. See Appendix III, p287, for additional lethal concentration data.

Table 8. Estimated toxicological parameters for L-Tryptophan: lethal concentration values and their associated 95% confidence intervals (italics).

Treatment	Treatment Levels (n)	Total larvae (n)	LC_1 (mol L^{-1})	LC_{50} (mol L^{-1})	LC_{99} (mol L^{-1})
L-Tryptophan	3	738	$7.0x10^{-8}$	$3.7x10^{-6}$	$1.9x10^{-4}$
			$3.0x10^{-8}$ – $1.4x10^{-7}$	*$2.9x10^{-6}$ – $4.6x10^{-6}$*	*$1.4x10^{-4}$ – $2.9x10^{-4}$*

4. DISCUSSION

4.1 GABA

Exposure of *P. canaliculus* larvae to GABA demonstrated that it has no ability to induce larval settlement in this species. In addition, this chemical inhibited settlement, likely due to the toxic effects observed in the mortality assays. The GABA concentrations chosen for induction trials were based on observations made in the literature with numerous marine invertebrate species. Although GABA was demonstrated to be inhibitive at higher concentrations, the treatment levels selected incorporated a concentration of $1x10^{-5}$ M which had no effect on larval settlement for *P. canaliculus* compared to the control assay. Therefore, the selection of concentrations (i.e. $1x10^{-5}$ M – $1x10^{-3}$ M) was well founded. The high mortality within high concentrations of GABA indicates that GABA has an effect on the biochemical physiology of these organisms. Therefore, *P. canaliculus* larvae must possess GABA receptors, either external transmembrane chemoreceptors or internal neuronal receptors. However, since GABA did not induce larval settlement it is unlikely these receptors are involved in the biochemistry of the settlement process for this species. The role of GABA receptors in *P. canaliculus* is unknown.

It has been suggested that exogenously applied neurotransmitters can act as natural settlement cue mimetics by activating external transmembrane chemoreceptors. A good example of this argument is that put forward by Morse and his associates (e.g. Trapido-Rosenthal & Morse 1985; 1986a,b; Woodwick & Morse 1991). They provide evidence through radio-ligand binding that the receptors involved in GABA reception for *Haliotis* spp. are specialised chemoreceptors located in the epithelial tissues of veliger larvae. Since Morse (i.e. Morse et al. 1979b, 1980, 1984; Morse & Morse 1984a,b; Morse 1991) has previously extracted various analogous

GABA mimetics from the surfaces of crustose corraline algae, they suggest that these molecules are the natural triggers for larval settlement for this genus.

The theorised mode of chemoreception in *Haliotis* spp. has been used as a model basis for other marine invertebrate species. However, alternative modes of biochemical action for exogenously applied neurotransmitters or compounds have not been considered. In *Haliotis* sp., larvae have been demonstrated to take up a variety of small molecules from seawater, including the transport of amino acids from the environment into their tissues (Manahan 1983; Jaeckle & Manahan 1989). Other marine invertebrates also have the ability to concentrate intracellular levels of amino acids from seawater in very high levels (Preston 1993). These compounds may have the ability to induce settlement or metamorphosis behaviours (Couper & Leise 1996). Therefore, perhaps GABA, being a small amino acid, also may have the ability to pass through epithelial membranes intracellularly or into extracellular spaces within the organism, directly acting on internal GABA receptors in the peripheral or central nervous system. With this alternative route in mind for the reception of exogenously applied treatments in larval settlement and mortality assays, it is suggested that the toxicity response to GABA observed in *P. canaliculus* is not due to the presence of epidermally bound chemoreceptors for perceiving a settlement cue, but the direct action upon GABA receptors in the nervous system. With the presence of such neuronal receptors, this species was still not induced to settle after treatment with GABA. However, since there are numerous types of GABA receptors with different mechanisms of action, it is entirely plausible that this neurotransmitter serves other unknown biological functions in *P. canaliculus*. For example, in the marine mollusc *Hermissenda crassicornis*, GABA is known to modulate phototaxis (Birmingham & Tauck 2003). In *H. crassicornis*, GABA binds to photoreceptors, stimulating the release of arachidonic acid, resulting in activation of protein kinase C (PKC). Protein kinase C has the ability to regulate a variety of biochemical processes from the stimulation of serotonin neurons (Rang 2003) to the contraction of smooth muscle cells (Horowitz et al. 1996). How GABA may function in *P. canaliculus*, and what biochemical pathways are involved, is currently uncertain. Although Baloun and Morse (1984) suggested that the biochemical

mechanism of settlement or metamorphosis induction by GABA in marine invertebrates would have to be one of excitory depolarisation, perhaps the mechanism/s involved in toxicity is one of inhibitory hyperpolarisation. This latter effect is by far the most common response to the receptor binding of GABA in most organisms (Enna & Mohler 2007). Hyperpolarisation of neuronal membranes would result in a decrease of nerve impulses being transmitted, thereby inhibiting signaling pathways which may result in death for the organism. Alternatively, GABA could act on receptors in non-neuronal cell membranes activating certain metabolites such as PKC, or lead to the deactivation of other metabolites affecting unknown biochemical processes and resulting in death.

There were three main reasons why GABA was chosen in this thesis to be investigated for its larval settlement inducing ability:

1. Active GABA analogues have been identified in red crustose coralline algae and *P. canaliculus* larvae and spat are known to be associated with these types of seaweeds.
2. GABA has long been known as a settlement inducer in numerous gastropod species.
3. GABA has recently been demonstrated to induce settlement in four species of bivalves; the clams *V. pullastra* and *R. philippinarum*, the oyster *O. edulis*, and most importantly the mussel *M. galloprovincialis*.

Because of the ineffectiveness of GABA as a larval settlement inducer for *P. canaliculus*, it would seem that the association this species has with crustose coralline algae is unlikely due to surface bound analogous GABA mimetic compounds. Perhaps *P. canaliculus* larvae are attracted to these seaweeds due to other surface bound compounds that have the ability to induce settlement. For example, a fluoroside-isethionic complex, produced by red algae, has the ability to induce colonisation of some marine invertebrates (Steinberg et al. 2002). Bacterial biofilms

also are commonly found on the surfaces of macroalgae and can induce larval settlement in numerous species (e.g. Steinberg et al. 2002; Hunag et al. 2007; Dworganyn & Pirozzi 2008), including *P. canaliculus* (Ganeesan et al. 2008). In addition, the physical properties of seaweeds are known to promote larval settlement and recruitment, and have been demonstrated to modulate larval substrate preference in *P. canaliculus* (Alfaro & Jeffs 2002). Therefore, perhaps the association young mussels have with red seaweed are due to one or more of these other inducing effects, rather than the presence of surface bound GABA mimetic compounds. The associations formed with red macroalgae by *P. canaliculus* and *Haliotis* spp. are very likely due to different reasons.

It has been suggested that larvae of the bivalve species *V. pullastra*, *R. philippinarum*, *O. edulis*, and *M. galloprovincialis* can be induced to settle upon exposure to GABA at $1x10^{-5}$ and $1x10^{-4}$ M (Garcia-Lavandeira et al. 2005). In contrast, GABA has been shown not to induce settlement in the marine mussel *Mytilus edulis* at concentrations of $1x10^{-6}$ to $1x10^{-4}$ M (Eyster & Pechenik 1987; Dobretsov & Qian 2003), and now for *P. canaliculus* between the range of $1x10^{-5}$ to $1x10^{-3}$ M. Since many pharmacologically active compounds have proven to be species specific in the literature, perhaps it is not surprising that GABA does not induce settlement in *P. canaliculus* and *M. edulis* but does in *M. galloprovincialis*. This could simply reflect differences in external chemoreceptors, or the involvement of GABA in endogenous signalling pathways between these marine mussels. Why *M. edulis* appears to be more similar to *P. canaliculus* than *M. galloprovincialis* in respect to GABA response is puzzling. This may suggest that the evolutionary divergence of these genera may be more complicated than previously thought. Whether mussels acquired or lost the 'GABA response' trait from a common ancestor is uncertain.

There is the possibility that larval settlement in response to GABA treatment in Garcia-Lavandiera et al's (2005) study may have been over-estimated. In their paper, there is no mention of toxicity effects for any of the compounds tested. It has been

demonstrated in this thesis that after 48 hours exposure to GABA, significant toxicity effects are present at $1x10^{-4}$ M for *P. canaliculus*. This concentration also produced significant acute toxic effects in *H. diversicolor* (Bryan & Qian 1998), and similarly in *H. discus hannai* (Yu & Bao-ling 1995). It is possible that in Garcia-Lavandiera et al's (2005) study, *M. galloprovincialis* mortality did not result from GABA exposure at the concentrations tested (i.e. $1x10^{-5}$ and $1x10^{-4}$ M). However, if toxic effects were apparent and not detected or revealed, some observations made of dead larva in this thesis may provide an explanation for the high settlement rates reported. When treated with GABA, *P. canaliculus* larvae that died released their tissues and gut contents past the shell margin, forming an apparent firm attachment to the substratum directly opposite the umbo. This attachment was solely due to the adhesive nature of the exogenously secreted tissues. In such cases larvae appeared to have settled. However, upon closer examination, no mucoid filamentous threads were detected, revealing that settlement had not occurred. This feature may lead to the overestimation of larval settlement in settlement inducing assays and should be carefully monitored and taken into account if it occurs.

Garcia-Lavandiera et al. (2005) performed settlement assays in triplicate in 90mm polystyrene Petri plates with approximately 500 individuals per plate. Larvae were considered attached if they could not be dislodged from the substrate with a stream of water. The number of individuals per plate is very high and would be difficult to quantify settlement with any degree of certainty within a reasonable time frame. Based on the present work, to determine larval settlement accurately in Petri plates and check for exogenous tissue release and preliminary mortality, at least 2 minutes observation under the microscope at 40X magnification is required per 10 individuals. Considering three treatment levels were used in Garcia-Lavandiera et al's (2005) study, 6000 individuals in 12 plates would need to have been enumerated. Due to the time required it is unlikely a water stream from a pipette under the microscope was used. Perhaps they poured water into, or over, the plate and very quickly and haphazardly counted those remaining under the microscope. In this case, and if acute toxicity effects were apparent, larvae would appear as if they were firmly attached to the substratum

and would not be dislodged with a water stream. For these reasons, perhaps the reported settlement results of Garcia-Lavandiera et al's (2005) study were overestimated. To be absolutely certain that larvae of *M. galloprovincialis* are induced to settle after exposure to GABA treatments, it is recommended that this mussel be tested again with careful observation of exogenous tissue release.

4.2 L-Tryptophan

Exposure of *P. canaliculus* larvae to the serotonin precursor L-Tryptophan, demonstrated that it does have an ability to induce larval settlement in this species. However, at all concentrations tested, high levels of mortality were observed after 48 hours exposure. This indicates that the concentration range selected was poor for settlement induction assays. It is therefore advised that this study be repeated at a later date incorporating lower treatment levels.

The mechanism of settlement induction by L-Tryptophan could be through bioconversion of the amino acid to serotonin. Serotonin may be an important neurotransmitter required in the biochemical pathways of settlement. However, the induction of larval settlement in *P. canaliculus* by this amino acid cannot be distinguished from serotonin since specific enzyme inhibitors were not used. It is likely that the observed activity resulted, at least partially, from the biochemical conversion of L-Tryptophan to serotonin. It is difficult to compare and draw conclusions between the various studies in the literature which investigate larval behaviour responses to serotonin. Numerous types of behaviours or processes (e.g. swimming, settlement, settlement behaviour, attachment, metamorphosis) are determined, often by using different techniques to apply the treatment (e.g. exogenous bath application of serotonin, enzyme inhibitors, receptor agonists/antagonists, or even injections in some cases). Making links between these studies is further compounded by the various definitions often used for the terms settlement, attachment, and metamorphosis. Since the role of serotonin in all marine invertebrate larvae is still unclear, and mechanisms of

L-Tryptophan induction in *P. canaliculus* are undetermined, it is suggested that a comprehensive study be performed on this species in the future. This study should involve investigation of settlement induction with: L-Tryptophan, tryptophan hydroxylase enzyme inhibitors, serotonin, and serotonin receptor agonists and antagonists. Also, levels of endogenous L-Tryptophan, tryptophan hydroxylase, and serotonin should be investigated during the normal development of the larvae before and after settlement and also after exposure to various regulatory serotonergic pathway treatments.

It has been demonstrated that an exogenously applied bath treatment of some marine invertebrate larvae to serotonin induces various ciliary activity (e.g. Goldberg et al. 1994; Bieras & Widdows 1995a; Christopher et al. 1996; Wada et al. 1997; Braubach et al. 2006). Therefore, serotonin may increase velar ciliary activity and swimming in veliger larvae. This may be corroborated by the observation that exposure of *P. canaliculus* larvae to L-Tryptophan seemed to increase motility within a few hours of T_0. Perhaps this is also an indication that L-Tryptophan was being converted to serotonin endogenously. In the barnacle *B. amphitrite*, Yanamoto et al. (2000) provides evidence that various serotonin agonists induce larval attachment and various serotonin antagonists inhibit larval attachment. However, no mention of ciliary activity is mentioned in Yanamoto's study and no information for this species could be sourced from the literature. It may be counter intuitive to suggest that attachment may be induced when swimming activity is increased at the same time. Although an observation made in this thesis was that firmly attached larvae often maintained a high degree of velar cilliary activity after 48 hour exposures to various treatments and control assays. Therefore, it is suggested that the activity of velar cilia and the larval attachment process may be independent.

It is possible that L-Tryptophan is a natural inducer of larval settlement for *P. canaliculus*. Dissolved free amino acids are prevalent in seawater (Amano et al. 1982), and are liberated by many marine animals (reviewed by Nicol 1960; Parry 1960;

Prosser & Brown 1961). Marine invertebrates, including mussels, are known to have the ability to uptake and endogenously concentrate amino acids from seawater (Manahan et al. 1982; Admiraal et al. 1984; Preston 1993; Zubkov et al. 2008). Larvae may extract dissolved L-Tryptophan from the ocean, and when required levels of serotonin are gained through bioconversion, the larvae undergo settlement. If true, the mode of action would have to be internally acting rather than through the binding of L-Tryptophan to external transmembrane chemoreceptors. This is because the bioconversion to serotonin cannot occur externally since the enzyme tryptophan hydroxylase would not be present. It also is possible that L-Tryptophan induces larval settlement in *P. canaliculus* by binding to external transmembrane chemoreceptors. However, since L-Tryptophan was observed to modulate velar ciliary activity in this mussel as previously mentioned, and serotonin is known to regulate beating in various types of cilia, it is more likely that L-Tryptophan was acting through bioconversion to the neurotransmitter. Furthermore, since L-Tryptophan was highly toxic to larvae, this suggests that the amino acid was affecting endogenous biochemical mechanisms in an adverse manner, probably through the over-production of serotonin.

Instead of obtaining L-Tryptophan from the water column, perhaps larvae preferentially select substrates with high levels of bound amino acids such as L-Tryptophan. Dissolved amino acids may adsorb to, and concentrate on, marine surfaces such as seaweeds. It was previously suggested in this chapter that the association young *P. canaliculus* mussels have with red macroalgae could be due to the presence of non-GABA mimetic surface bound settlement cues. Perhaps for *P. canaliculus*, L-Tryptophan bound to the surfaces of coralline algae may provide such a cue. However, whether L-Tryptophan concentrates at all, or adsorbs more, to the surfaces of these types of seaweeds than others is uncertain. Marine biofilms coat almost all surfaces in the ocean and are complex structures consisting of small bound molecules, macromolecules, diatoms, bacteria, fungi, and protozoa (Decho 2000). These matrices have varying compositions depending on the substrate on which they form (Hung et al. 2009). There may be something unique about the biofilms which cover the surfaces of red macroalgae, they may provide surface characteristics that can regulate levels of

adsorbed amino acids such as L-Tryptophan. Alternatively, the biofilms might secrete L-Tryptophan. For example, it is known that the marine bacterium *Photobacterium phosphoreum*, a common constituent in the microbial communities on macroalgal surfaces, produces the amino acid histamine (Fujii et al. 1997), and has been suggested to naturally induce larval settlement of the sea urchin, *Holopneustes purpurascens*, through this amino acid (Swanson et al. 2004).

Interestingly, L-Tryptophan has recently been discovered to be a natural attractant compound in the marine environment, promoting chemotaxis. The eggs of *Haliotis* spp. release L-Tryptophan into seawater to attract sperm (Zimmerman et al. 2009). Zimmerman et al. (2009) suggested that the amino acid is produced by the egg to signal to the sperm that it can manufacture high levels of serotonin. This may create an evolutionary advantage for these organisms since the neurotransmitter is vital for early larval development. Perhaps L-Tryptophan gamete signaling is more prevalent in the marine environment, and not just limited to *Haliotis* spp. Also, perhaps the importance of the genetic sequences in the sperm, which code for molecular components (e.g. receptor proteins) in the L-Tryptophan signaling pathway, are conserved after fertilisation, and continue to be active within the embryo's genome. If so, although purely hypothetical, these features may provide an explanation for why L-Tryptophan acts as an environmental signaling molecule in *P. canaliculus*, attracting the larvae to a suitable substrate and inducing settlement. This is an interesting concept, and one which should be investigated in the future.

In summary: (1) GABA does not induce larval settlement in *P. canaliculus*. (2) It is unlikely that this mussel possesses external epidermally bound GABA chemoreceptors. (3) GABA may act on internal receptors in the central or peripheral nervous system, serving unknown biochemical processes. (4) The toxic effects of GABA may indicate that the neurotransmitter regulates important neuronal pathways through the inhibitory hyperpolarisation of cell membranes. (5) Surface-bound GABA mimetic compounds are probably not responsible for the substrate association that young *P. canaliculus* mussels

have with red macroalgae. (6) The association of this mussel with red seaweeds must be regulated by a factor other than presence of GABA mimetic compounds such as: macroalgal physical structure, presence of biofilms, or other surface-bound compounds. (7) In settlement inducing assays, exogenous tissue release as a response to toxicity may lead to the overestimation of larval settlement. (8) L-Tryptophan induces larval settlement in *P. canaliculus*. (9) L-Tryptophan is highly toxic to larvae at $1x10^{-5}$ M. (10) L-Tryptophan likely induces settlement via bioconversion to the neurotransmitter serotonin. (11) L-Tryptophan probably does not act at external chemoreceptor sites. (12) L-Tryptophan is likely transported into extracellular spaces, or into the cytoplasm, where conversion to serotonin can take place. (13) A possible source of L-Tryptophan in the marine environment could be from dissolved amino acids in seawater, from surface bound amino acids on macroalgal surfaces, or from mixtures of amino acids released by biofilms. (14) An L-Tryptophan signaling pathway which controls chemotaxis of spermatozoa in some marine invertebrate species may be present in *P. canaliculus*, and may be conserved throughout larval development. (15) The presence of such a pathway may explain why L-Tryptophan can mediate larval behaviors of this mussel.

CHAPTER 5

EFFECT OF EXCESS POTASSIUM IONS ON LARVAL SETTLEMENT

1. INTRODUCTION

This chapter details the investigation of excess potassium ions, in various forms, as an inductor of larval settlement in *P. canaliculus*.

Potassium ions are essential for the normal functioning of cells. Within cells, potassium ions regulate many biological processes. They act by maintaining electrical gradients across cell membranes (Purves et al. 2008). It has been demonstrated in larvae of numerous marine invertebrate species that exogenously applied potassium ions can induce a variety of responses, including settlement and metamorphosis. Potassium ions have been demonstrated to induce metamorphosis of larvae from seven Phyla (summarised by Hermann 1995; Woolacott & Hadfield 1996). The location at which potassium operates in the larvae is unknown. It has been suggested that potassium acts by depolarising external excitable cells in the epithelial membrane which are normally responsible for binding chemical cues (e.g. Baloun & Morse 1984; Yool et al. 1986; Leitz & Klingmann 1990; Herrman 1995; Woolacott & Hadfield 1996; Carizo-Ituarte & Hadfield 1998). These external cells are thought to be associated with the peripheral nervous system (PNS), the component of the nervous system that commonly receives and transmits environmental information to the central nervous system (CNS) or transmits information from the CNS to various cells within the organism. Another suggestion is that exogenously applied potassium ions act internally and directly on the CNS, or some intermediary site in the PNS, by crossing external epithelial membranes of the organism (Herrman 1995; Carizo-Ituarte & Hadfield 1998; Hadfield 2000).

Varying results of the settlement or metamorphosis inducing capabilities of excess potassium ions exist among different species. For example, in the bryozoans

Bugula stolonifer, *B. turrita*, and *B. somplex*, optimal potassium ion concentrations for induction of settlement are 5–10 mM, 10–15 mM, and 10–25 mM, respectively (Wendt & Wollacot 2005). The ascidian *Herdmania momus* is maximally induced to undergo metamorphosis after exposure to 40 mM KCl (Denegan et al. 1997). Within the class gastropoda, Li et al. (2006) demonstrated that larvae of the Taiwanese abalone, *Haliotis diversicolor supertexta*, can be induced to settle with low levels of excess extracellular potassium ions (1–10 mM); however, Baloun & Morse (1984) found that treatment with 4mM KCl had no effect on larval settlement of the red abalone, *Haliotis rufescens*. They also suggested that treatment with 12 mM KCl increases metamorphosis significantly with a rate-limiting effect being apparent around 20 mM. Larvae of the Pacific abalone *Haliotis discus hannai* are not induced to settle after treatment with 14–19 mM KCl, and significant inhibition is observed at concentrations greater than 24 mM (Kang et al. 2003). Larvae of the whelk *Astraea undosa* are maximally induced to settle with 10 mM excess extracellular potassium ions with inhibitive effects observed between 15–20 mM (Yool et al. 1986).

Within the class bivalvia, larvae of the pearl oyster, *Pinctada fucata martensii*, are induced to settle after exposure to 20 mM potassium chloride (Yu et al. 2008). Also, in the green mussel *Perna viridis*, a close relative of *P. canaliculus*, treatment with excess potassium ions significantly increased larval settlement between 6–9 mM (Ke et al. 1998). In contrast, treatment with 5–40 mM excess potassium ions in the blue mussel *Mytilus edulis* had no effect on larval settlement (Eyster & Pechenik 1987; Dobretsov & Qian 2003). From the marine invertebrate species investigated, representatives of bivalvia are the least tested for settlement induction with potassium ions.

Much of the literature relies only on treatment with KCl to determine the effects of excess potassium ions on larval settlement (e.g. Baloun & Morse 1984; Yu & Bao-Ling 1995; Matsumura et al. 1999; Dobretsov & Qian 2003). However, sodium, calcium, and

chloride ions also are associated with many cellular processes, including neuronal signalling (Purves et al. 2008). Since the chloride ion also is a key regulator of neuronal mechanisms, the inductive effect of KCl cannot be solely attributed to the potassium ion with any degree of certainty. In this study, settlement induction assays were performed with potassium sulphate and potassium metabisulphite to check if potassium was the main ion involved in the settlement process.

2. METHODS

2.1 Organisms

Larvae for these assays were sourced from the Sealord Ltd. hatchery facility in Ruakaka (northern New Zealand) and from the Cawthron Institute (South Island, New Zealand). Organisms were reared and transported according to the methods outlined in Chapter 3, p60. For details of larval source and age for each settlement assay performed, see Appendix II, p285.

2.2 Settlement assays

Settlement assays were conducted using the same methods and environmental parameters outlined in Chapter 3, p62. Briefly, assays were performed at 17±1°C in sterile polystyrene Petri plates under diffuse light with 20–30 larvae per plate (from a larval solution pre-calibrated to 20–30 larvae/ml). Static conditions were maintained, no food provided, and FSW/treatment solutions were well oxygenated immediately prior to initiating assays, but not aerated during the assay. Ten replicates were used for each treatment and control. The number of settled larvae was detected after 48 hours for each Petri plate and using the suction-by-pipette technique.

2.2.1 Treatments

Potassium chloride (KCl), potassium sulphate (K_2SO_4), and potassium metabisulphite ($K_2S_2O_5$) were dissolved in 0.45 μm filtered seawater (FSW), respectively. Stock solutions of each treatment were prepared immediately prior to all settlement assays. Following serial dilution in FSW, treatment solutions were prepared as 10X concentrates. Controls consisted of 9 ml FSW and 1 ml larval solution. Treatments consisted of 8 ml FSW, 1 ml larval solution, and 1ml treatment solution. Final exposure concentrations for treatments were: KCl; 5, 10, 15, 20, 40 & 60 mM, K_2SO_4; 5, 10, 15, 20 & 40 mM, $K_2S_2O_5$; 5, 10, 15, & 20 mM.

2.3 Mortality assays

Mortality assays were performed in accordance with the methods outlined for settlement assays. Mortality detection incorporated visual observations of velum, foot, or gut movement and the use of neutral red, a vital stain (for further details see Methods section in Chapter, p88). Due to time constraints and larval availability, the acute toxicity effects of only KCl and $K_2S_2O_5$ were determined.

2.3.1 Treatments

Solutions of KCl and $K_2S_2O_5$ were prepared as previously described for settlement assays. The final treatment concentrations used in the mortality assays were: KCl; 20, 40, 60, & 80 mM, $K_2S_2O_5$; 5, 10, 15, & 20 mM

2.4 Statistics

2.4.1 Larval settlement & mortality

Statistical methods used for settlement induction assays and mortality assays were the same as those outlined in the methods section in Chapter 3, p64. Briefly, all percent data were arcsin transformed and analysed using statistical software, Minitab v.15. Where data satisfied the assumptions of parametric analysis, 1-way ANOVA with Tukey's multiple comparisons test was used. Non-parametric data were analysed using the Kruskal-Wallis test, and multiple comparisons made with a specialised Dunn's macro enabled for multiple comparisons of medians. Actual *p*-values for all comparisons can be found in Appendix I, p277.

2.4.2 Larval toxicology

To determine the toxicity of treatment compounds, various lethal concentration values were calculated from the raw mortality data using the same techniques as those outlined in the methods section in Chapter 4, p91. Briefly, where data fitted the assumptions of the model, a probit analysis was performed using the EPA Probit Analysis Program Used for Calculating LC/EC Values Version 1.5. Where data were not suitable for this model, the LC_{50} value was calculated manually by the Spearman-Karber method.

3. RESULTS

3.1 Larval settlement

In order to determine the effect of excess potassium ions, a known inducer of settlement in many marine invertebrate species, treatment with various potassium salts was assayed.

3.1.1 Potassium chloride

Potassium chloride was first trialled using 20–60 mM treatment concentrations (Figure 21). This concentration range was initially selected for a pre-screen study and contains the effective concentrations proven to induce larval settlement or metamorphosis in many marine invertebrate species, including representatives within: Bivalvia (Yu et al. 2008), Gastropoda (Pechenik & Heyman 1987; Gallardo & Sanchez 2001), Ascidiacea (Degnan et al. 1997; Matsumura et al. 1999; Huang et al. 2003), and Echinodermata (Cameron et al. 1989). Increased external potassium ions via addition of KCl did not effectively induce larval settlement over the three concentrations assayed, however significant difference was detected among treatments (ANOVA; $F_{3,36}=17.09$; $p<0.01$). At the lower concentrations, 20 and 40 mM, no differences in settlement were detected between the control and treatment levels (Tukey test; $p>0.05$ in each comparison). However, at a higher concentration a negative, or inhibitory, effect was observed with 0% larval settlement resulting from exposure to 60 mM excess potassium ions.

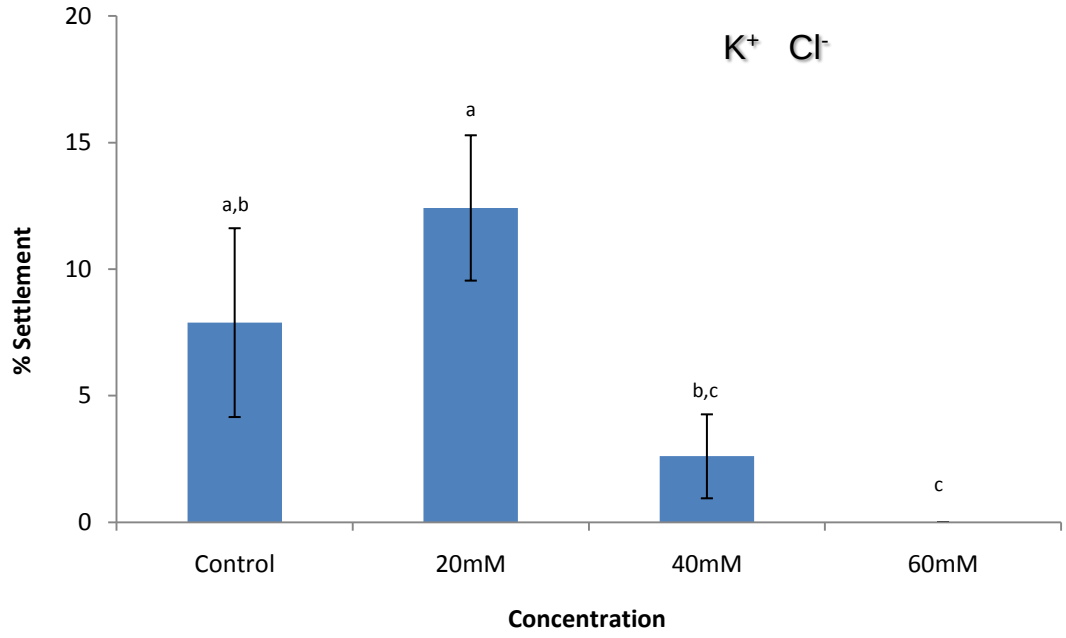

Figure 21. Effect of excess potassium ions (KCl) at high concentrations on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Since some bivalve molluscs are induced to settle in the presence of lower concentrations of KCl (e.g. Yool et al. 1986; Ke et al. 1998), the treatment concentrations were decreased to 5–20 mM and the assay repeated (Figure 22). The results of this analysis reveal a typical dose response curve with significant difference detected among treatments (ANOVA; $F_{4,45}=13.19$; $p<0.001$). Exposure to potassium chloride concentrations at 5, 10, & 15 mM significantly increased larval settlement compared to control assays (Tukey test; $p<0.01$ in each comparison), with a settlement response peak maxima at 10 mM.

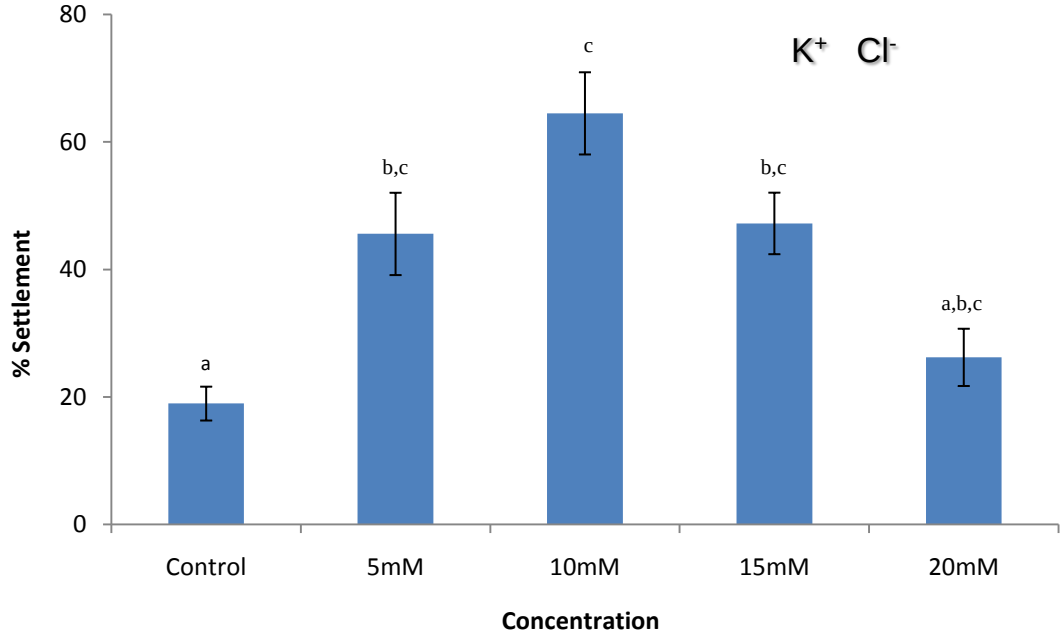

Figure 22. Effect of excess potassium ions (KCl) at low concentrations on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.2 Potassium sulphate

KCl, being a chloride salt, contains the chloride anion in solution. Therefore to determine which of the two ions are primarily responsible for the induction ability of KCl previously described, the sulphate salt, K_2SO_4, was trialled (Figure 23). Potassium sulphate contains 2 moles of K^+ for every mole of compound. Therefore, since the potassium ion is the main component of interest, treatment concentrations of K_2SO_4 expressed in Figure 23 relates to the concentration of K^+ in the experimental medium after complete ionic dissociation of the salt, rather than the concentration of the undissociated compound.

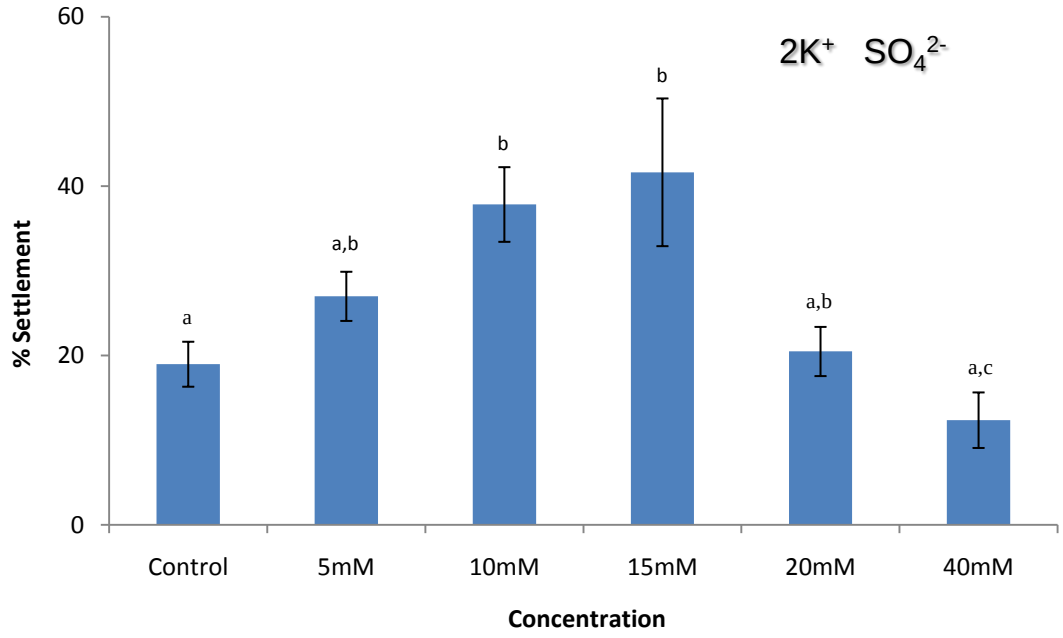

Figure 23. Effect of excess potassium ions (K_2SO_4) on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Induction of settlement with excess K^+ from K_2SO_4 revealed a typical dose response curve, similar to the trend given by KCl with significant difference detected among treatments (ANOVA; $F_{5,54}=6.75$; $p<0.001$). However, the mean settlement rates were lower than those induced by the chloride salt and the settlement response peak maxima occurs at a higher concentration of 15 mM excess K^+ from K_2SO_4. Induction of larval settlement at 10 and 15 mM were significantly different from control assays (Tukey test; $p<0.05$ in each comparison), but were not different from one another (Tukey test; $p>0.05$). Unlike induction with KCl, a concentration of 5 mM excess K^+ showed no significant capacity to induce larval settlement. However, at 5 mM the trend revealed a mean increase over the control assays.

3.1.3 Potassium metabisulphite

To provide further evidence for the induction ability of potassium ions a third salt, potassium metabisulphite, was trialled (Figure 24). Non-parametric analysis of settlement data revealed significant difference between treatments (Kruskal-Wallis; $p<0.05$). At a concentration of 5 mM, an increase in mean settlement was observed compared to the control assay. However, this increase was not significant (Dunn's test; $p>005$). At concentrations of 10 mM and higher, inhibitory effects were inferred from the mean settlement data. Significant differences were observed between larvae exposed to 5mM $K_2S_2O_5$ and all other concentrations assayed (Dunn's test; $p<0.05$ in each comparison). It should be noted that the non-parametric Dunn's test statistic for the comparisons of medians is extremely conservative and the statistic is based on variance adjusted for ties.

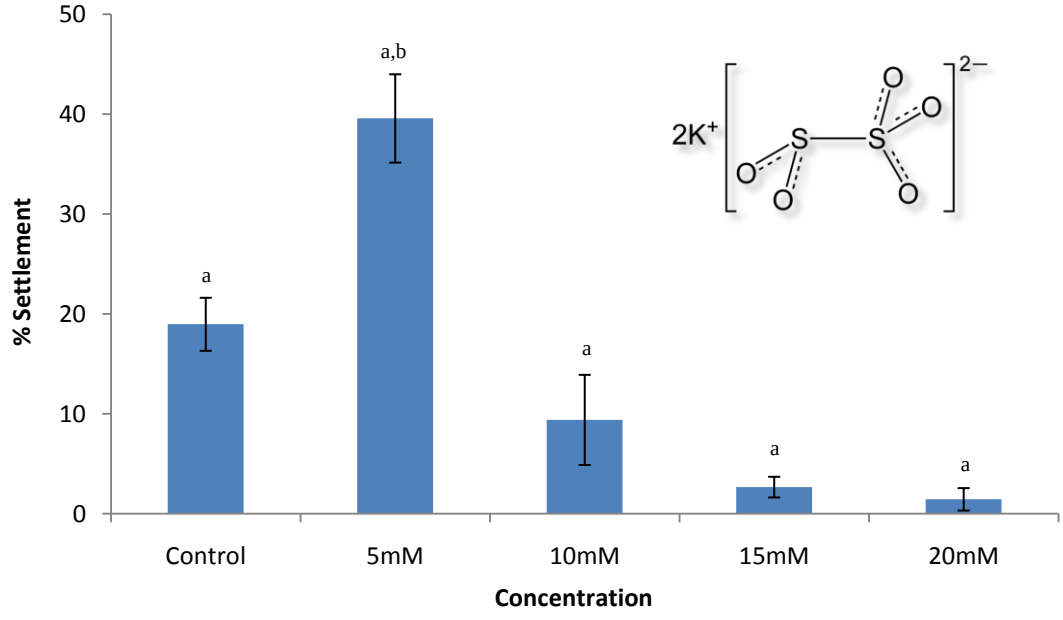

Figure 24. Effect of excess potassium ions ($K_2S_2O_5$) on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.2 Mortality & toxicology

The acute toxicity effects of KCl and $K_2S_2O_5$ were determined and corresponding estimated LC_x values calculated.

3.2.1 Potassium chloride

Exposure to KCl for 48 hours reveals toxic effects at all four concentrations assayed (Figure 25). All concentrations were significantly different from the control (Tukey test; $p<0.05$ in each comparison), with almost 100% mortality occurring at 60 mM.

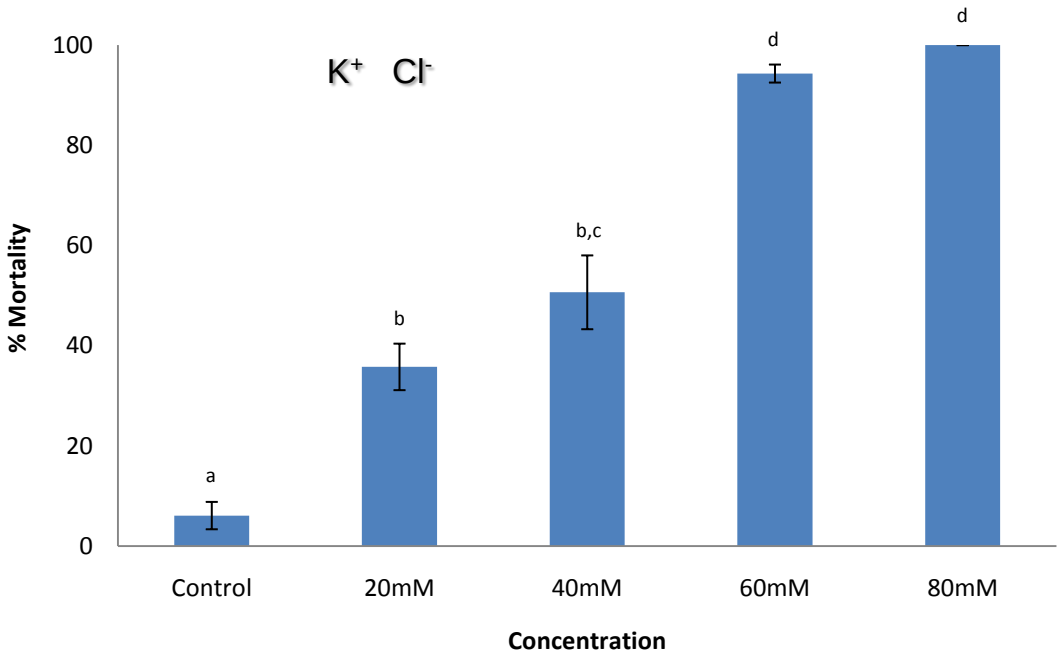

Figure 25. Effect of excess potassium ions (KCl) on larval mortality after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Lethal concentration values were obtained from the raw mortality data by performing further statistical calculations based on the mortality data. The estimated LC_1, LC_{50}, and LC_{99} values are shown in Table 9. Mortality data failed to satisfy the assumptions of the probit model so the Spearman-Karber method was employed. Since the Spearman-Karber method can only calculate an LC_{50} estimate, results of the probit model also are represented. However, confidence intervals could not be computed. The LC_{50} values reveal that a relatively high concentration of around 34 mM KCl kills 50% of the population compared to the compounds tested in Chapter 4. The first signs of acute toxicity calculated by the Spearman-Karber analysis are estimated at a concentration of around 9 mM – this value should be interpreted with caution.

Table 9. Estimated toxicological parameters for KCl: lethal concentration values.

Treatment	Treatment Levels (n)	Total larvae (n)	LC_1 (mmol L^{-1})	LC_{50} (mmol L^{-1})	LC_{99} (mmol L^{-1})
KCl	5	1393	-	33.8[a]	-
			9.2[b]	31.5[b]	108.2[b]

[a] Spearman-Karber estimate
[b] EPA probit analysis estimates

3.2.2 Potassium metabisulphite

Exposure to $K_2S_2O_5$ for 48 hours at four concentrations results in high levels of acute toxicity at all treatment levels assayed (Figure 26). The concentrations expressed are in terms of K^+ to be consistent with the settlement assay. Therefore, actual values of compound concentrations are half of those expressed and are represented in parentheses on the graph. The lowest concentration of the compound, 2.5 mM, significantly increased the mortality rate compared to the control (Tukey test; $p<0.05$), with 100% being detected at 7.5 mM. However, no significant difference in mortality rate was detected between 5 and 7.5 mM (Tukey test; $p>0.05$).

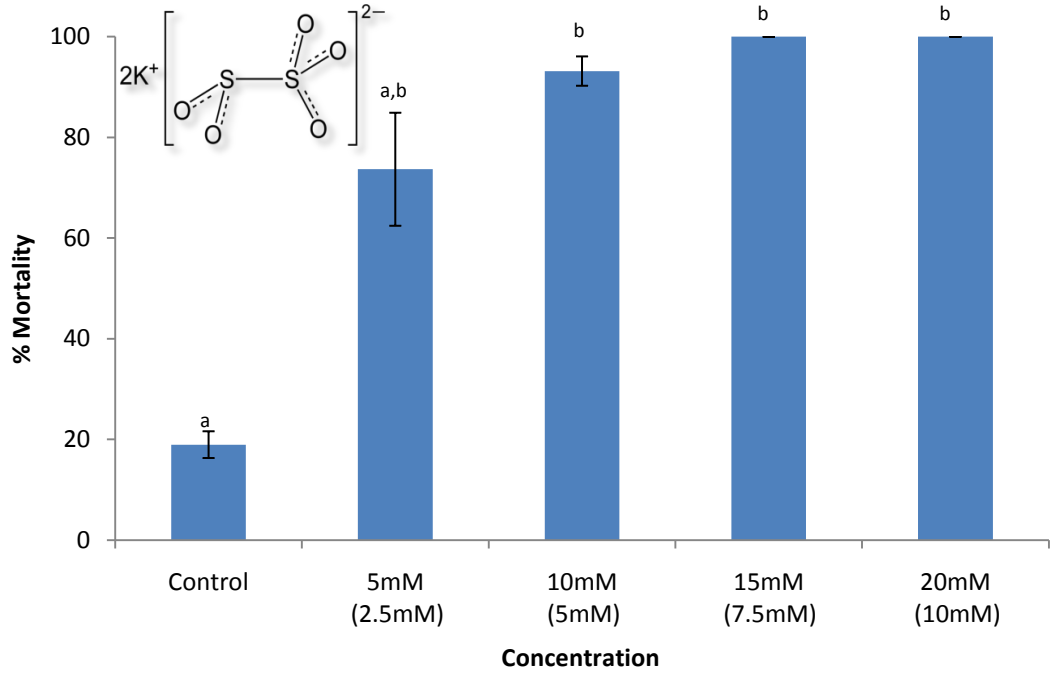

Figure 26. Effect of excess potassium ions ($K_2S_2O_5$) on larval mortality after 48 hours incubation. Concentrations are expressed in terms of [K^+] with compound concentration below in brackets. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

From the mortality data, estimated lethal concentration values for potassium metabisulphite, and their association 95% confidence intervals, were calculated (Table 10). The estimated LC values reveal that on average, $K_2S_2O_5$ is approximately fifteen times more toxic than KCl.

Table 10. Estimated toxicological parameters for $K_2S_2O_5$: lethal concentration values and their associated 95% confidence intervals (italics). Concentrations are expressed in terms of compound, not [K^+].

Treatment	Treatment Levels (n)	Total larvae (n)	LC_1 (mmol L^{-1})	LC_{50} (mmol L^{-1})	LC_{99} (mmol L^{-1})
K_2SO_4	5	1117	0.5	1.9	7.7
			>0.1 – 1.7	*0.3 – 2. 9*	*4.9 – 123.0*

4. DISCUSSION

The selective movement of ions across specialised membranes is a fundamental mechanism in the function of excitable cells including neurons. The cation potassium is one of the primary regulators of these processes (Purves et al. 2008). A variety of induced responses, including settlement and metamorphosis, can be induced by exogenous treatment with potassium in numerous marine invertebrate species (e.g. Morse et al. 1984; Yool et al. 1986; Pechenik & Heyman 1987; Cameron et al. 1989; Degnan et al. 1997; Ke et al. 1998; Matsumura et al. 1999; Gallardo & Sanchez 2001; Huang et al. 2003; Kang et al. 2003; Li et al. 2006). Potassium ions have now been demonstrated to have the ability to induce settlement in the larvae of *P. canaliculus*.

Results of the settlement assays demonstrate a peak of 10 mM excess potassium ions applied exogenously to the external medium, as chloride salt, increased settlement rates significantly. At 15 mM inhibition of induction was observed, and at a concentration of 20 mM, induction was reduced with significant differences being detected over the control assay. Settlement inhibition was inferred after treatment with 40 mM KCl from the reduction in mean settlement compared to the control, however inhibition was only statistically determined after exposure to 60 mM. Since the concentration for the estimated first observable signs of acute toxicity (LC_1) was around 10 mM, and induction of settlement was observed between 5–15 mM KCl, it strongly indicates that KCl is acting in some way as a chemical cue for settlement at low concentrations. Again, induction of settlement was achieved with 5–15 mM excess K^+ from the sulphate salt with an induction rate limiting concentration of 15 mM. A third potassium ion treatment, potassium metabisulphite ($K_2S_2O_5$), corroborated previous results that potassium ions induce settlement by indicating inductive effects at 5 mM excess K^+, with a mean percent increase in settlement. However, due to the increased toxicity of the compound, concentrations greater than 5 mM proved ineffective at inducing larval

settlement. The toxic effects observed at low concentrations with $K_2S_2O_5$ likely arose due to chemical degradation breakdown products when in contact with water, primarily the production of toxic sulphur dioxide. In light of these combined findings there is evidence to suggest that excess K^+ ions applied exogenously to the medium at concentrations of between 5–15 mM certainly affect the signal transduction mechanism of larval settlement in *P. canaliculus*.

In two separate studies, larvae of the blue mussel *M. edulis* were not induced to settle at concentrations between 5–40 mM excess K^+ (Eyster & Pechenik 1987; Dobretsov & Qian 2003). In the green mussel *P. viridis*, a close relative of *P. canaliculus*, treatment with excess potassium ions significantly increased larval settlement between 6–9 mM with a rate limiting concentration of 9–12 mM and inhibitive effects being observed over 20 mM (Ke et al. 1998). For *P. canaliculus*, settlement was induced between 5–15 mM with a rate limiting concentration of 10–15 mM and inhibitive effects inferred at 40 mM. These results correlate quite well at low concentrations with those obtained by Ke et al. (1998). However, differences were apparent at higher concentrations. In the case of *Haliotis* spp., it is well documented that differences in response to K^+ exist between species (e.g. Baloun & Morse 1984; Li et al. 2006). Although differences have been demonstrated between mussel genera (i.e. *P. viridis vs*. *M. edulis*) the results of the present study for *P. canaliculus* adds further weight to this observation, and also for the first time, differences in potassium ion induction between mussels at a species level have been identified (i.e. *P. viridis vs*. *P. canaliculus*)

Differences occurred between the optimal mean settlement percentages obtained after treatment with KCl and K_2SO_4. After standardising against the control, these values were 47.4% and 24.5%, respectively. The larvae used for both induction trials came from the same hatchery reared cohort population at the same age. Therefore, these differences cannot be due to age, batch, or hatchery effects. The only difference between the two assays was the identification of the anionic component of the salts, Cl^- and $SO4^{2-}$. This suggests that chloride may also affect the signal transduction mechanisms of settlement. The concentration at which maximum induction occurred

with KCl was 10 mM, settlement peak maxima of K_2SO_4 induction was observed at a higher concentration of excess potassium ions at 15 mM. If chloride also induces settlement, then the lower induction concentration for KCl makes sense, causing an additive effect. Treatment with potassium chloride induced almost double the percent of larval settlement than treatment with potassium sulphate. Although K_2SO_4 has double the amount of potassium ions than KCl, this was taken into consideration when calculating treatment doses and final concentrations were expressed only in terms of excess K^+. Therefore, this suggests that Cl^- also may induce larval settlement in *P. canaliculus*. If so, this may be the first time in which it has been demonstrated that the chloride ion has the ability to induce larval settlement in marine invertebrates. For this reason, it is highly recommended that larval settlement induction trials performed on any species should not solely use KCl as an inducer to determine effects of exogenously applied K^+ ions. Otherwise, there is the possibility that any suggestions and conclusions based on KCl results may be erroneous.

Few studies which investigate the effects of neuroactive induction of settlement in marine invertebrate larvae attempt to explain the biochemical mechanisms behind their observations. By reviewing the process of ionic control across the cell membrane, and action potential formation within the nervous system, an explanation for the results of settlement induction and inhibition with excess extracellular potassium ions in *P. canaliculus* is presented.

Potassium ions are principal ions involved in delivering nerve impulses, or action potentials, which send a wave of electrochemical signals from the neurons through nerve axons for extracellular communication (Bullock et al. 1977; Purves et al. 2008). Active ion pumps within the membrane of a neuron cause sodium and potassium ions to form concentration gradients across the cell membrane. Positively charged potassium ions are found in excess inside the cell, whereas positively charged sodium ions dominate the external medium (Steinbach & Spiegelman 1943; Hodgkin 1951). The two active ion pumps responsible for maintaining these gradients move three

sodium ions out of the cell for every two potassium ions replaced within the cell (Caldwell et al. 1960). This causes a slight difference in the magnitudes of positive charges across the membrane. To amplify this effect, non-gated passive potassium ion channels within the cell membrane allow a slow 'leak' of potassium ions back into the extracellular medium (Nicholls et al. 2001). The presence of proteins containing strong anionic properties within the cell further amplifies the differences in charge across the membrane. The result is a net negative charge within the cell and a net positive charge outside the cell. In this polarised state, neurons are inactive and have a polarised resting potential of about -70 mV. Nerve impulses are triggered creating communication pathways when an event causes a depolarisation across the cell membrane and the membrane potential increases beyond a threshold level (Nicholls et al. 2001).

The spike of the action potential is initiated when voltage gated sodium ion channels are activated by a stimulus (e.g. chemical or physical cue) causing a voltage change across the membrane. Excess sodium ions enter the neuron thereby creating a depolarising current. This depolarisation is balanced by the opening of voltage gated potassium ion channels, triggered by the influx of sodium ions, causing the positively charged potassium ions to exit the cell along the concentration gradient (Gutkin & Ermentrout 2006). However, there is a lag effect between sodium ions entering and potassium ions leaving. It is this lag which causes the depolarisation for a split moment in time. It is during this period in which the nerve impulse is fired. Once repolarised by the exiting potassium ions, the active ion pumps slowly exchange the sodium and potassium ions across the cell membrane to restore the original concentration gradients. Now back in its polarised state, if the stimulus is still present to re-open the voltage gated sodium ion channels then the cycle is repeated and nerve impulses continue to fire. Without the stimulus the cell will remain polarised and therefore inactive (Nicholls et al. 2001). This process is illustrated in Figure 27 & Figure 28.

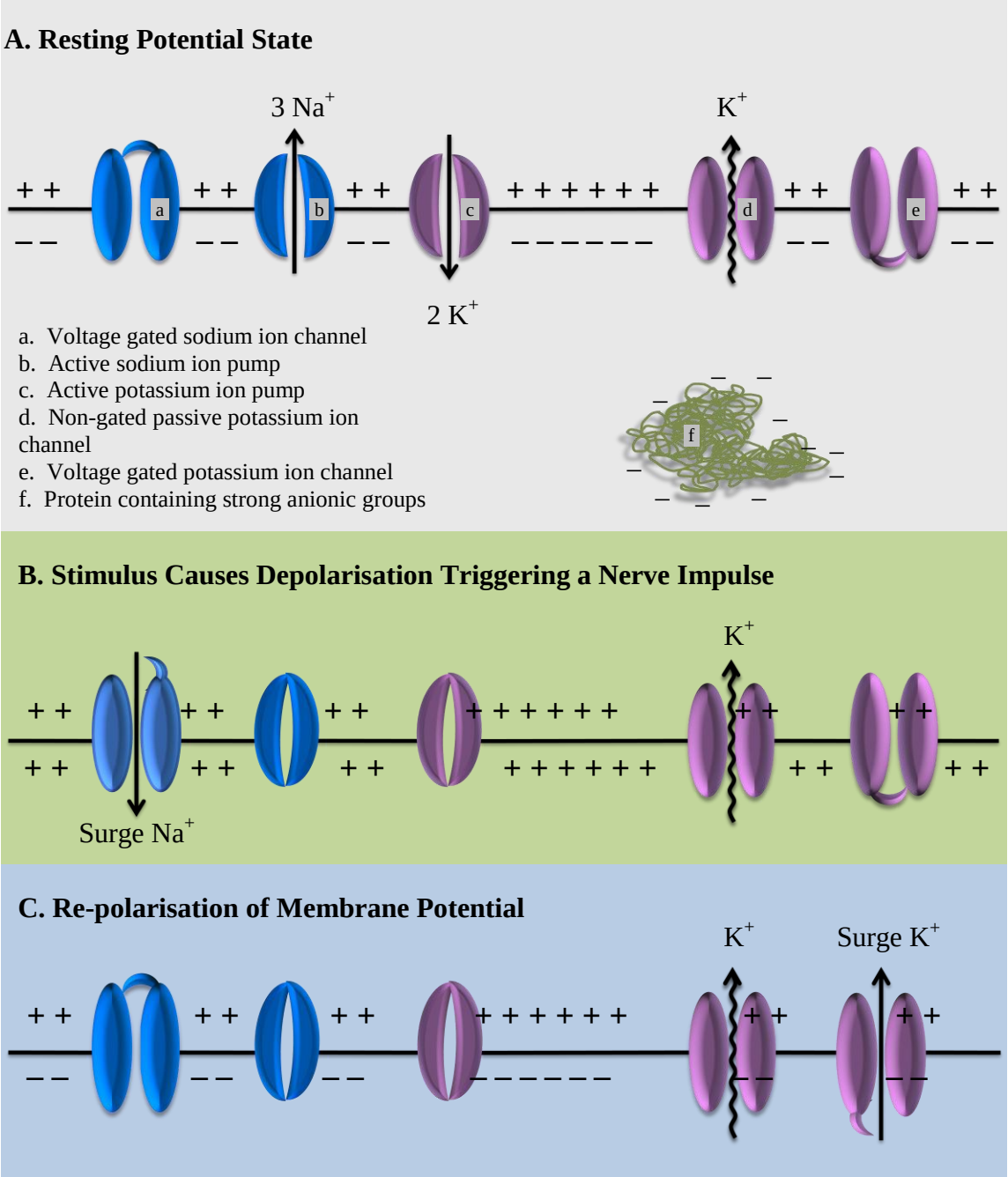

Figure 27. Mechanism of cell membrane depolarisation. A: Polarised resting potential state - active sodium and potassium ion channels create and maintain the electrical gradient. When polarised, there is a net positive charge outside the cell and a net negative charge inside the cell. Gated passive ion channels are closed and a small amount of potassium, represented by the wavy arrow, leaks through non-gated ion channels. B: Once a stimulus opens the voltage gated sodium ion channels, sodium ions surge across the membrane by diffusion causing a net positive charge within the cell and depolarisation is achieved. An increase in the membrane potential occurs and once beyond the threshold level of around -50mV a nerve impulse is fired. C: After a brief lag, the influx of sodium ions initiate the opening of gated passive potassium ion channels, causing a re-polarisation of the membrane potential. Completing the cycle, the active sodium and potassium ion channels are activated once again, thereby restoring the original ion concentration gradient (A).

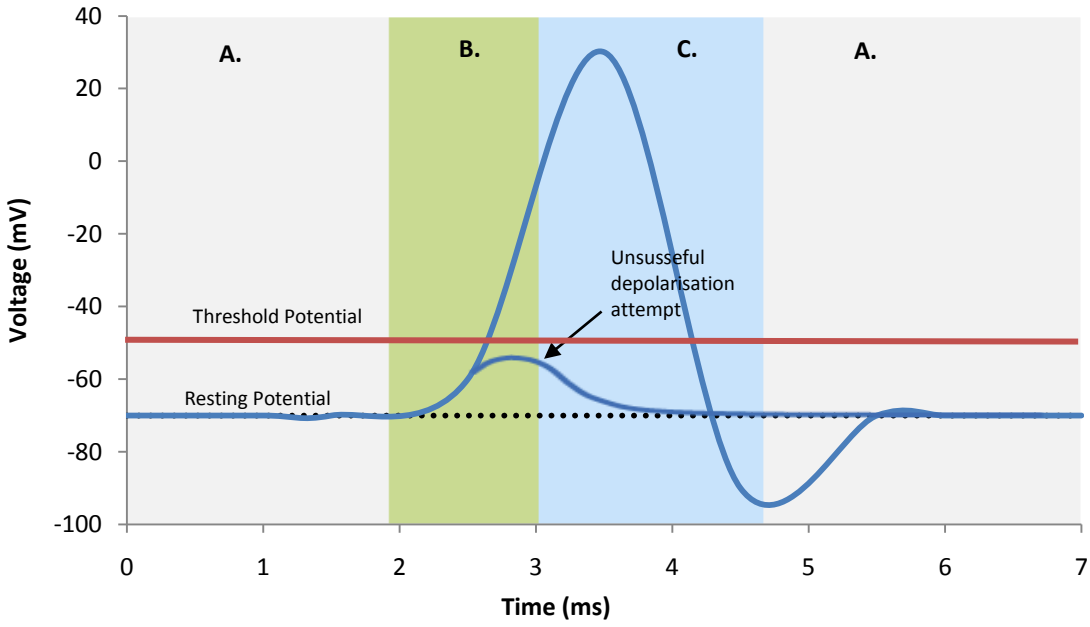

Figure 28. Mechanism of action potential formation. A: polarised resting potential state – active ion pumps maintain electrical gradient. B: stimulus causes slight depolarisation causing sodium ions to enter the cell – if this is great enough to rise above the threshold potential, sodium ions surge into the cell and the membrane becomes fully depolarised (failed attempts often occur when the initial stimulus is not great enough). C: after a brief lag, potassium ions exit the cell causing a polarising flux. A slight overshoot in voltage reduction occurs here but is quickly restored by the re-introduction of the active sodium and potassium pumps.

Numerous studies of neuronal cells in various organisms, including bivalves, have shown that treatment with low levels of excess potassium ions cause an increase in the resting potential of the cell membrane (Ling & Gerard 1950; Wilkins 1977; Weiss 1996). In muscle cells of the brown mussel, *Perna perna*, a close relative of *P. canaliculus*, an increase in the resting potential after such treatment also is observed (Ferreira & Salomão 2000). Although the resting potential is increased, there is no effect on the threshold potential. Fortune and Lowery (2007) demonstrated that an increase in external K^+ causes a reduction of the action potential amplitude at neuro-muscular junctions. An increase of 8 mM extracellular K^+ was also found to reduce action potential amplitude and also decreased the duration of re-polarisation (Qin & Li 2008).

When the resting potential is increased due to potassium ion treatments and the threshold potential remains the same, the voltage difference between the two potentials decrease. This decrease could be due to the effect of excess extracellular K^+ on the flow of K^+ though the passive ion channels (Figure 29). During the resting potential state, small increases in extracellular K^+ concentration will reduce the concentration gradient across the membrane thereby reducing the constant slow efflux of potassium outside of the cells through the passive ion channels. Since the active potassium and sodium ion pumps are still regulating the inward and outward movements of the positively charged ions in different ratios, the net result is a slight hypopolarisation due to an intracellular increase of K^+.

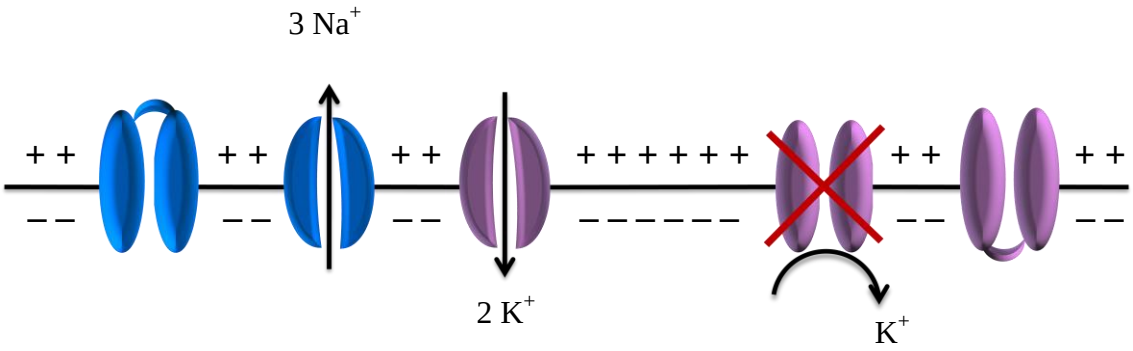

Figure 29. Effect of increased extracellular potassium ions on slow efflux of potassium through passive ion channels at the resting potential. Note: a complete inhibition of K^+ efflux is unlikely but this efflux will be reduced therefore increasing the intracellular K^+ concentration and inducing membrane hypopolarisation.

It is suggested here that perhaps the potassium ion inductions of larval settlement and metamorphosis, observed in numerous marine invertebrate species, is caused by an increase in the number of action potentials being generated. If true, the action potentials would have to be directly involved in the signalling pathways of these important life history events. A low increase in extracellular K^+ decreases the difference between the

resting and threshold potentials therefore increasing the excitability of the cell and making action potential formation much easier to achieve with less stimulus than is normally required (e.g. chemical or physical settlement cue). Also, since there may be a decrease in the duration of re-polarisation, perhaps the frequency of action potentials involved in the settlement signal transduction mechanism are escalated further. This effect may substantially increase the numbers of action potentials being produced and delivered to synaptic clefts where the signal is transmitted further producing resulting in a variety of biochemical processes. These nerve impulses may trigger the release of neurotransmitters from presynaptic neurons, which travel across the synaptic cleft and bind with receptors on postsynaptic terminals. Depending on the neurotransmitters involved (e.g. serotonin, acetylcholine, L-DOPA, epinephrine), the initiation or inhibition of further action potential production in receiving axons, or signal transductions in neuro-muscular postsynaptic cells, are regulated. This induced signal transmission may result in a variety of biochemical processes (e.g. production of cyclic adenosine monophosphate). This will be discussed in later chapters.

Although settlement induction was observed with after treatment with low levels of excess K^+, settlement was inhibited at higher concentrations. Perhaps the inhibitory action caused by large increases in external K^+ concentration (i.e. 40+ mM) effectively increases the initial level of polarisation of neurons involved in the signalling pathway of settlement. This would cause a decrease in the resting membrane potential and increase the difference between the resting and threshold potentials. Consequently, the threshold potential would be more difficult to achieve by the necessary stimuli. Although membrane hypopolarisation may occur with small increases of extracellular K^+ due to the intracellular build up previously discussed, a large extracellular increase in levels of the positively charged cation would effectively overpower this impact and cause hyperpolarisation. Hence, a disruption in the formation of action potentials would follow by maintaining the neuron membrane in a constant state of depolarisation. The induction of settlement rate limiting concentration of 10–15 mM K^+ could be the concentration range at which the effects of high excess concentrations of external potassium ions (hyperpolarisation) are first initiated thereby reducing the effects of low

concentrations (hypopolarisation). At 20 mM no settlement induction was observed with percent settlement similar to the control (i.e. hyperpolarisation = hypopolarisation effects). At 40+ mM mean settlement inhibition was observed (i.e. hyperpolarisation > hypopolarisation effects).

The concentration at which significant settlement inhibition was observed over the control occurred at 60 mM. However, this concentration is well above that which induced significant mortality (20+ mM) indicating that toxic response is completely separate from settlement transduction mechanisms and that dose dependant K^+ concentration may affect these processes in a different manner.

In summary: (1) exogenously applied K^+ induces larval settlement in *P. canaliculus*. (2) These settlement responses are different to those observed in the blue mussel *M. edulis*. (3) The settlement responses of *P. canaliculus* to K^+ are similar to those observed in the green mussel *P. viridis* at low concentrations, but different at higher concentrations. (4) Chloride ions also may play a role in the induction of larval settlement and contribute an additive induction effect to that of potassium ions when using KCl as a determining treatment. (5) Small increases in external K^+ concentration may cause an increase in action potentials involved in the signalling pathways of settlement by decreasing the difference between the resting and threshold potentials, making these neuronal cells more excitable – the more excitable the cells are, the less stimulus they require (e.g. physical or chemical settlement cue). (6) A large increase in external K^+ may cause hyperpolarisation thereby decreasing sensitivity to settlement cues and inhibiting action potentials involved in the signalling pathway of settlement. (7) The regulation of action potential formation may affect secretion of neurotransmitters (e.g. epinephrine) and other biochemical processes (e.g. production of cyclic adenosine monophosphate) involved in the signalling pathway. (8) Settlement inhibition is not caused by acute toxicity effects, indicating differences in the biochemical pathways of settlement, and those which result in mortality.

CHAPTER 6

EFFECTS OF ACETYLCHOLINE AND ATROPINE ON LARVAL SETTLEMENT (RECEPTOR IDENTIFICATION)

1. INTRODUCTION

This chapter details the investigation of the effects of acetylcholine on larval settlement of *P. canaliculus*. The effect of an acetylcholine receptor antagonist (atropine) on settlement of this mussel also is examined.

Acetylcholine, an ester of acetic acid and choline, is a neurotransmitter in the peripheral nervous system (PNS) and the central nervous system (CNS) of many organisms, including humans (Martinez-Murillo & Rodrigo 1994). Acetylcholine is biosynthesised in neuronal cells from choline and acetyl-CoA by the enzyme choline acetyltransferase (Squire et al. 2003). In the PNS, acetylcholine, released by presynaptic neurons into neuromuscular junctions, has the ability to modulate muscular activities. Depending on the type of acetylcholine receptors involved, binding of acetylcholine to receptors promotes contractions of muscle fibres in some cases (e.g. Muneoka et al. 1991), and inhibits contractions in others (e.g. Yang et al. 1993). In the CNS, acetylcholine often acts as an excitory neurotransmitter (e.g. Belousov et al. 2001). However, acetylcholine also can be inhibitory (e.g. Kelly et al. 1979). The endogenous vesicle release of acetylcholine by presynaptic neurons into synaptic clefts leads to the transmission of a nerve impulse signal from one nerve cell to another (Squire et al. 2003). Crossing the synaptic cleft acetylcholine binds to acetylcholine receptors located in the membranes of other neurons. As an excitory neurotransmitter this transmission stimulates the firing of further nerve impulses, or action potentials, in particular signalling pathways (Lodish 2007). Once the signal has been transmitted the acetylcholine molecule is released by the receptor and is quickly degraded back into choline and acetate by the enzyme acetylcholinesterase (Squire et al. 2003). This process is especially important in neuromuscular junctions because excess acetylcholine in the extracellular spaces can cause muscular paralysis (Sullivan & Kreiger 2001). The primary excitory mode of acetylcholine is through depolarisation of cell membranes. Binding of the ligand to a postsynaptic acetylcholine receptor stimulates an inward cellular

flux of sodium ions and an outward flux of potassium ions (Mathews 2001). However, the inward flux of sodium is greater than the outward flux of potassium, reducing the membrane potential, hence depolarising the cell membrane.

There are two primary classes of acetylcholine receptors, muscarinic receptors (mAChRs) and nicotinic receptors (nAChRs). These receptors were named after the ligands that were discovered to activate them, muscarine (from mushrooms: *Inocybe* spp., and *Clitocybe* spp.) and nicotine (from the nightshade family of plants: Solanaceae) (Decker et al. 1995). Muscarinic acetylcholine receptors are G protein-coupled receptors composed of a single protein, and are indirectly linked with ion channels through G proteins as second messenger molecules (Figure 30). There are five subtypes of mAChRs, the M1, M2, M3, M4, and M5 configurations, all with slightly different protein sequences, with each type stimulating various biochemical functions. For example, activation of the M4 configuration inhibits the enzyme adenylyl cyclase, causing a reduction in levels of cyclic adenosine monophosphate (Brown et al. 1985), whereas the M1 configuration has the ability to activate protein kinase C (Eglen 1997). Nicotinic acetylcholine receptors are ionotropic receptors – ligand-gated ion channels that are permeable to potassium, sodium, calcium, and sometimes chloride ions (Corringer et al. 2000; Bolsover et al. 2003) (Figure 30). Nicotinic acetylcholine receptors are pentameric receptors consisting of five protein subunits (Cooper et al. 1991). There are four subfamilies of nAChRs, three neuronal types, and one muscular type – further divisions exist based on protein compositions, all being involved in different cellular functions (Novere & Changaux 1995; Genetics Home Reference 2009). Activation of nicotinic receptors produces a variety of physiological responses in animals. For example, activation in presynaptic neurons facilitates the release of a number of neurotransmitters, including dopamine, norepinephrine, epinephrine, serotonin, γ-aminobutyric acid, glycine, aspartate, and glutamate – many of which have been implicated in modulating diverse animal behaviours (Yaded et al. 1992; Decker et al. 1995; Lopez et al. 2001; Quarta et al. 2007). Activation of nAChRs also has been demonstrated to mediate the uptake of dopamine (Zhu et al. 2009). Although acetylcholine was the first molecule to be identified as a neurotransmitter in 1921 (see Yusuf 1992), deciphering all of the physiological manifestations connected with

the actions of this compound has been difficult – there are many questions around cholinergic neurotransmission that are unanswered, and many debates that remain unresolved (Gainetdinov & Caron 1999; Wells 2008). Each of the two classes of acetylcholine receptors demonstrates a large amount of heterogeneity, further complicating the allocation of specific physiological functions.

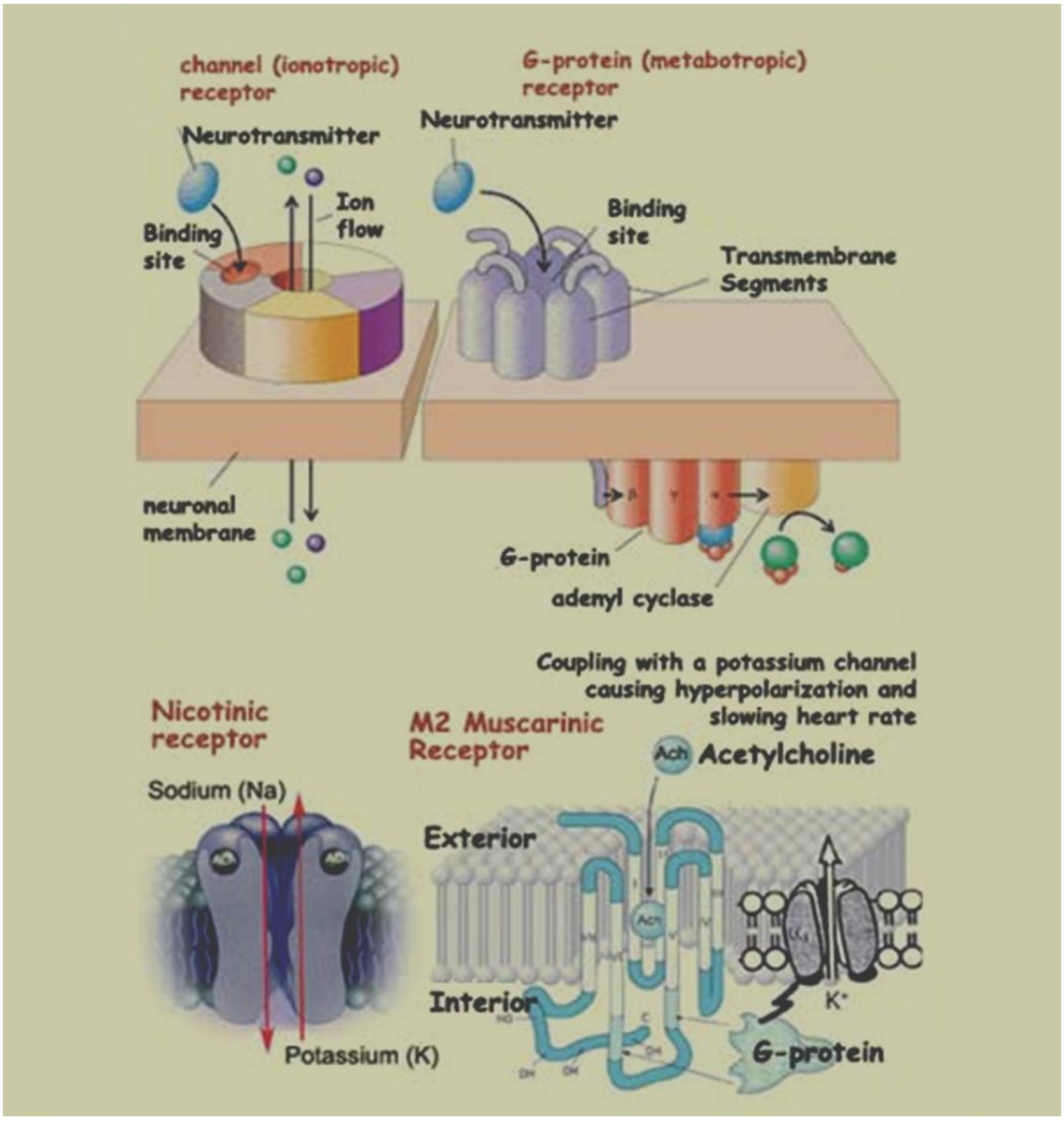

Figure 30. Acetylcholine receptors. Top left: generalised ionotropic receptor. Top right: generalised metabotropic G protein-coupled receptor. Bottom left: nicotinic acetylcholine receptor. Bottom right: muscarinic acetylcholine receptor. Modified from McGill University 2009.

Acetylcholine is involved in the neuroethologies of numerous terrestrial and marine invertebrate species. For example, acetylcholine mediates escape behaviour in flies (*Drosophila* spp.) through nicotinic-type acetylcholine receptors (Fayyazuddin et al. 2006), and also is known to modulate swimming behaviour in the marine mollusc *Clione limacina* (Panchin et al. 1995). Furthermore, acetylcholine has been demonstrated to induce larval settlement and metamorphosis in many marine invertebrate species. For example, acetylcholine induces settlement and/or metamorphosis in the Japanese short-neck clam *Ruditapes philipinarum* (Fusetani 2004; Urrutia et al. 2004), the Pacific oyster *Crassostrea gigas* (Beiras & Widdows 1995), the pearl oyster *Pinctada fucata martensii* (Yu et al. 2008), the goldlip pearl oyster *Pinctada maxima* (Zhao et al. 2003), the blue mussel *Mytilus edulis* (Dobretsov & Qian 2003), the barnacle *Balanus amphitrite* (Faimali et al. 2003), and the ascidian *Ciona intestinalis* (Coniglio et al. 1998). In contrast to the inductive affects of acetylcholine, this neurotransmitter does not induce larval settlement in the Indian oyster *Crassostrea madrasensis* (see Flemming et al. 2009). This indicates that the signalling pathways involved in the larval settlement behaviour of this oyster, compared with *C. gigas*, and other marine invertebrate species, are different. Also, exposure of the bryozoan *Bugula neritina* to acetylcholine actually inhibits larval attachment to substrata (Yu et al. 2007). Furthermore, larval metamorphosis is not induced by acetylcholine treatment in the gastropods *Haliotis rufescens* (Morse et al. 1979) and *H. discus hannai* (Akashige et al. 1981), suggesting differences in the biochemistries of these organisms with other marine invertebrates that can be induced by this compound. Since the acetylcholine precursor choline has been shown to induce larval settlement or metamorphosis in a variety of marine invertebrate species, it has been suggested that this infers an acetylcholine involvement. For example, treatment of larvae with choline induces larval settlement in the nudibranch *Phestilla sibogae* (Hirata & Hadfield 1986), and the marine snail *Ilyanassa obsoleta* (see Pawlik 1990). Larval metamorphosis can also be induced with choline in the nudibranchs *Adalaria proxima* (Todd et al. 1991) and *P. sibogae* (Hadfield 1984), and the polychaete worm *Phragmatopoma lapidosa californica* (Pawlik 1990). However, acetylcholine does not induce metamorphosis in *P. sibogae* or *P. lapidosa californica* (Pawlik 1990). This indicates that the positive effects of choline

are not due to the bioconversion of this compound to the neurotransmitter acetylcholine, but through another mechanism entirely.

In the central nervous system of mammals, nAChRs, located within presynaptic neuronal cell membranes, predominately regulate presynaptic neurotransmitter release, rarely being involved in fast direct synaptic transmission of nerve impulses (Clarke & Reuben 1996; Alkondon et al. 1998; Kaiser & Wonnacott 2000; Reuben & Clarke 2000; Wonnacott et al. 2000; Grady et al. 2001). However, in the molluscan central nervous system, nAChRs 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 nAChRs (Vulfius et al. 1967; Chiarandini & Gerschenfield 1967; Chiarandini et al. 1967; Watchel & Kandel 1971; Chiarandini et al. 1982; Chemeris et al. 1982). It has been suggested that nAChR subtypes in molluscs are comparable in structure to those found in vertebrates, although display a much higher functional complexity (Neirop et al. 2006). In the gastropod mollusc *Lymnaea stagnalis*, it has been suggested that virtually all of the neurons in the CNS respond to acetylcholine through nicotinic-type receptors (Zeimal & Vulfius 1967; Vulfius et al. 1967). This may highlight the importance of these receptors in molluscan neuronal transmissions. However, the extent to which nAChRs are involved in the nervous systems of other molluscan species is unknown. Although a number of studies have been performed investigating the effects of acetylcholine on larval settlements, very few, if any, attempts have been made to distinguish which class or subtype/s of acetylcholine receptors are involved in the biochemical mechanisms of these settlement inductions.

A number of acetylcholine receptor agonists/antagonist compounds exist. Many of these molecules are highly specific, targeting receptor subtypes within the two classes of acetylcholine receptors (see Appendix IV, p294). However, there also are a number of

compounds which are less specific, having the ability to agonise or antagonise the mAChRs or nAChRs. For example, α-bungarotoxin, a neurotoxin from the venom of the elapsid snake *Bungarus multicintus*, selectively and competitively blocks nicotinic type acetylcholine receptors (Young et al. 2003). On the other hand, atropine, a tropane alkaloid from plants of the Solanaceae family (e.g. deadly nightshade and mandrake), is a competitive antagonist of acetylcholine at muscarinic type receptors (Goldfrank et al. 2006). Acetylcholine receptor agonist and antagonist molecules are useful tools for determining which type/s of receptors are involved in specific biochemical mechanisms, and are commonly used for such purposes (e.g. Lee et al. 1972; Verbitsky et al. 2000; Park et al. 2004)

In this chapter, the effect of acetylcholine on *P. canaliculus* larval settlement was determined. Due to the inductive effects observed in those experiments (see Results section, p144), further assays were performed to establish if the settlement response was due to one, or both, of the two classes of acetylcholine receptors. To do this, atropine was used to block the muscarinic type acetylcholine receptors.

2. METHODS

2.1 Organisms

Larvae for these assays were sourced from the Sealord Ltd hatchery facility in Ruakaka only. Organisms were reared and transported according to the methods outlined in Chapter 3, p60. For details of larval source and age for each settlement assay performed, see Appendix II, p285.

2.2 Settlement assays

Settlement assays were conducted using the same methods and environmental parameters outlined in Chapter 3, p62. Briefly, assays were performed at 17±1°C in sterile polystyrene Petri plates under diffuse light with 20–30 larvae per plate (from a larval solution pre-calibrated to 20–30 larvae/ml). Static conditions were maintained, no food provided, and FSW/treatment solutions were well oxygenated immediately prior to initiating assays, but not aerated during the assay. Ten replicates were used for each treatment and control. The number of settled larvae was detected after 24 and/or 48 hours for each Petri plate and using the suction-by-pipette technique.

2.2.1 Treatments

Acetylcholine chloride and atropine sulphate were dissolved in 0.45 μm filtered seawater (FSW), respectively. Stock solutions of each treatment were prepared immediately prior to all settlement assays. Following serial dilution in FSW, treatment

solutions were prepared as 10X concentrates. Controls consisted of 9 ml FSW and 1 ml larval solution. Treatments consisted of 8 ml FSW, 1ml larval solution, and 1 ml treatment solution. Final exposure concentrations for treatments were: acetylcholine; $1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$ M, atropine; $1x10^{-5}$ M.

2.3 Mortality assays

Mortality assays were performed in accordance with the methods outlined for settlement assays. Mortality detection incorporated visual observations of velum, foot, or gut movement and the use of neutral red, a vital stain (for further details see Methods section, Chapter 4, p88). Only mortality assays were performed for acetylcholine.

2.3.1 Treatments

Solutions of acetylcholine were prepared as previously described for settlement assays, using the same concentrations (i.e. $1x10^{-5}$, $1x10^{-4}$, and $1x10^{-3}$ M).

2.4 Statistics

2.4.1 Larval settlement & mortality

Statistical methods used for settlement induction assays and mortality assays were the same as those outlined in the methods section in Chapter 3, p64. Briefly, all percent data were arcsin transformed and analysed using statistical software, Minitab v.15. Where

data satisfied the assumptions of parametric analysis, 1-way ANOVA with Tukey's multiple comparisons test was used. Non-parametric data were analysed using the Kruskal-Wallis test, and multiple comparisons made with a specialised Dunn's macro enabled for multiple comparisons of medians. Actual *p*-values for all comparisons can be found in Appendix I, p277.

2.4.2 Larval toxicology

To determine the toxicity of acetylcholine, various lethal concentration values were calculated from the raw mortality data using the same techniques as those outlined in the methods section in Chapter 4, p91. Briefly, where data fitted the assumptions of the model, a probit analysis was performed using the EPA Probit Analysis Program Used for Calculating LC/EC Values Version 1.5. Where data were not suitable for this model, the LC_{50} value was calculated manually by the Spearman-Karber method.

3. RESULTS

3.1 Larval settlement

3.1.1 Acetylcholine

Exposure of mussel larvae to solutions of acetylcholine for 24 and 48 hours showed settlement inductive effects (Figure 31). A significant difference in larval responses among all treatments was detected after 48 hours exposure (ANOVA; $F_{3,36}$=23.06; p<0.001). Compared to the control assays, significant increases in larval settlements were detected for every acetylcholine concentration tested (Tukey test; p<0.01 in each comparison). The settlement peak maximum occurred after exposure of mussels to $1x10^{-4}$ M acetylcholine solutions. At a higher concentration (i.e. $1x10^{-3}$ M), this induction of settlement was reduced, but was still higher than in the control.

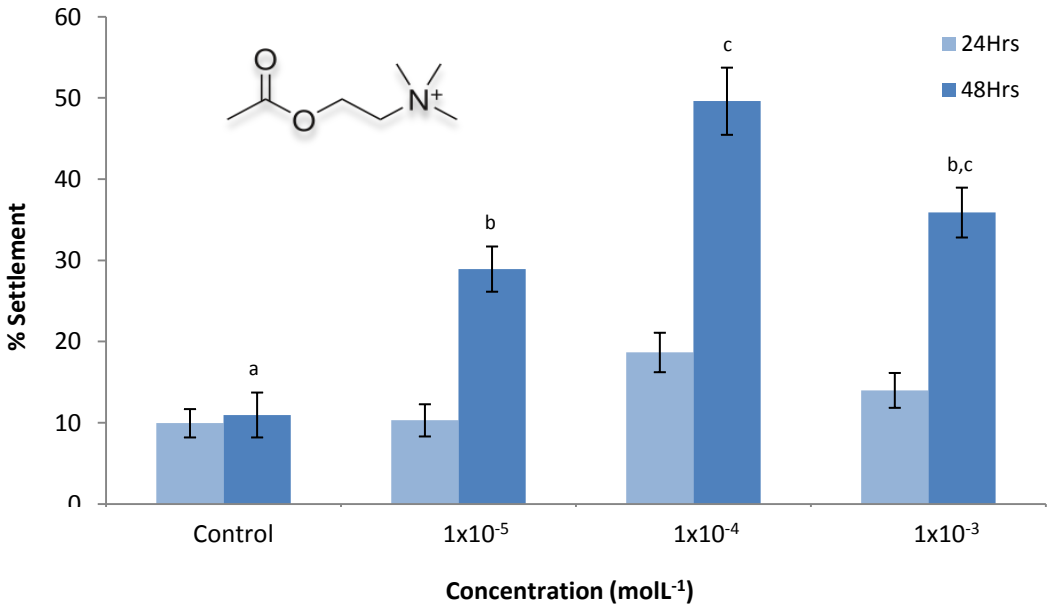

Figure 31. Effect of acetylcholine on larval settlement after 24 and 48 hour exposures. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.2 Acetylcholine & atropine

To determine which of the two classes of acetylcholine receptors (i.e. mAChRs or nAChRs) are involved in the acetylcholine induced settlements, the muscarinic type receptor antagonist atropine was used to competitively block the mAChRs (Figure 32). Treatments for this experiment consisted of: a negative control (FSW), a positive control ($1x10^{-4}$ M acetylcholine solution), atropine ($1x10^{-5}$ M solution), and a combined treatment of acetylcholine and atropine. After exposure of mussel larvae to treatments for 24 and 48 hours, mean percent settlements for all treatments were consistently higher after the longer exposure time. However, the general trends between exposure times were similar. After 48 hours, significant difference in larval settlement was detected among all treatments assayed (ANOVA; $F_{3,35}=26.41$; $p<0.001$).

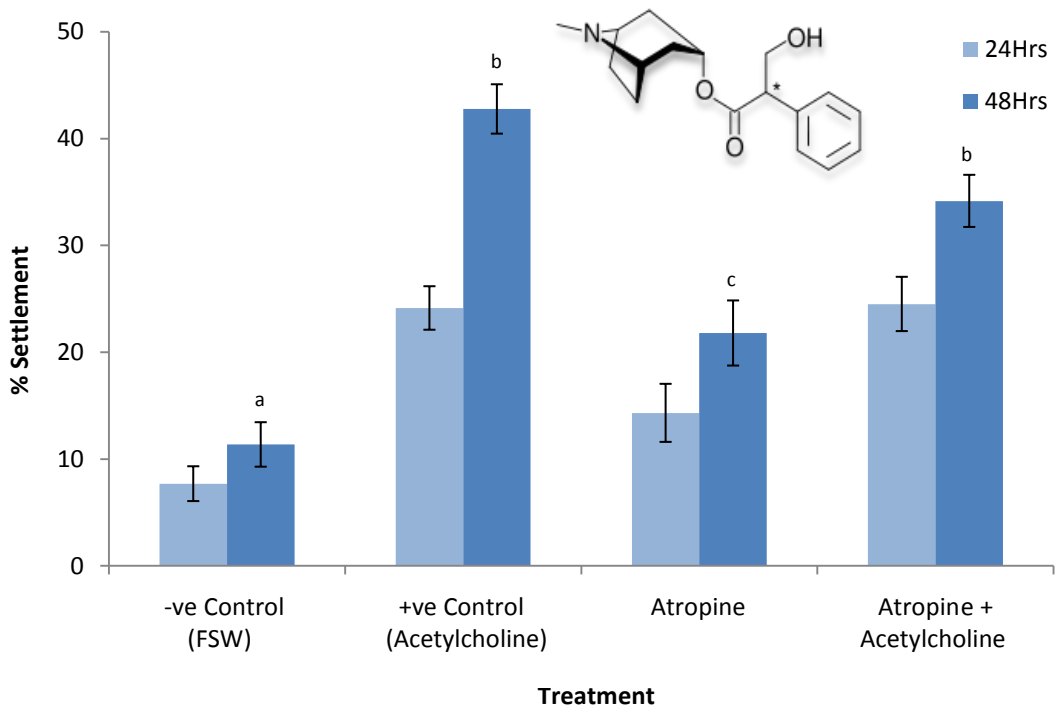

Figure 32. Effect of atropine ($1x10^{-5}$ M) on acetylcholine ($1x10^{-4}$ M) induced larval settlement after 24 and 48 hour exposures. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Exposure of larvae to the positive control treatment ($1x10^{-4}$ M acetylcholine) resulted in a significant increase in larval settlement after 48 hours compared to the control assays (Tukey test; $p<0.001$), substantiating the results gained in the previous experiment. A significant increase in settlement compared to the control was detected after 48 hour exposures to atropine ($1x10^{-5}$ M) (Tukey test: $p<0.05$), with this settlement being significantly lower than that observed after treatment with acetylcholine (Tukey test: $p<0.001$). Exposure of larvae to the combined treatment of acetylcholine and atropine for 48 hours resulted in significantly higher percent settlements than in the control assays (Tukey test: $p<0.001$). No significant difference in larval settlement was detected between the positive control treatment (acetylcholine) and the combined acetylcholine/atropine treatment after 48 hours (Tukey test; $p>0.05$).

3.2 Mortality & toxicology

Due to time constraints and larval availability, only the acute toxicity effects of acetylcholine were determined in mortality assays, and subsequent toxicological calculations for estimating LC_x values.

3.2.1 Acetylcholine

Exposure of mussel larvae to acetylcholine produced a typical dose response curve (Figure 33), with significant difference detected among treatments (ANOVA; $F_{4,45}>50$; $p<0.001$). No significant difference in larval mortalities were detected between the control assays and treatment with acetylcholine at concentrations of $1x10^{-5}$ M and $1x10^{-4}$ M (Tukey test; $p>0.05$ in each comparison). Maximal 100% mortality was observed after exposure to acetylcholine at $1x10^{-2}$ M.

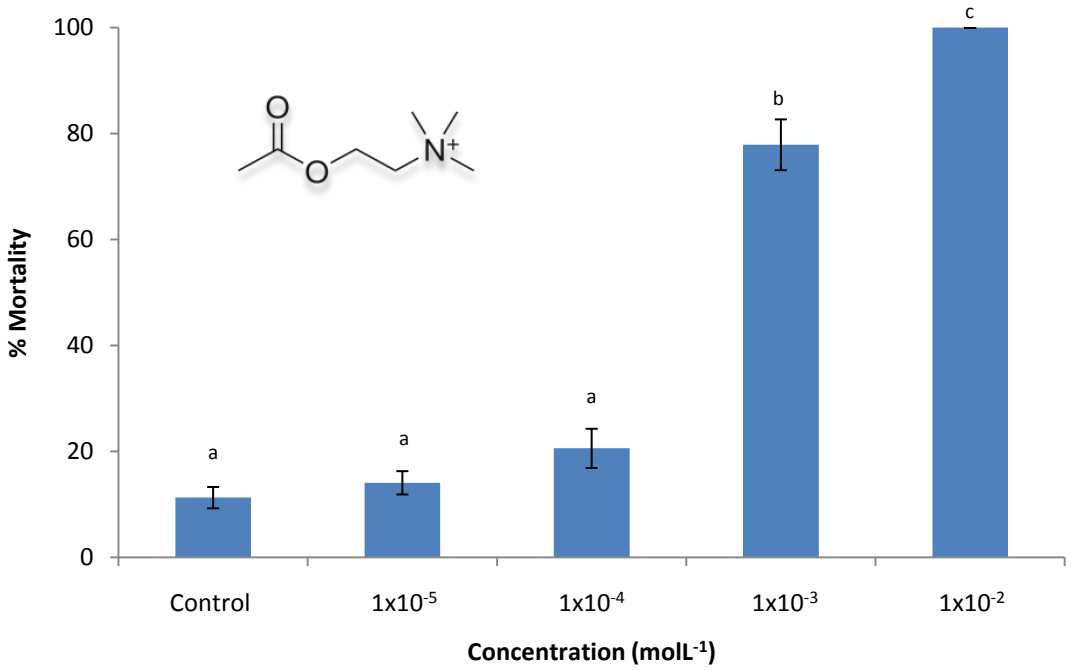

Figure 33. Effect of acetylcholine on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Results of the probit analysis of the raw percent mortality data estimates that the first observable acutely toxic effect of acetylcholine would be apparent after exposure of *P. canaliculus* larvae to a $3.7x10^{-5}$ M solution of acetylcholine (i.e. LC_1 value) (Table 11). This concentration is less than half of the optimal concentration previously determined to induce maximal settlement in this species (i.e. $1x10^{-4}$ M). However, at almost double the optimal concentration for inducing larval settlement, it is estimated that 50% of the population would die after exposure to acetylcholine at $4.7x10^{-4}$ M.

Table 11. Estimated toxicological parameters for acetylcholine: lethal concentration values and their associated 95% confidence intervals (italics).

Treatment	Treatment Levels (n)	Total larvae (n)	LC_1 (mol L^{-1})	LC_{50} (mol L^{-1})	LC_{99} (mol L^{-1})
L-DOPA	3	1024	$3.7x10^{-5}$	$4.7x10^{-4}$	$6.0x10^{-3}$
			$2.6x10^{-5}$ – $4.9x10^{-5}$	*$4.2x10^{-4}$ – $5.2x10^{-4}$*	*$4.9x10^{-3}$ – $7.5x10^{-3}$*

4. DISCUSSION

The neurotransmitter acetylcholine is known to have the ability to induce larval settlements in numerous marine invertebrate species (e.g. Beiras & Widdows 1995; Dobretsov & Qian 2003; Faimali et al. 2003; Zhao et al. 2003; Fusetani 2004; Urrutia et al. 2004; Yu et al. 2008). In this thesis, acetylcholine has now been demonstrated, for the first time, to induce larval settlement in the New Zealand marine mussel, *P. canaliculus*. Exposure of mussel larvae to acetylcholine produced maximal settlement responses at a concentration of $1x10^{-4}$ M, with minimal acute toxicity effects. Treatment of larvae with $1x10^{-3}$ M acetylcholine solution significantly increased settlement rates over the control assay, but was lower than when treated with $1x10^{-4}$ M acetylcholine – this was almost certainly due to the toxicity of this compound.

In order to identify which class of acetylcholine receptors were responsible for the inductive effects of acetylcholine, the muscarinic-class receptor antagonist, atropine, was used to competitively block the mAChRs. Since settlement inhibition was not observed with atropine treatment, this infers, on its own, that the receptor class responsible for acetylcholine induced settlement is of the nicotinic type. Treatment with atropine actually increased larval settlement compared to the control treatment. The increase in the observed settlement response with atropine could have been due to two possible causes. Firstly, it is feasible that by blocking muscarinic-type receptors in the organism, there was an increase in available endogenous acetylcholine to act on the nicotinic-type receptors, thereby inducing settlement. Secondly, it is possible that muscarinic-type receptors regulate (perhaps through inhibition) components of the biochemical pathways involved in larval settlement behaviour. For example, the M4 mAChR subtype has the ability to inhibit the enzyme adenylyl cyclase, thereby decreasing intracellular levels of cyclic adenosine monophosphate (c-AMP) (Brown et al. 1985). It has been suggested that high endogenous levels of c-AMP stimulate larval settlements in many marine invertebrate species, such as the barnacle *Balanus*

amphitrite (Rittschof et al. 1986; Clare et al. 1995; Clare 1996; Holm et al. 2000; Li 2007), the mussel *Mytilus edulis* (Dobretsov & Qian 2003), and the oysters *Pinctada maxima* (Zhao et al. 2003) and *Pinctada fucata martensii* (Yu et al. 2008). Therefore, it is possible that by deactivating the mAChRs with atropine, a decrease in endogenous levels of c-AMP resulted. In this thesis, the role of c-AMP in *P. canaliculus* larval settlement is determined and discussed in Chapter 9. However, it also is possible that antagonisation of the mAChRs by atropine resulted in the stimulation, inhibition, production, or degradation of other molecules that have not currently been identified as important components in the signalling mechanisms of larval settlement for any marine invertebrate species. After 24 hour exposures, the combined treatment of acetylcholine and atropine resulted in a mean percent settlement that was practically identical to that observed when treated with acetylcholine only (i.e. 24.5% *vs*. 24.1% respectively). After 48 hour exposures, larval settlement responses to the combined treatment were not significantly different from those observed after acetylcholine treatment. This supports the suggestion that nAChRs are the receptor class responsible for larval settlement inductions of *P. canaliculus*. Had mAChRs been involved, it would have been expected that atropine would block the inductive action of acetylcholine. However, this was not the case.

Although it has been suggested that nicotinic acetylcholine receptors are predominantly responsible for the fast and direct synaptic transmissions of nerve impulses in molluscan species (e.g. Yeoman et al. 1993; Woodin et al. 2002), the mode of acetylcholine action on *P. canaliculus* larval settlement induction is unclear. Due to the complexities of molluscan nicotinic-type receptors (see Neirop et al. 2006), and since little is known about them compared to those in vertebrates, it would be too speculative to discuss, in detail, the possible modes of action of acetylcholine inductions of larval settlement in this mussel. However, in this thesis, five possible modes of action are outlined, and one possible role of acetylcholine in larval settlement is discussed.

It is possible that exogenously applied acetylcholine imitates endogenous presynaptic release of this neurotransmitter (by making its way into neuron-neuron synapses) and acts on postsynaptic acetylcholine receptors, thereby stimulating nerve impulses. The possible biochemical and behavioural outcomes of such action are inconceivably great, and cannot be commented on. Alternatively, acetylcholine may be acting on presynaptic neurons, stimulating the release of other neurotransmitters, such as dopamine, norepinephrine, epinephrine, serotonin, glycine, aspartate, and glutamate. The first four of which have been demonstrated to be effective inducers of larval settlement in numerous marine invertebrate species (e.g. Coon et al. 1985; Yanamoto et al. 1996; Sumin et al. 2006). It also is possible that the communication of neurons with non-neuronal cells may regulate a diverse range of physiological activities leading to the modulation of larval behaviours. For example, the mode of action of acetylcholine in larval settlement could involve binding of the neurotransmitter to nAChRs at ciliary junctions, causing innervations of velar cilia, leading to modulations in swimming behaviour. Acetylcholine also may act through binding to receptors at neuromuscular junctions. This could potentially modulate exploratory behaviours of substrata through the pedal organ. Lastly, acetylcholine may act at goblet cell junctions leading to the secretion of extracellular mucous, providing adhesive material to assist in the attachment of the larvae to substrata. However, it is just as likely that acetylcholine could modulate such behavioural activities through binding to nAChRs in presynaptic or postsynaptic neurons (as previously mentioned), leading to the release and subsequent effects of other neurotransmitters (e.g. dopamine, epinephrine etc), or stimulation of action potentials further upstream in the signalling pathway.

There are potentially many possible biochemical roles of acetylcholine in larval settlement inductions, such as modulation of velum cilia, pedal organ stimulation, and mucoid secretion. Acetylcholine is known to have an ability to innervate ciliary activity in various organisms (e.g. cilia in mouse trachea; Konig et al. 2009, ciliary bands on perioral arms of sea urchin; Yokota et al. 2002, palate cilia in frogs; Slaughter & Aiello 1982). To complicate matters, acetylcholine is known to increase cilia beat frequency in some cases, and inhibit it in others (Bulbring et al. 1953). Furthermore, current knowledge of the acetylcholine receptors associated with cholinergic ciliary innervations suggests a muscarinic-type involvement

(e.g. Zagoory et al. 2001; Zagoory et al. 2002; Klein et al. 2009). Therefore, this option as a possible role of acetylcholine in *P. canaliculus* larval settlement behaviour is a difficult one to discuss – further research would need to be carried out in order to determine such a role. Regulation of pedal organ stimulation also is difficult to discuss since little, if any, research has been carried out in the literature which may implicate an acetylcholine involvement in substrate exploration. There is however evidence which may support the suggestion that acetylcholine, and associated nAChRs, could be involved in the adhesion of *P. canaliculus* larvae to substrata through mucoid secretions. Goblet cells are specialised mucus secretory cells with a large variety of transmembrane receptors. These receptors control intracellular signalling pathways, which regulate the secretion of mucous material (Laboisse et al. 1996). Although much research has been performed over the years in order to understand the regulation of mucous secretion, very little is known about the chemical transmitters and the intracellular transduction pathways involved in this regulation, and remains to be poorly understood. However, it is at least known that acetylcholine can play an important role in the secretion of mucous-like material in various organisms. In many higher mammals and birds, submucosal glands in nasal, tracheal, and bronchial air ways secrete large quantities of mucus when stimulated by acetylcholine (Widdicombe 1978; Quinton 1979; Ueki et al. 1980; Trout et al. 1998). Also, exogenously applied acetylcholine has been shown to induce the secretion of mucous in rabbit intestinal goblet cells (Specian & Neutra 1980). Furthermore, a specific role of nicotinic-type acetylcholine receptors in mucous secretions has been demonstrated. For example, nAChRs were identified in human small and large bowel mucosal epithelium (Richardson et al. 2001; Richardson et al. 2003), and have been shown to modulate mucus secretion (Labiosse et al. 1996). The activation of nicotinic acetylcholine receptors also leads to mucin synthesis and secretion in other regions of the human gastrointestinal tract (Wu & Cho 2004), and stimulates mucus secretion in lung bronchi (see Clementi et al. 2000). In the frog *Rana pipiens*, the stimulation of nicotinic acetylcholine receptors substantially increases the quantity of mucus produced by palate goblet cells, and also increases the proportion of goblet cells that are secreting the mucus (Slaughter & Aiello 1982). Due to the diversity of mucus secretory cells in the literature, and the organisms from which they come from, it is feasible that the goblet cells responsible for mucoid-like secretions in *P. canaliculus* larvae are

stimulated through nAChRs. Although there is no direct evidence in the literature, or in this thesis, to show that acetylcholine is involved in mucoid-like secretions of larval adhesives, it seems like a promising area for future research.

In this chapter, acetylcholine proved to have a highly inductive ability for increasing larval settlement rates in *P. canaliculus*. Almost a 500% increase in settlement over the control treatment was observed after 48 hours exposure to acetylcholine at a concentration of $1x10^{-4}$ M. Due to this inductive ability, combined with the comparatively low toxicity of this molecule compared to other compounds tested in this thesis, this neurotransmitter may have applications in commercial aquacultural practices. Hatchery production of *P. canaliculus* larvae is limited in New Zealand, partially due to the expense involved. However, there is considerable interest in expanding and developing hatchery production of this mussel species in order to minimise the dependency on natural stocks. At the Sealord Ltd. hatchery in Ruakaka, northland, New Zealand, larvae are cultured in large tanks in a rotational batch process. The larvae are generally removed from the culturing tanks between 24 to 28 days post-fertilisation and placed into 'setting' tanks where ropes are available for the young mussels to attach to. This attachment process may take up to 10 days, depending on the development stage of the larvae. Acetylcholine may have applications for 'setting' the larvae sooner, and faster, than is currently practiced. This may decrease the time between hatchery batch productions of larvae, hence improving economical viability and sustainability of this important industry. Due to this possible commercial application, a provisional patent was applied for, and granted, for the use of acetylcholine in aquaculture (i.e. "Method and Compound for use in Aquaculture"; Young et al. 2008).

In summary: (1) Acetylcholine is an effective inducer of larval settlement in *P. canaliculus*. (2) The effects of atropine on larval settlement provides evidence to suggest that the receptor class involved in the biochemical signalling pathway of the induced settlement behaviour is of the nicotinic type. (3) Muscarinic acetylcholine

receptors may regulate larval settlement, through inhibitory mechanisms. (4) The mode of action of acetylcholine could be through activation of postsynaptic nAChRs leading to firing of nerve impulses, activation of presynaptic nAChRs resulting in release of other neurotransmitters (e.g. dopamine, norepinephrine, epinephrine, serotonin, glycine, aspartate, and glutamate), or the activation of nAChRs on other cells at ciliary, muscular, or goblet cell junctions. (5) The role of acetylcholine in larval settlement inductions could be through innervations of velar cilia (regulating swimming behaviour), stimulation of the pedal organ (modulating substrate exploratory behaviour), or secretion of mucoid-like material to provide an adhesive for attaching to substrata. (6) Acetylcholine may have important commercial applications in the aquaculture of this native New Zealand mussel species.

CHAPTER 7

EFFECT OF EPINEPHRINE AND PRECURSORS ON LARVAL SETTLEMENT

1. INTRODUCTION

This chapter details the investigation of the ability of the catecholamine epinephrine to induce larval settlement in *P. canaliculus*. The biosynthesis precursor amino acids L-DOPA, L-Tyrosine and L-Phenylalanine are also examined.

Epinephrine, also known as adrenalin, is a hormone and neurotransmitter belonging to a class of compounds called catecholamines, along with norepinephrine and dopamine. These molecules are all neuroactive with distinct structures containing a benzene ring, two hydroxyl groups, an intermediary ethyl carbon chain and an amine terminal group. Catecholamines serve important roles in the regulation of various cellular processes and hence behavioural responses in all vertebrate and invertebrate organisms (Squire et al. 2003; Bohlen & Halbach 2006). Catecholamines are biosynthesised from the amino acids L-Phenylalanine and L-Tyrosine (Figure 34). The first step in the synthesis pathway involves the conversion of L-Phenylalanine to L-Tyrosine by the enzyme phenylalanine hydroxylase. Tyrosine hydroxylase then converts L-Tyrosine to the amino acid 3,4-dihydroxy-L-phenylalanine (L-DOPA). L-DOPA is decarboxylated to the first catecholamine, dopamine, by DOPA decarboxylase (aromatic L-amino acid) – the same enzyme involved in the conversion of L-Tryptophan to serotonin. Dopamine, which is readily active in particular neurons, can be further oxidised to norepinephrine by dopamine β-hydroxylase requiring a cofactor, L-Ascorbic acid. The final step in the biosynthesis pathway is the production of epinephrine from methylation of the primary distal amine of norepinephrine by the enzyme phenethanolamine N-methyltransferase (Mason 1984; Squire et al. 2003; Bohlen & Halbach 2006). As a source of L-Phenylalanine and L-Tyrosine for this biosynthesis pathway, apart from dietary, many marine invertebrate species including mussels have been shown to have the ability to uptake free amino acids from the external environment (e.g. Ferguson 1977; Jorgensen 1983; Revital & Yehunda 2000).

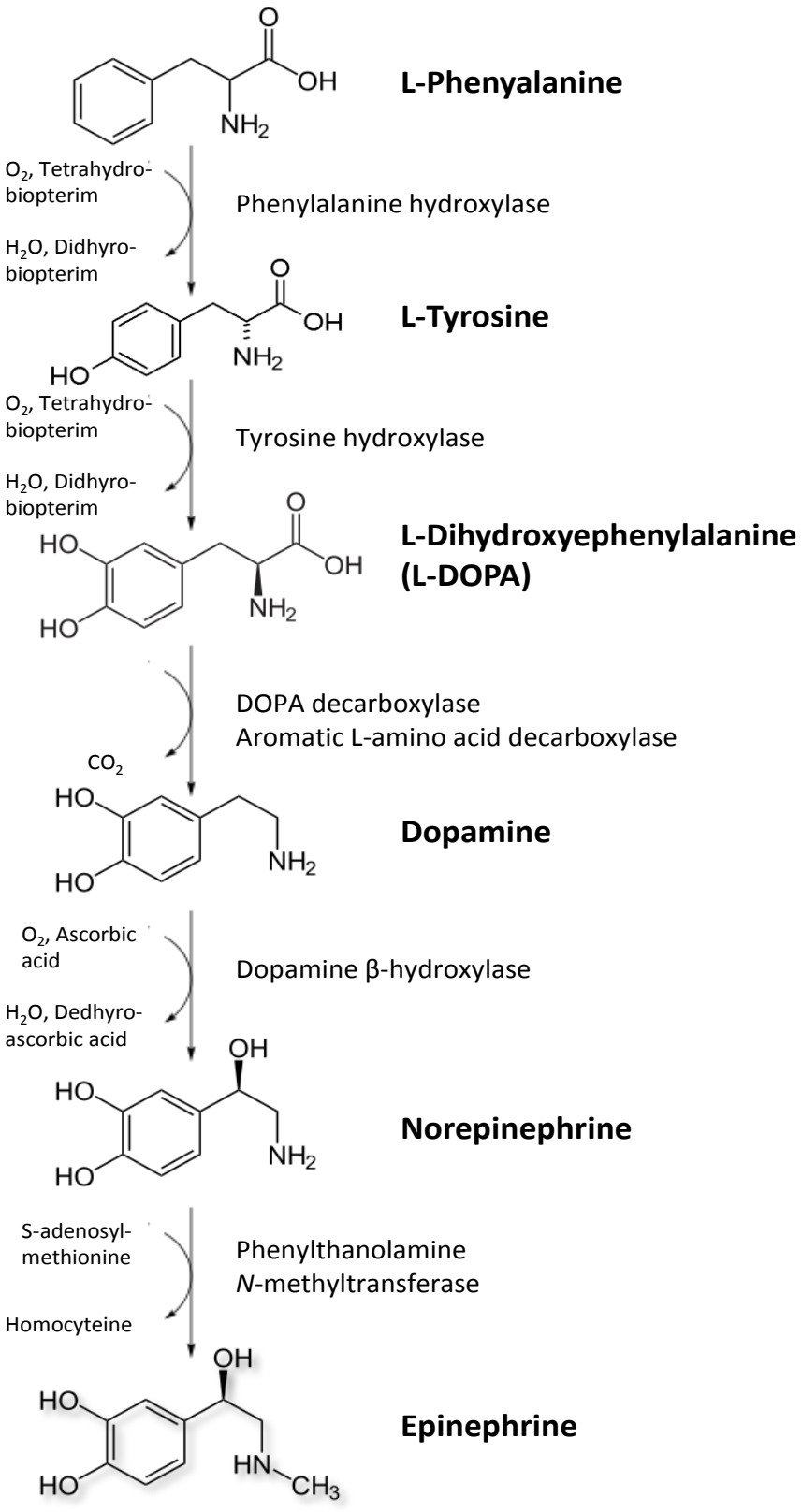

Figure 34. Epinephrine biosynthesis pathway.

The catecholamines norepinephrine and dopamine have been found to be present in appreciable quantities in the larvae and spat of the oyster *Crassostrea gigas* (Coon & Bonar 1986). The amount of these chemicals in the animal change throughout the early development stages, and it is suggested that increases in norepinephrine levels, synthesised from dopamine, plays an important role in mediating metamorphosis (Coon & Bonar 1986). Also, norepinephrine and dopamine levels increase substantially during early development of the nudibranch gastropod *Phestilla sibogae* (Pires et al. 1997). It was similarly suggested that levels of these compounds are involved in the process of metamorphosis for this organism. Increases in endogenous dopamine are likely caused by internal transformations of the precursor amino acids L-Phenylalanine and L-Tyrosine, rather than from an exogenous input of dopamine. Marine invertebrate larvae are known to have the ability to uptake dissolved free amino acids from seawater (e.g. Manahan et al. 1983; Jaeckle & Manahan 1989), therefore providing a mechanism for precursor inputs, and subsequent bioconversion to the neuroactive catecholamines. Pharmacological manipulations of catecholamine biosynthesis had a significant effect on metamorphosis of the nudibranch *P. sibogae* (Pires et al. 2000). By treating larvae of this species with the catecholamine precursor L-DOPA, endogenous levels of dopamine were increased more than two-fold and norepinephrine levels increased 26-fold. In *P. sibogae*, Pires et al. (2000) found that stimulating endogenous dopamine production, by exogenous treatment with L-DOPA, causes an increase in metamorphic responses to a natural chemical inducer (i.e. an aqueous extract of the coral *Porites compressa* of unknown chemical composition). Furthermore, treating *P. sibogae* larvae with α-methyl-DL-*m*-tyrosine (α-MMT), an inhibitor of the catecholamine generative enzyme tyrosine hydroxylase, resulted in a decrease of endogenous catecholamine levels and inhibition of metamorphic responses to the coral extract (Pires et al. 1997). It would appear that endogenous levels of catecholamines modulate metamorphosis in larvae of *P. sibogae*, although they may not be part of a primary signal transduction mechanism. However, catecholamines in the nervous system of this nudibranch may be involved in the gating or enabling of a primary signalling pathway which relies chiefly on external chemical cues (Pires et al. 2000).

Epinephrine has been demonstrated to induce larval settlement and/or metamorphosis in numerous marine invertebrate species. For example, representatives of the Class Bivalvia include: the scallops *Chlamys varia* (Mesias-Gansbiller et al. 2008) and *Pecten maximus* (Pawlik 1990), the oysters *C. gigas* (Coon et al. 1985; McAnally-Salas et al. 1989; Beiras & Widdows 1995) and *Ostrea edulis* (Garcia-Lavandeira et al. 2005), the mussel *Mytilus galloprovincialis* (Garcia-Lavandeira 2005; Satuito et al. 2005; Yang et al. 2008), and the clams *Venerupis pullastra* and *Ruditapes philippinarum* (Garcia-Lavandeira et al. 2005). Sumin et al. (2006) confirmed the results in Garcia-Lavandeira's 2005 study who demonstrated that larvae of the manila clam, *R. philippinarum*, are optimally induced to settle and undergo metamorphosis by exogenous application to epinephrine at $1x10^{-6}$ M. Using differential display reverse transcription PCR, Sumin et al. (2006) also provided evidence that significant alterations in gene expression occurred after exposure to epinephrine. The other two catecholamines, norepinephrine and dopamine, also demonstrate an ability to induce larval settlement or metamorphosis in a variety of marine invertebrate species (e.g., the gastropod *Ilyanassa obsolete*; Levantine & Bonar 1986, the bivalve *C. gigas*; Coon et al. 1985; McAnally-Salas et al. 1989, and the echinoid *Dendraster excentricus*; Burke 1983). In contrast to the positive inducing abilities of the catecholamines previously described, Droudi and Southgate (2002) found that epinephrine and norepinephrine had no significant effect on either larval settlement or crawling behaviour of the tropical pearl oyster (*Pinctada margaritifera*). Norepinephrine also does not induce settlement in the mussel *M. galloprovincialis* (Satuito et al. 1999) or metamorphosis in the nudibranch *P. sibogae* (Hadfield 1984). These dissimilarities in the larval responses to exogenously applied epinephrine treatments suggest that differences in the physiochemical pathways (i.e. neurotransmission and signal transduction mechanisms) of settlement behaviour exist between species.

L-DOPA, a precursor of epinephrine, has the ability to induce metamorphosis in the polychaete *Hydroides ezoensis* (Okamoto et al. 1995, 1998), and larval settlement in the oyster *C. gigas* (Bonar et al. 1985) and the mussel *Mytilus edulis* (Pawlik 1990).

L-DOPA also has previously been inferred by Buchanan (1999) to act as an effective inducer of larval settlement for *P. canaliculus* larvae. However in Buchanan's study, settlement was defined as non-swimming behaviour, which is different from the definition of settlement in this thesis (i.e. firm attachment to a substrate). He also provided evidence that the concentrations assayed were toxic to *P. canaliculus* larvae. Therefore, it is highly likely that the reason why larvae were considered 'settled' was because they were dead, and hence not swimming. This highlights the importance of defining the terms used when describing larval behaviours. Due to Buchanan's results, an opportunity is presented to determine whether L-DOPA does actually have the ability to induce larval settlement (firm attachment to the substratum) in *P. canaliculus*.

The amino acids L-Tyrosine and L-Phenylalanine have been less investigated for their abilities to induce larval settlement in marine invertebrates. However, it has been shown that L-Tyrosine induces settlement in larvae of the oyster *Crassostrea virginica* and the mussel *Mytilus edulis* (Pawlik 1990). L-Tyrosine has been demonstrated to induce larval settlement in the barnacle *Balanus amphitrite*. However, L-Phenylalanine failed to show inductive effects on larvae of this arthropod species (Mishra & Kitamura 2000). This may infer that a mechanism to convert L-Phenylalanine to L-Tyrosine in barnacles is not present – perhaps these organisms do not possess the enzyme phenylalanine hydroxylase. In *Haliotis discus hannai*, treatment with L-Phenylalanine and L-Tyrosine actually inhibits larval settlement (Kang et al. 2003). Since epinephrine can induce settlement in *Haliotis* spp. (e.g. Degnan & Morse 1995) the negative effects of the catecholamine precursors, L-Phenylalanine and L-Tyrosine, may reflect some very different aspects in the neuroethologies of these gastropods compared to bivalves such as *C. virginica* and *M. edulis*. In *Haliotis* spp., perhaps these amino acids are involved in other biosynthesis pathways, resulting in the manufacture of unknown metabolites which are undesirable for the larval settlement process.

Although unrelated to neuronal stimulations and signal transduction mechanisms, a point of interest may be the importance of L-DOPA in the formation of marine adhesive proteins. The byssal plaques in the marine mussels *Mytilus californianus* and *M. edulis* contain a number of adhesive foot proteins, all of which contain modified L-DOPA (Taylor et al. 1994; Suci & Geesey 1995; Baty et al. 1997; Saby & Loung 1998; Vreeland et al. 1998; Zhao et al. 2006). Through oxidation to quinone, L-DOPA is believed to be accountable for the rapid curing ability and water resistant nature of these proteins (reviewed by Silverman & Roberto 2007). Exogenously applied labelled L-tyrosine (L-[*ring*-4-^{13}C]tyrosine and L-[*ring*-d_4]tyrosine) can be incorporated into the proteins of byssus threads and plaques (25% of all tyrosine related structures) of *M. edulis* proving that the amino acid can be taken up from the environment to aid in adhesive secretions (Klug et al. 1996). Although not specifically identifying L-DOPA as a constituent, *in situ* attenuated total reflection-infrared spectroscopy on the byssal plaques of *M. galloprovincialis* and *P. canaliculus* revealed that the compounds secreted are similar to those produced by other *Mytilus* spp. (Gao et al. 2007). The attachment ability of *P. canaliculus* larvae to hydrophobic surfaces is different from that observed in the adults, and although larvae which are just becoming competent to settle have not yet developed the pedal organ which is responsible for the secretion of L-DOPA, it is not possible to rule out the presence of this amino acid in the secreted adhesive (Petrone et al. 2008).

In this chapter, L-Phenylalanine, L-Tyrosine, L-DOPA, and epinephrine were tested for their potential inductive effects on larval settlement in *P. canaliculus*.

2. METHODS

2.1 Organisms

Larvae for these assays were sourced from the Sealord Ltd. hatchery facility in Ruakaka (northern New Zealand) and from the Cawthron Institute (South Island, New Zealand). Organisms were reared and transported according to the methods outlined in Chapter 3, p60. For details of larval source and age for each settlement assay performed, see Appendix II, p285.

2.2 Settlement assays

Settlement assays were conducted using the same methods and environmental parameters outlined in Chapter 3, p62. Briefly, assays were performed in sterile Petri plates with 20–30 larvae per plate. The physical parameters included: 17±1°C, static water conditions, no food provided, and FSW/treatment solutions were well oxygenated at T_0, but not aerated during the assay. Ten replicates were used for each treatment and control. The number of settled larvae was detected after 24 and/or 48 hours for each Petri plate using the suction-by-pipette technique.

2.2.1 Treatments

Epinephrine and L-DOPA were first dissolved with dilute NaOH in FSW at 30°C, adjusted to pH 8.1 with dilute HNO_3, then made up to the desired concentration with filtered seawater (FSW). The amino acids L-Tyrosine and L-Phenylalanine were dissolved in 0.45 μm filtered seawater (FSW) only. Stock solutions of each treatment were prepared immediately prior to all settlement assays. Following serial dilution in FSW, treatment solutions were prepared as 10X concentrates. Controls consisted of 9 ml FSW and 1 ml larval solution. Treatments consisted of 8 ml FSW, 1 ml larval solution, and 1 ml treatment solution. Final exposure concentrations for all treatments were $1x10^{-5}$, $1x10^{-4}$, and $1x10^{-3}$ M.

2.3 Mortality assays

Mortality assays were performed in accordance with the methods outlined for settlement assays. Mortality detection incorporated visual observations of velum, foot, or gut movement and the use of neural red, a vital stain (for further details see methods section in Chapter 4, p88).

2.3.1 Treatments

Treatment solutions of epinephrine, L-DOPA, L-tyrosine, and L-phenylalanine were prepared as previously described for settlement assays and using the same concentrations.

2.4 Statistics

2.4.1 Larval Settlement & mortality

Statistical methods for analysis of settlement induction and mortality data were the same as those outlined in the methods section in Chapter 3, p64. Briefly, all percent data were arcsin transformed and analysed using statistical software, Minitab v.15. Where data satisfied the assumptions of parametric analysis, 1-way ANOVA with Tukey's multiple comparisons test was used. Non-parametric data were analysed using the Kruskal-Wallis test, and multiple comparisons made with a specialised Dunn's macro enabled for multiple comparisons of medians. Actual *p*-values for all comparisons can be found in Appendix I, p277.

2.4.2 Larval Toxicology

To determine the toxicity of treatment compounds, various lethal concentration values were calculated from the raw mortality data using the same techniques as those defined in the methods section in Chapter 4, p91. Briefly, where data fitted the assumptions of the model, a probit analysis was performed using the EPA Probit Analysis Program Used for Calculating LC/EC Values Version 1.5. Where data were not suitable for this model, the LC_{50} value was calculated manually by the Spearman-Karber method. Results of the L-Phenylalanine and L-Tyrosine mortality assays did not fit all of the criteria set for calculation of LC values (i.e. the control assay must result in ≤10% mortality, at least one treatment level must result in <80% mortality, and at least on treatment level must result in 100% mortality, see Chapter 4, p91). Therefore, lethal concentration values were only determined for epinephrine and L-DOPA treatments.

3. RESULTS

3.1 Larval settlement

3.1.1 Epinephrine

Epinephrine was found to be an effective inducer at $1x10^{-5}$ M after 24 and 48 hour exposures, however failed to induce at higher concentrations (Figure 35). After 48 hours, significant differences in settlement were detected among treatments (ANOVA; $F_{3,36}>50$; $p<0.001$). All treatments were significantly different from one another (Tukey test; $p<0.001$ in each comparison), except between the control and $1X10^{-4}$ M (Tukey test; $p>0.05$). Epinephrine showed a negative and inhibitive effect at $1X10^{-3}$ M.

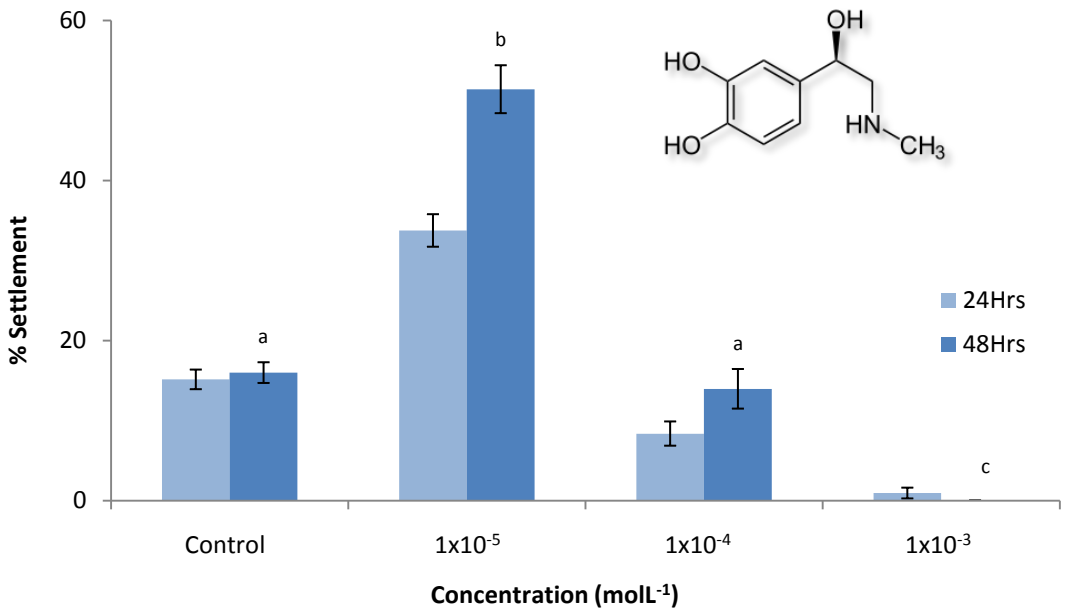

Figure 35. Effect of epinephrine on larval settlement after 24 and 48 hours incubations. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences in 48 hour exposure data (Tukey test).

3.1.2 L-DOPA

The larval settlement response after 48 hours exposure to the amino acid L-DOPA, a precursor of epinephrine, reveals a very similar trend to that of epinephrine over all concentrations tested (Figure 36). Analysis with 1-way ANOVA revealed significant differences among treatments (ANOVA; $F_{3,36}>50$; $p<0.001$). At a lower concentration of $1x10^{-5}$ M, significant induction of settlement was observed (Tukey test; $p<0.001$). An inhibitive effect resulted from exposure to $1x10^{-3}$ M with 0% settlement being detected.

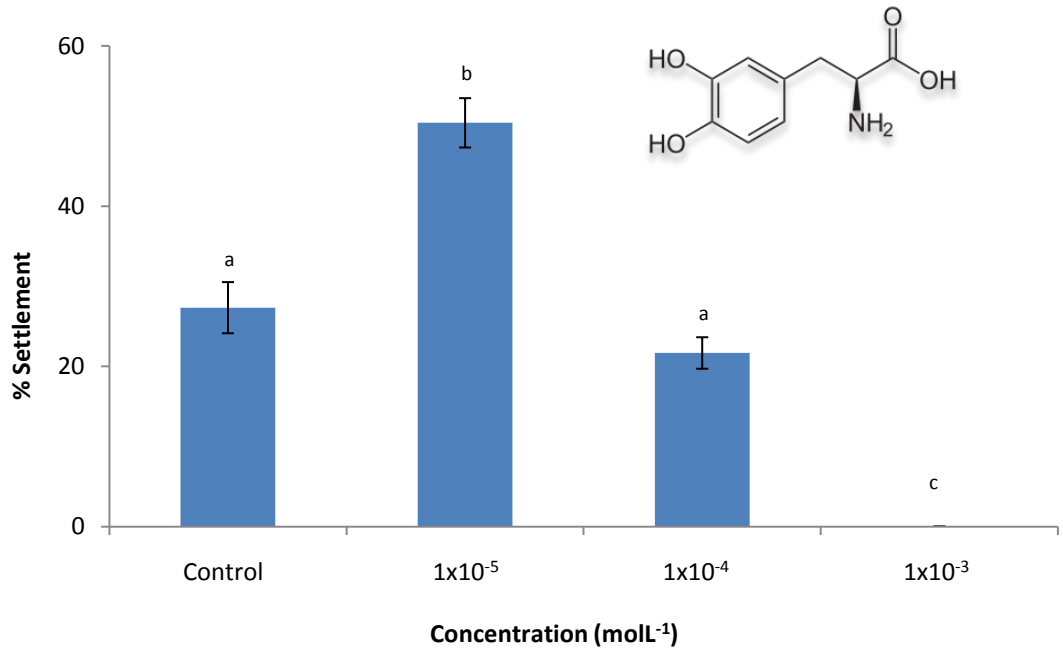

Figure 36. Effect of L-DOPA on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.3 L-Tyrosine

L-Tyrosine also demonstrated an ability to induce larval settlement in *P. canaliculus* after 48 hours exposure (Figure 37). Although significant differences in settlement were detected among treatments (ANOVA; $F_{3,36}>50$; $p<0.001$), results revealed a different trend in induction capabilities of L-Tyrosine over the three concentrations assayed. At the lower concentration of $1x10^{-5}$ M, L-Tyrosine induced approximately 50-60% settlement, similar with the L-DOPA and epinephrine treatments. However, at higher concentrations of $1x10^{-4}$M and $1x10^{-3}$ M, settlement was increased to around 80%. No inhibitive effects were observed. All concentrations assayed were significantly different from the control (Tukey test; $p<0.001$ in each comparison). However, no differences were observed between the two highest concentrations assayed (Tukey test; $p>0.05$).

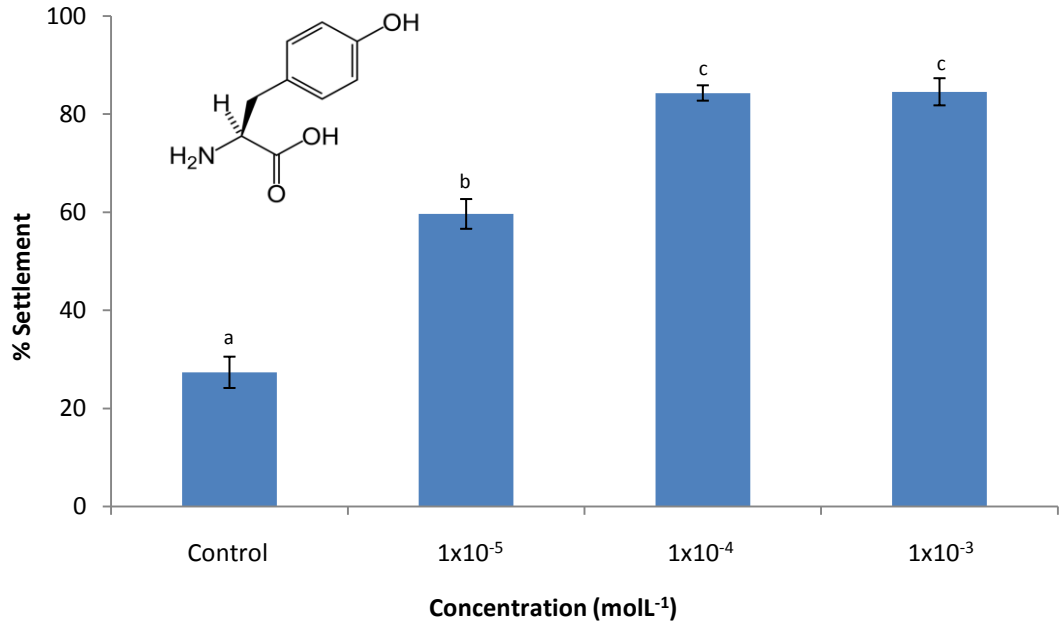

Figure 37. Effect of L-Tyrosine on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.4 L-Phenylalanine

L-Phenylalanine also demonstrated a high capacity to induce larval settlement in *P. canaliculus* after 48 hours exposure (Figure 38). Results clearly show considerable inductive effects with all treatment levels producing over 80% larval settlement and all being significantly different from the control assay (Tukey test; $p<0.001$ in each comparison)

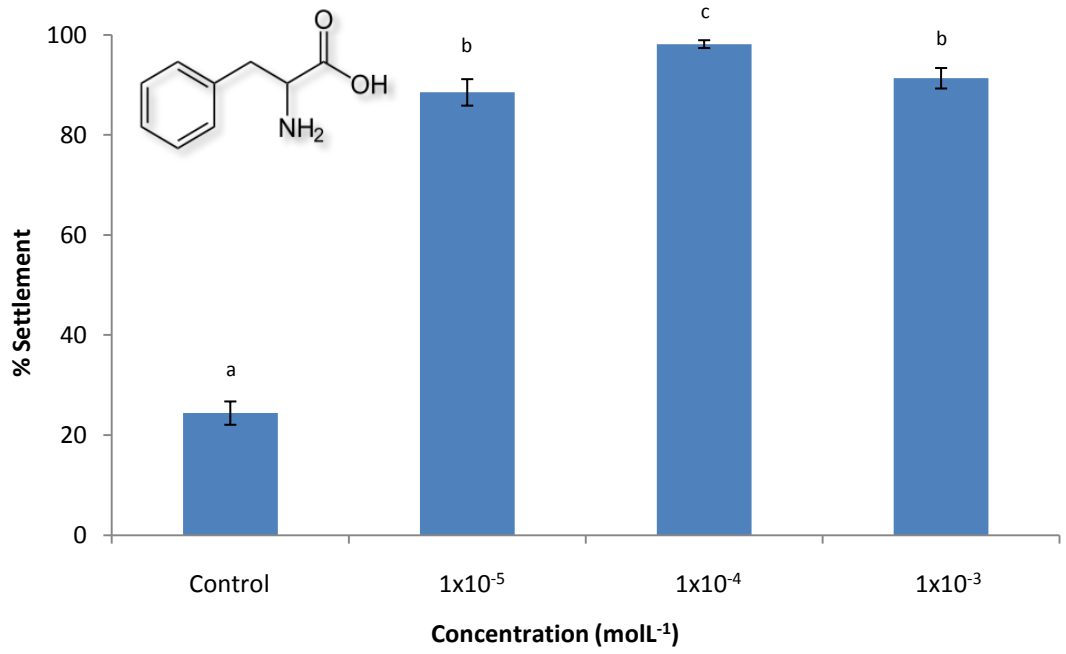

Figure 38. Effect of L-Phenylalanine on larval settlement after 48 hours incubation. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.2 Mortality & toxicology

3.2.1 Epinephrine

Exposure to epinephrine produced toxic effects at $1x10^{-4}$ M (Figure 39). No significant difference in mortality was detected between the control and at the lowest concentration assayed, $1x10^{-5}$ M (Tukey test; $p>0.05$). Maximal 100% mortality was observed after exposure to epinephrine at $1x10^{-3}$ M.

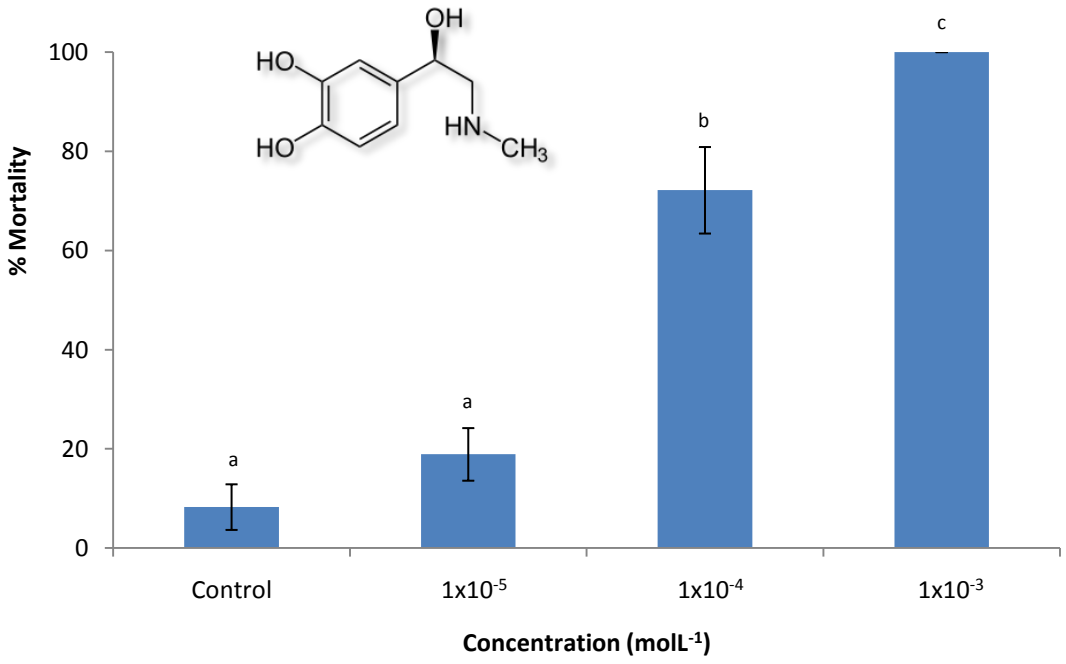

Figure 39. Effect of epinephrine on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

For calculating estimated lethal concentration values, the mortality data failed to satisfy all of the assumptions of the probit model so the Spearman-Karber method was used to determine the LC_{50} value and associated 95% confidence intervals (Table 12). Since the Spearman-Karber method can only estimate the concentration which kills 50% of the population, results of the probit model also are represented. However, confidence intervals for these estimates could not be computed and values should be interpreted with caution.

Table 12. Estimated toxicological parameters for epinephrine: lethal concentration values.

Treatment	Treatment levels (n)	Total larvae (n)	LC_1 (mol L^{-1})	LC_{50} (mol L^{-1})	LC_{99} (mol L^{-1})
Epinephrine	3	961	-	$5.1x10^{-5}$ [a] *$4.2x10^{-5}$ – $6.1x10^{-5}$*	-
			$3.0x10^{-6}$ [b]	$5.0x10^{-5}$ [b]	$8.3x10^{-4}$ [b]

[a] Spearman-Karber estimate with 95% CI below (italics)
[b] EPA probit analysis estimates

3.2.2 L-DOPA

Exposure to L-DOPA for 48 hours revealed a similar trend as epinephrine over the three concentrations assayed (Figure 40). However, significant difference in percent mortality between the control assay was first observed at a lower concentration of $1x10^{-5}$ M (Tukey test; $p<0.05$). This indicates a higher degree of toxicity for L-DOPA. Results of the lethal concentration probit analysis also show L-DOPA is more toxic than epinephrine, with a lower LC_{50} value (Table 13).

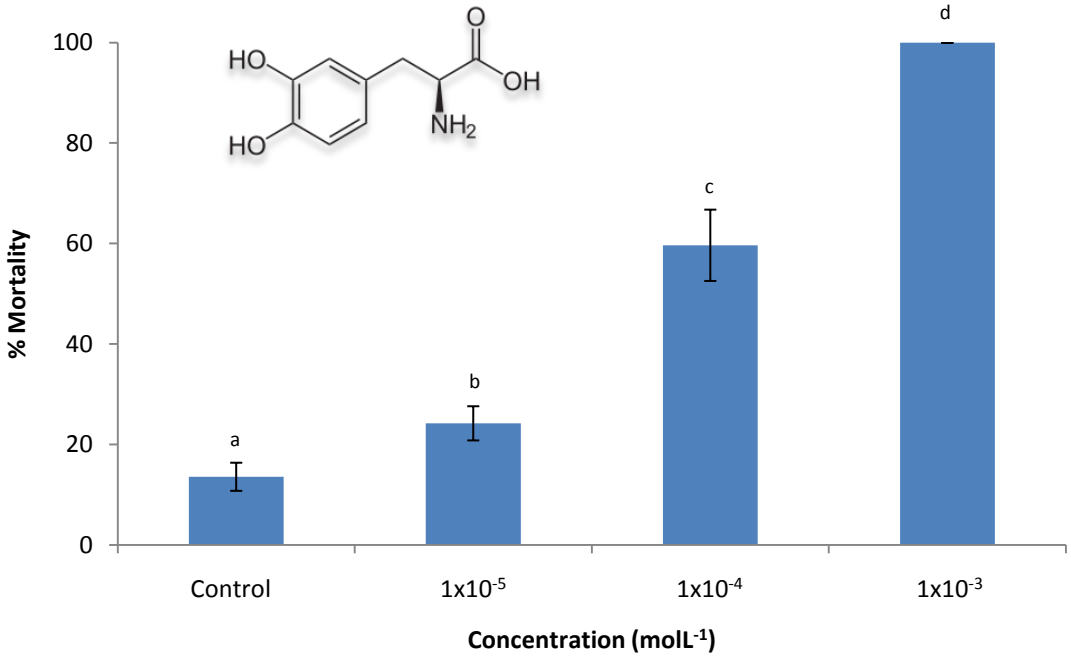

Figure 40. Effect of L-DOPA on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

Table 13. Estimated toxicological parameters for L-DOPA: lethal concentration values and their associated 95% confidence intervals (italics).

Treatment	Treatment Levels (n)	Total larvae (n)	LC_1 (mol L^{-1})	LC_{50} (mol L^{-1})	LC_{99} (mol L^{-1})
L-DOPA	3	1024	$3.1x10^{-6}$	$3.3x10^{-5}$	$3.6x10^{-4}$
			$2.2x10^{-6} – 4.0x10^{-6}$	*$3.0x10^{-5} – 3.7x10^{-5}$*	*$3.0x10^{-4} – 4.4x10^{-4}$*

3.2.3 L-Tyrosine

Exposure to L-Tyrosine resulted in very high levels of mortality at all concentrations assayed (Figure 41). Significant difference in mortality was detected between the control assays and exposure to L-Tyrosine at a concentration of $1x10^{-5}$ M (Tukey test; $p<0.001$). Maximal 100% mortality occurred at a treatment concentration of $1x10^{-4}$ M. Lethal concentration values were not computed since mortality in the control was over 10% and no treatment level resulted in mortality rates under 80%.

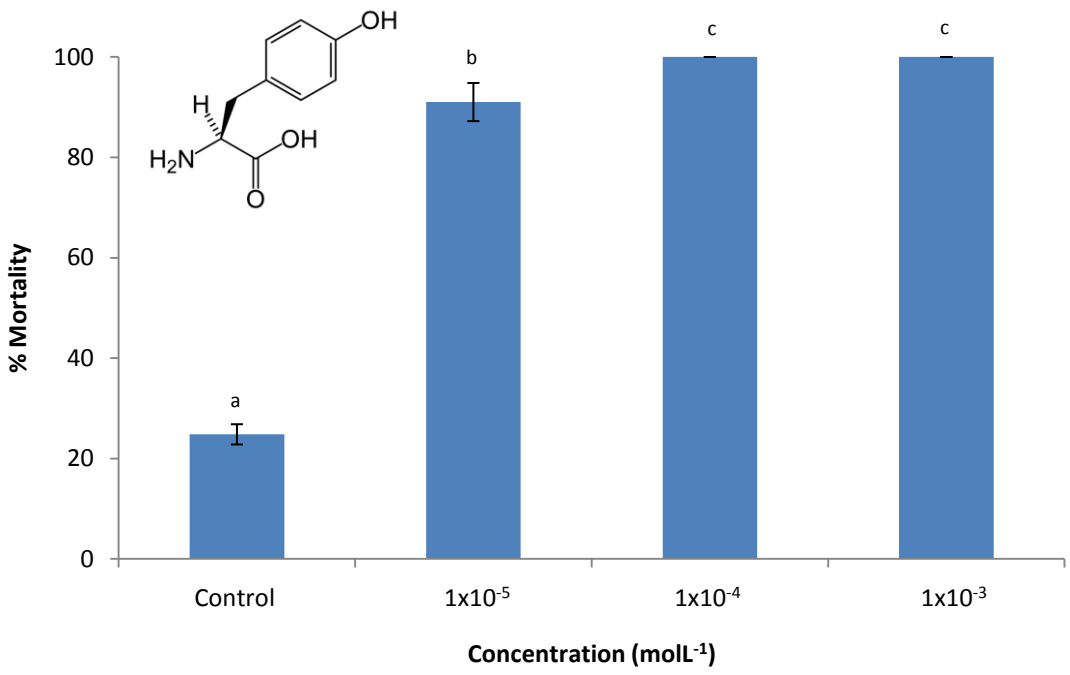

Figure 41. Effect of L-Tyrosine on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.2.4 L-Phenylalanine

Exposure to L-Phenylalanine also resulted in very high levels of mortality at all concentrations assayed (Figure 42), similar to the results of L-Tyrosine exposure. Significant difference in mortality was detected between the control assay at a concentration of $1x10^{-5}$ M (Tukey test; $p<0.001$). However, the mean percent mortality for L-Phenylalanine at this concentration was slightly lower than that observed after exposure to L-Tyrosine (84 vs. 91% respectively). Lethal concentration values were similarly not computed since mortality in the control was over 10% and no treatment level resulted in mortality rates under 80%.

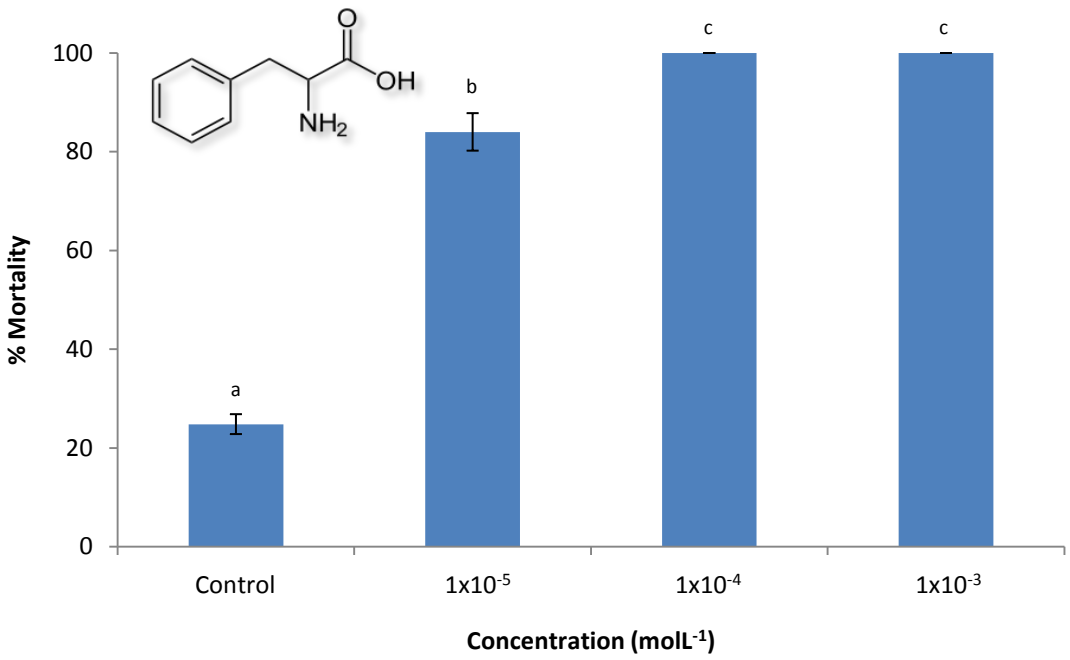

Figure 42. Effect of L-Phenylalanine on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

4. DISCUSSION

Epinephrine, L-DOPA, L-Tyrosine, and L-Phenylalanine all demonstrate significant abilities to induce larval settlement in *P. canaliculus*. Epinephrine and L-DOPA produced inductive responses after 48 hours exposure to $1x10^{-5}$ M. However, they failed to induce at higher concentrations. Comparisons between the inducing abilities of these compounds cannot be made since the larvae assayed were sourced from different hatchery facilities, which have previously been shown to affect settlement responses (see Chapter 3, p69). It was noticed that treatment solutions of epinephrine and L-DOPA changed colour within 2 hours after T_0. This indicates that breakdown products were being formed through oxidation. Although light conditions during the assay were kept low, photo-oxidation or other oxidation processes were evidently still occurring. Most of the literature examining the effects of epinephrine and L-DOPA on larval settlement, using the same concentrations assayed in this thesis, does not mention oxidative breakdown products. However, it seems a very worthy point to note. If these compounds are producing other compounds how can their inductive abilities be solely attributed to their native forms? This will be investigated and discussed in the next chapter.

Regardless of oxidation effects, some interesting suggestions can still be inferred about the inducing abilities of epinephrine and L-DOPA. Whatever the compounds are that have the ability to encourage settlement, comparisons between other studies can be made since the conditions in which those assays were performed are the same as those employed in the current study. Firstly, it can be concluded that L-DOPA does induce larval settlement in *P. canaliculus* after 48 hours exposure when exogenously applied. Buchanan (1999) suggested that L-DOPA induced larval settlement in *P. canaliculus*. In his study, high percent settlement was claimed since larvae ceased swimming, rather than detection of attachment to the substrate. However, the treatments were demonstrated to be acutely toxic at the concentrations assayed which would

undoubtedly cause the larvae to cease being mobile. This would suggest that Buchanan's claim was mistaken. However, based on results in the current study, L-DOPA was indeed found to induce larval settlement (i.e. firm attachment to the substratum) in *P. canaliculus*. Therefore, this is the first time in which L-DOPA has been reported to induce actual settlement in mussels of the genus *Perna*.

Toxic effects were apparent at the L-DOPA concentration which induced settlement, $1x10^{-5}$ M. This suggests that L-DOPA (or its oxidation products) was detrimentally affecting internal cellular processes. Therefore, L-DOPA could be inducing settlement by direct action on the central nervous system. The induction of metamorphosis in oysters by L-DOPA is delayed when compared to the induction caused by a natural inducer of metamorphosis (i.e. bacterial exudates of *Alteromonas colwelliana* and *Vibrio cholerae*). This observation may support the suggestion that L-DOPA acts internally. Perhaps the delay in response is due to the time it takes for L-DOPA to pass through epidermal tissue into the central nervous system or to intermediary sites in the peripheral nervous system.

For epinephrine, significant toxic effects were not observed except for concentrations at $1x10^{-4}$ M and $1x10^{-3}$ M, which leaves limited interpretations. Epinephrine (or oxidation products) could be acting on epidermal chemoreceptors or on internal receptors. Epinephrine is known to have the ability to cross the blood-brain barrier in vertebrates (Bohlen & Halbach 2006), indicating an affinity to easily pass through cell membranes such as those in the epidermis of larvae. Exogenous application of epinephrine can induce metamorphosis in *R. philippinarum* and exert changes in gene expression (Sumin et al. 2006). Nonetheless, this does not provide evidence of internal action since it is possible that binding of epinephrine (or oxidation products) to external chemoreceptors could lead to endogenous release of other metamorphosis stimulating compounds. The positive settlement inducing effects of epinephrine in *P. canaliculus* do, however, reveal a response similarity with the marine mussel *M. galloprovincialis*, indicating related signal transduction mechanisms of settlement between the two genera.

In summary for L-DOPA and epinephrine: (1) exogenously applied L-DOPA is an inducer of larval settlement in *P. canaliculus.* (2) Perhaps the site of L-DOPA action is internal. (3) Exogenously applied epinephrine induces larval settlement in *P. canaliculus*. (4) Similarities exist in the control of larval settlement between *Mytilus* sp. and *Perna* sp. (5) Oxidative products are formed quickly in solutions of L-DOPA and epinephrine, which may affect the settlement response observed. This last point suggests the need to assay a comprehensive selection of treatment compounds when investigating larval settlement in response to L-DOPA or any of the three catecholamine neurotransmitters. Experimental design must be carefully considered to provide meaningful results which can be interpreted with a degree of certainty. This point also questions the findings of many studies in the literature which investigate catecholamine induction and the subsequent discussions and suggestions that are made.

L-Phenylalanine and L-Tyrosine resulted in very high larval settlement responses compared to L-DOPA and epinephrine. Perhaps one reason for this could be due to them being natural externally active inducers which bind to epithelial transmembrane chemoreceptors. These molecules could be present in seawater as dissolved free amino acids, or associated with algal or biofilm surfaces. It may be interesting to note that the chemotactic response in the unicellular marine alga *Dunaliella tertiolecta* is induced with L-Phenylalanine, L-Tyrosine, and L-Tryptophan – the same amino acids which have shown an ability to induce larval settlement in *P. canaliculus*. These are also the only three amino acids out of all others which induce chemotaxis in this alga (Sjoblad et al. 1978). Coincidently, this organism also is attracted to ammonium ions, which have long been known as inducers of settlement and metamorphosis in numerous marine invertebrate larvae (e.g. Berking 1988; Coon et al. 1990; Siefker et al. 2000). Therefore, *D. tertiolecta* apparently possesses chemoreceptors that can bind all four of these molecules. Although unicellular algae are genetically very far removed from bivalves, this does not necessarily mean that particular chemoreceptor proteins are as well. Hypothetically, perhaps some marine invertebrates retained this trait from a common ancestor or they developed it through convergent evolution. Despite this

possibility, other explanations exist which may be more likely. Perhaps the actions of these amino acids are due to the internal transportation into the nervous system and subsequent conversion to the neuroactive catecholamines. However, textbook neuroscience detailing the biochemistry of higher organisms would dismiss this notion. Traditional concepts reveal that dopamine, norepinephrine, and epinephrine are not capable of crossing the blood-brain barrier in vertebrates, whereas L-Phenylalanine, L-Tyrosine and L-DOPA can cross this barrier. In vertebrates, L-DOPA is converted to dopamine by the enzyme DOPA decarboxylase which is not a rate limiting enzyme, and higher concentrations of L-DOPA enhances synthesis of catecholamines (Masserano & Weiner 1984; Bohlen & Halbach 2006). It is however known that tyrosine hydroxylase is the rate limiting enzyme of vertebrates in the generation of the catecholamines in both the central and peripheral nervous system. Since it operates at maximal catalytic capacity, an increase in the concentration of L-Tyrosine does not lead to a greater production of L-DOPA or the three catecholamines (Bohlen & Halbach 2006). However, recent evidence in invertebrates reveals that treatment of organisms with L-Tyrosine can lead to an increase in endogenous levels of catecholamines. For example, injected L-Tyrosine increases dopamine levels in the mussel *M. edulis*. Furthermore, exogenously applied L-Tyrosine to pedal ganglia preparations causes a significant increase in dopamine levels (Zhu et al. 2005). Therefore, this indicates that tyrosine hydroxylase is not a rate limiting enzyme responsible for the biosynthesis of catecholamines and may not operate at maximal capacity in marine invertebrates. This leads to the question: what is the rate limiting component of the catecholamine biosynthesis pathway in marine invertebrates? Perhaps it could be levels of L-Phenylalanine or L-Tyrosine available in the nervous system. Supporting this suggestion, Pires et al. (2000) found that treatment of *P. sibogae* larvae with L-DOPA caused an increase in the numbers of cells containing catecholamines. These cells already contained levels of the enzyme tyrosine hydroxylase which indicates that levels of the enzyme are not critical for synthesising catecholamines but the substrate L-Tyrosine is. If these simple amino acids are natural inducers of larval settlement through entry into the nervous system, and endogenous catecholamine biosynthesis regulates settlement and metamorphosis, then it makes sense that tyrosine hydroxylase

is not rate limiting for these organisms. This is an interesting model which should be investigated further.

Linked to the last discussion, as a newly discovered bioconversion mechanism of these two amino acids, is the possibility of production of endogenous morphine. If exogenously applied L-Phenylalanine and L-Tyrosine are being converted in the nervous system to dopamine by tyrosine hydroxylase, then there is a high chance that dopamine is further converted to morphine rather than, or as well as, being used as a precursor for synthesis of the other two catecholamine neurotransmitters, norepinephrine and epinephrine. It has long been thought that morphine production is limited to plants. However, due to the similarities in the synthesis steps and having L-DOPA and dopamine as precursors, it has also been suggested that this pathway in animals has been conserved through evolution and they too can biosynthesise this neuroactive alkaloid *de novo*. This hypothesis is now proven (reviewed by Kream & Stefano 2006; Stefano et al. 2008). Furthermore, recent findings by Zhu et al. (2005) have demonstrated the presence of morphine biosynthesis in the mussel *M. edulis*. An injection of this organism with L-Tyrosine increases endogenous levels of morphine. Exogenous application of L-Tyrosine to excised pedal ganglia also increases endogenous morphine levels. Zhu et al. (2005) also provides evidence that morphine and dopamine synthesis are coupled, and inhibition of the catecholamine biosynthesis mechanisms leads to greater synthesis of morphine. The activity of morphine in *M. edulis* has been demonstrated to inhibit cilia movements in gill tissues (Mantione et al. 2006). Therefore, this also leads to the possibility that endogenous morphine levels may regulate velar ciliary activity in mussel larvae, hence partly controlling larval settlement behaviour. The involvement of morphine in the signal transduction mechanisms of settlement in marine invertebrates has so far been wholly unexplored. A significant opportunity is therefore presented to determine these effects. Perhaps in doing so, the mystery of the role of catecholamines and their precursors in settlement and metamorphosis may be further unravelled providing greater insights into these vital processes in marine invertebrate larvae. It is suggested that morphine biosynthesis and

its function in larval settlement is investigated comprehensively – this could lead to groundbreaking discoveries of the neuroethology in these organisms.

A fourth possibility exists for the high levels of settlement induction observed after exposure to L-Phenylalanine and L-Tyrosine. Perhaps these amino acids are being converted to thyroid hormones such as thyroxine. L-Tyrosine is important in the biosynthesis of these molecules and it is well known that thyroid hormones are critical regulators of metabolism in all vertebrates (Hulbert 2000; Yen 2001; Valverde et al. 2004; Heyland & Moroz 2005). For example, thyroid hormones are important for governing metamorphosis in amphibians (Yaoita & Brown 1990; Shi et al. 1996) and fish (Manzon & Youson 1997; Manzon et al. 2001). They also have been shown to orchestrate morphological and behavioural transformations associated with habitat selection in larvae of the marine fish *Hexagrammos agrammus* (Matsumoto & Tanaka 1996). Furthermore, Ogasawara et al. (1996a,b) have demonstrated the presence of thyroid hormones in marine invertebrate ascidians by providing evidence for the gene expression of a primary enzyme involved in the biosynthesis pathway. In a number of echinoderm species, thyroid hormones also have been established to play a key role in the early larval development of these organisms (e.g. Heyland & Hodin 2004; Heyland et al. 2004; Heyland et al. 2006). Additional evidence which may support the suggestion that thyroid hormones may regulate larval settlement in *P. canaliculus* is the observed induction of metamorphosis in the gastropod molluscs *Haliotis discus discus* and *Haliotis gigantea* (Fukezawa et al. 2001). Similar to the possible morphine induction, the involvement of thyroid hormones in larval settlement in marine molluscs also have never been explored, which may provide a niche for future research in this area. Existence of such involvement also may bridge gaps between the roles of L-Phenylalanine and L-Tyrosine in catecholamine biosynthesis and hormone induced behaviour modulations.

In summary for L-Phenylalanine and L-Tyrosine: (1) these simple amino acids are active inducers of settlement in *P. canaliculus* larvae. (2) Although the mode of action

was not investigated, it is suggested that L-Phenylalanine and L-Tyrosine may act on external chemoreceptors, or internally by the endogenous conversion to catecholamines, morphine, or thyroid hormones. (3) For marine invertebrates such as *P. canaliculus*, the enzyme tyrosine hydroxylase may not be a rate limiting factor in the bioconversion of L-Tyrosine to L-DOPA, as in their vertebrate counterparts. (4) It is evident that further research must be conducted to determine the mode/s of action which may result in a significant increase in the knowledge of external chemoreceptor systems and endogenous signalling pathways of marine invertebrate larval settlement.

The large mortality differences observed between L-Phenylalanine/L-Tyrosine and L-DOPA/epinephrine may be explained by the fact that the latter underwent oxidation to form other products. This would indicate that these breakdown compounds were not as toxic as the simple amino acids. Perhaps L-DOPA and epinephrine are endogenously toxic at the concentrations assayed. However, because a substantial decrease in the concentrations of the native forms resulted from oxidation, this toxicity was not realised. If L-DOPA and epinephrine do produce toxic effects on various internal cellular processes at the concentrations assayed, perhaps the high degree of mortality observed from exposure to L-Phenylalanine and L-Tyrosine was due to the endogenous bioconversion of these amino acids to these derivative molecules. This again highlights the importance of determining what the real triggers are that induce larval settlement when using L-DOPA, or any of the three catecholamines as exogenous treatments. This problematic cause for error which is mostly not discussed in the literature forms the basis for the next chapter.

CHAPTER 8

EFFECTS OF CATECHOLAMINE OXIDATION PRODUCT AND ANTIOXIDANT ON LARVAL SETTLEMENT

1. INTRODUCTION

This chapter investigates the effect of hydrogen peroxide, a by-product of L-DOPA and epinephrine oxidation, and the antioxidants ascorbic acid and sodium metabisulphite.

As discussed in the previous chapter, treatment solutions of L-DOPA and epinephrine were observed to change colour within two hours after T_0, forming black/brown or red/brown solutions respectively. This colour change is indicative of oxidation products being formed. Therefore, how can the inductive effects of L-DOPA and epinephrine be solely contributed to the native forms of these compounds? This question challenges some of the implications suggested by authors in the literature that use L-DOPA and epinephrine, or other catecholamines, as treatments for larval settlement assays.

Epinephrine undergoes photo-oxidation, and auto-oxidation in basic conditions, to form the adrenochrome breakdown product. During this process, oxygen is reduced to hydrogen peroxide (Bonevski et al. 1978; Jahnke & Frenkel 1978; Bindoli et al. 1999; NIIRB 2004). Further oxidation can lead to the production of melanins (Cordell et al. 2003). Hydrogen peroxide, being an oxidant itself, may further the oxidation of epinephrine. The settlement experiments trialled with epinephrine in this thesis were designed to control photo-oxidation effects by decreasing light levels. However, seawater is slightly basic, being readily maintained at around pH 8.4, and alterations of seawater pH is not advisable since even small deviations in pH are known to adversely affect marine invertebrate larvae (e.g. Clark et al. 2009). Therefore, it is impossible to control oxidation of an epinephrine treatment solution unless an antioxidant additive is incorporated to minimise this effect. Similarly, L-DOPA is oxidised in air and in slightly basic conditions to form melanins, which can undergo further oxidation to produce hydrogen peroxide as a by-product (Carlberg 1990; Stroomer et al. 1990;

Korytowski et al. 1997). Therefore, the effect of the hydrogen peroxide breakdown product is investigated in this thesis to determine the influence of L-DOPA and epinephrine oxidation in settlement assay treatment solutions. This investigation should provide evidence as to whether the inductive ability of L-DOPA and epinephrine are caused by their native forms, or by degradation into other compounds.

In order to minimise the oxidation of L-DOPA and epinephrine, antioxidant additives could be incorporated into treatment solutions. There is a wide range of antioxidant molecules that could be used. For medical use, epinephrine is commonly added to epidurally administered drugs and has been shown to prolong the duration, and improve the quality, of both epidural local anesthetic block and epidural opioid analgesia (Kern & Bernards 1997). However, oxidation of epinephrine is a major concern. The epinephrine concentration most frequently used in epidurals is $2.3x10^{-5}$ M, more than double the concentration found to induce larval settlement in *P. canaliculus* ($1x10^{-5}$ M). At this concentration, the addition of sodium metabisulphite ($Na_2S_2O_5$), between 2.7–8.3 mM, reduces oxidation of epinephrine and produces a much more stable solution (Kern & Bernards 1997; NIIRB 2004). This suggests that $Na_2S_2O_5$ may be an effective antioxidant to use for settlement assays involving epinephrine treatment solutions. In medicine, endogenous oxidation by the enzyme catechol-O-methyl transferase (COMT) also is problematic. Although sodium metabisulphite does limit this type of oxidation somewhat, Kern & Bernards (1997) found that by adding ascorbic acid (commonly known as vitamin C) to epinephrine solutions, oxidation is further reduced significantly. For ascorbic acid to work, it must be present at much higher concentrations than epinephrine. At pH 7.4, an effective concentration which will limit oxidation of a $2.3x10^{-5}$ M epinephrine solution is 1 mM.

In biological systems, L-DOPA also undergoes oxidation by enzymes, and forms melanin oligimers through the reactive intermediate dopaquinone. The enzyme responsible for this conversion is tyrosinase. It has been shown that rat striatal neurons

exposed to L-DOPA and epinephrine (from $3x10^{-6}$ M to $3x10^{-5}$ M) result in dose-dependent neurocytoxicity. This cell death can be stopped by treatment with ascorbic acid at $2x10^{-5}$ M, indicating that the toxicity observed is caused by oxidative breakdown products (Cheng et al. 1996). In another study, L-DOPA was demonstrated to be toxic to dopamine and non-dopamine neurons in cultures from rat mesencephalon. The damage of non-dopamine neurons was partly due to auto-oxidation of L-DOPA to quinones whereas the damage to dopamine neurons took place before rises in quinones were detected. Ascorbic acid and sodium metabisulphite prevented quinone formation as well as cell death of non-dopamine neurons (Pardo et al. 1995). In industrial processes for the production of L-DOPA, sodium metabisulphite is also commonly added to prevent L-DOPA auto-oxidation of product solutions (Fiechter et al. 2000). Therefore, these antioxidants partly inhibit oxidation of endogenous L-DOPA, but more importantly inhibit oxidation of aqueous solutions. In this chapter, ascorbic acid and sodium metabisuplphite are investigated for their effect on larval settlement to determine if they would be suitable antioxidant candidates to reduce oxidative breakdowns of L-DOPA and epinephrine in settlement treatment solutions. Potassium metabisulphite may have been another antioxidant contender, however, since excess potassium ions have previously demonstrated an ability to induce larval settlement, this compound was not considered (see Chapter 4).

2. METHODS

2.1 Organisms

Larvae for these assays were sourced only from the Cawthron Institute (South Island, New Zealand). Organisms were reared and transported according to the methods outlined in Chapter 3, p60. For details of larval source and age for each settlement assay performed, see Appendix II, 285.

2.2 Settlement assays

Settlement assays were conducted using the same methods and environmental parameters outlined in Chapter 3, p62. Briefly, assays were performed at 17±1°C in sterile polystyrene Petri plates under diffuse light with 20–30 larvae per plate (from a larval solution pre-calibrated to 20–30 larvae/ml). Static conditions were maintained, no food provided, and FSW/treatment solutions were well oxygenated at T_0 but not aerated during the assay. Ten replicates were used for each treatment and control. The number of settled larvae was detected after 48 hours for each Petri plate using the suction-by-pipette technique.

2.2.1 Treatments

Ascorbic acid and sodium metabisulphite were dissolved in 0.45 µm filtered seawater (FSW). A hydrogen peroxide stock solution was first prepared by dilution of 30% H_2O_2. Stock solutions of each treatment were prepared immediately prior to all settlement

assays. Following serial dilution in FSW, treatment solutions were prepared as 10X concentrates.

Exposure to exogenously applied potassium metabisulphite previously produced toxic responses at 5+ mM (see Chapter 4). Therefore, a lower 1 mM solution of $Na_2S_2O_5$ was incorporated into the concentration range selected. In support of this concentration having some effective antioxidising power, Kern & Bernards (1997) showed that sodium metabisulphite at 2.7 mM was successful at significantly reducing oxidation of $2.3x10^{-5}$ M epinephrine solutions, and since the effective concentration of epinephrine found to induce *P. canaliculus* larval settlement was less than half of this value, a 1 mM solution of $Na_2S_2O_5$ also would likely reduce oxidation in settlement treatment solutions. The concentration of ascorbic acid which reduces oxidation of a $2.3x10^{-5}$ M solution of epinephrine is 1 mM at pH 7.4 (Kern & Bernards 1997). However, since the breakdown of epinephrine is significantly amplified in slightly basic conditions, such as seawater (pH 8.4), this ascorbic acid concentration was increased to a minimum of 5 mM for settlement assays in this chapter.

Controls consisted of 9 ml FSW and 1 ml larval solution. Treatments consisted of 8 ml FSW, 1 ml larval solution, and 1ml treatment solution. Final exposure concentrations of treatments were: H_2O_2; 400 and 800 ppm, ascorbic acid; 5, 10, 15 and 20 mM, sodium metabisulphite; 1, 5, 10, 15, and 20 mM.

2.3 Mortality assays

Mortality assays were performed in accordance with the methods outlined for settlement assays. Mortality detection incorporated visual observations of velum, foot, or gut movement and the use of neutral red, a vital stain (for further details see methods section in Chapter 4, p88).

2.3.1 Treatments

Treatment solutions of hydrogen peroxide, ascorbic acid and sodium metabisulphite were prepared as previously described for settlement assays and using the same concentrations. Only the maximum concentration which resulted in 100% mortality is represented on the mortality graphs in the results section.

2.4 Statistics

2.4.1 Larval settlement & mortality

Statistical methods used for settlement induction assays and mortality assays were the same as those outlined in the methods section in Chapter 3, p64. Briefly, all percent data were arcsin transformed and analysed using statistical software, Minitab v.15. Where data satisfied the assumptions of parametric analysis, 1-way ANOVA with Tukey's multiple comparisons test was used. Non-parametric data were analysed using the Kruskal-Wallis test, and multiple comparisons made with a specialised Dunn's macro enabled for multiple comparisons of medians. Actual *p*-values for all comparisons can be found in Appendix I, p277.

2.4.2 Larval toxicology

Lethal concentration values were not calculated since the acute toxicity data obtained from the mortality assays were not suitable for any of the three treatments i.e. controls resulted in >10% mortality and/or exposure to all treatment levels resulted in >80% mortality and/or no treatment level resulted in 100% mortality.

3. RESULTS

3.1 Larval settlement

3.1.1 Hydrogen peroxide

Treatment with hydrogen peroxide, a product of epinephrine and L-DOPA oxidation, induced larval settlement (Figure 43) with significant differences detected among treatments (ANOVA; $F_{2,27}$=4.80; p<0.05). After 48 hours exposure to 400 ppm H_2O_2 solution, a significant increase in settlement was detected over the control assay (Tukey test; p<0.05). However although mean settlement was higher than the control assay, at a treatment concentration of 800 ppm, no significant difference in settlement was detected between the control (Tukey test; p>0.05).

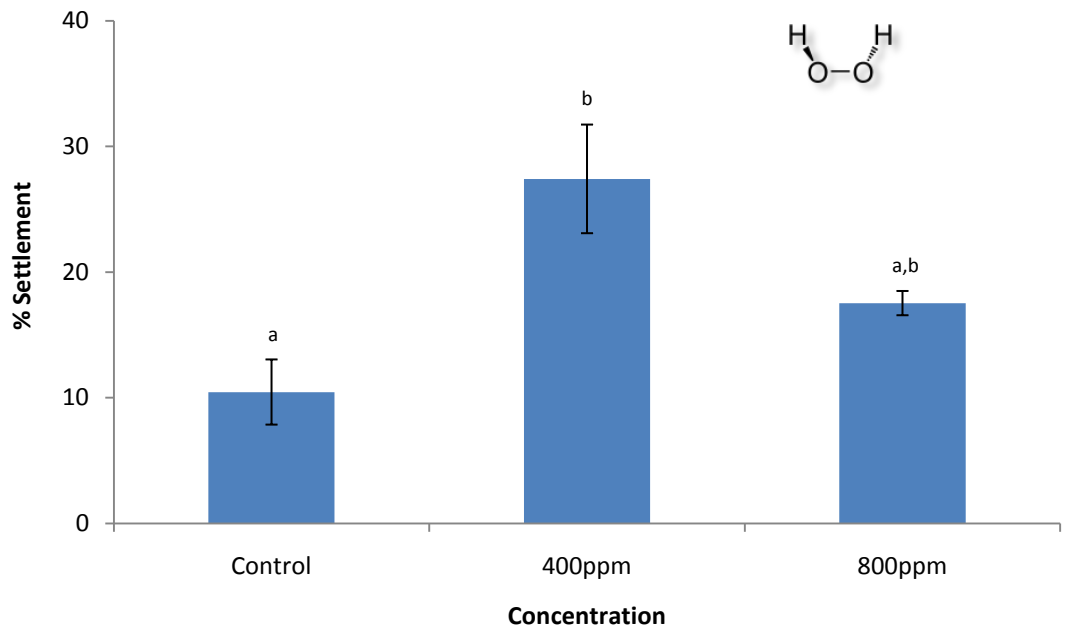

Figure 43. Effect of hydrogen peroxide on larval settlement after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.2 Ascorbic acid

To determine if ascorbic acid may be a suitable antioxidant to incorporate into epinephrine and L-DOPA treatment solutions to minimise oxidation, this vitamin was tested for any inadvertent settlement inducing ability (Figure 44). Analysis with 1-way ANOVA revealed significant differences among treatments (ANOVA; $F_{3,36}=11.71$; $p<0.001$). At all concentrations assayed, a significant increase in settlement over the control assay was detected (Tukey test; $p<0.05$ in each comparison), with an induction peak maximum of around 50% settlement occurring at 5 mM.

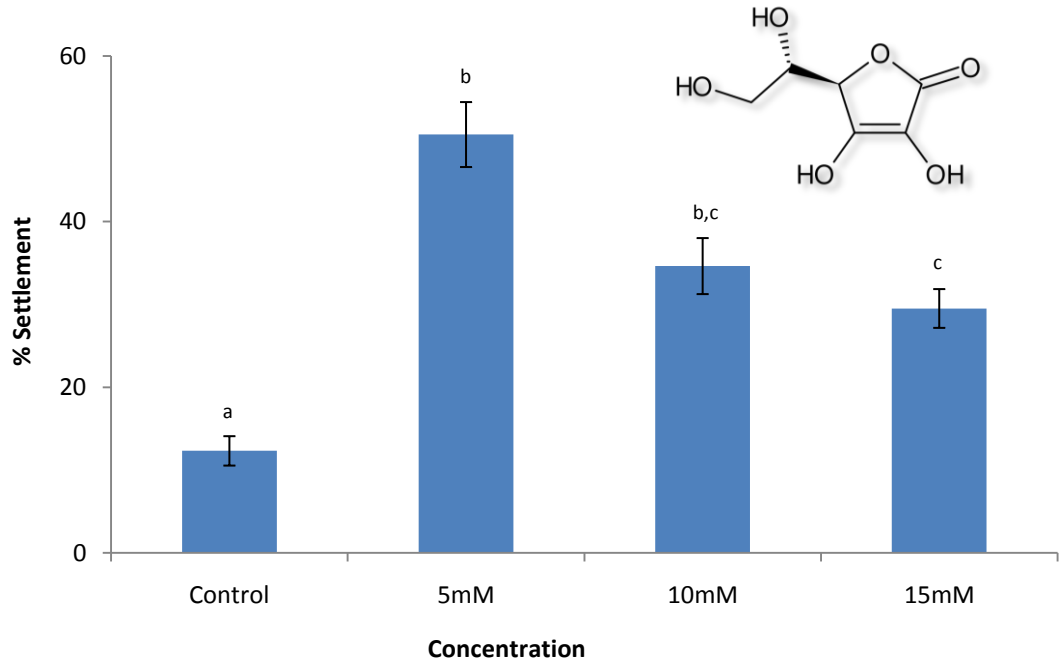

Figure 44. Effect of ascorbic acid on larval settlement after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.3 Sodium metabisulphite

Similarly, to determine if sodium metabisulphite may be a suitable antioxidant to incorporate into epinephrine and L-DOPA treatment solutions, this compound was tested for any inadvertent settlement inducing ability (Figure 45). Significant differences in larval settlement was detected among treatments (ANOVA; $F_{5,54}$=19.77; p<0.001). At 1 mM, a suitable concentration to minimise oxidation, neither induction nor inhibition was observed. However, at 5 mM a significant increase in settlement was detected compared to the control assay (Tukey test; p<0.001). Higher concentrations resulted in significant settlement inhibition over the control assay (Tukey test; p<0.05 in each comparison).

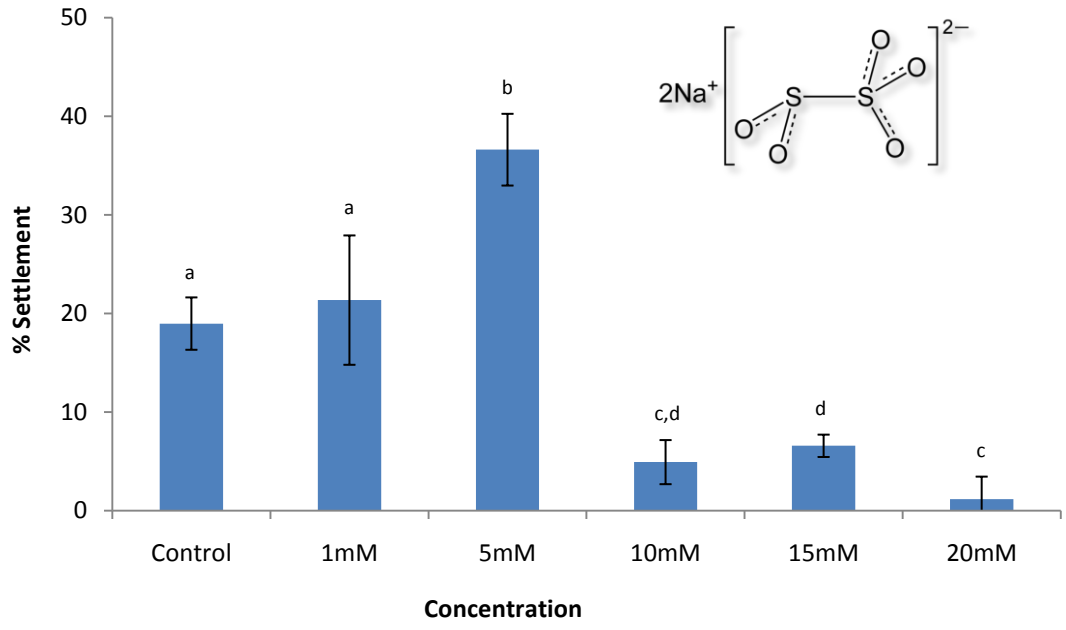

Figure 45. Effect of sodium metabisulphite on larval settlement after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.2 Mortality & toxicology

3.2.1 Hydrogen peroxide

Exposure of larvae to hydrogen peroxide revealed no difference in larval mortality among treatments (ANOVA; $F_{2,27}$=1.89; p>0.05). At a concentration of 800 ppm, in an increase in mean mortality was observed compared to the control, indicating toxic effects were apparent (Figure 46). However, due to variations in the data, this acute toxicity was not significant.

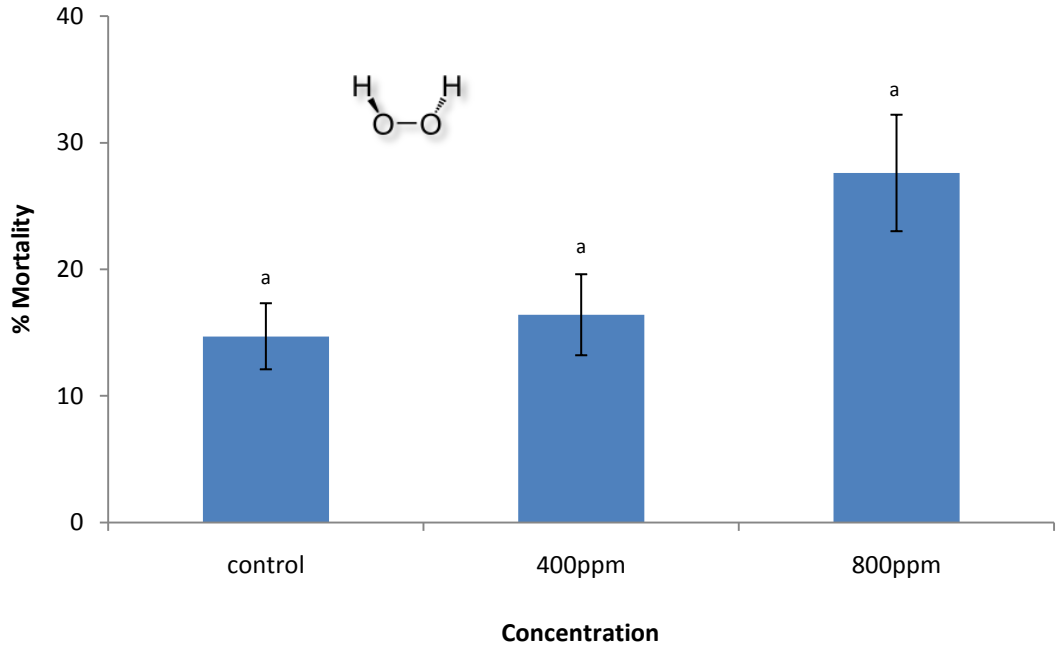

Figure 46. Effect of hydrogen peroxide on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.2.2 Ascorbic acid

After 48 hours exposure of larvae to ascorbic acid, very high levels of mortality were observed (Figure 47). At the lowest concentration assayed (5 mM), almost 100% of larvae died. No significant difference in settlement was detected between the two treatment levels (Tukey test; $p>0.05$)

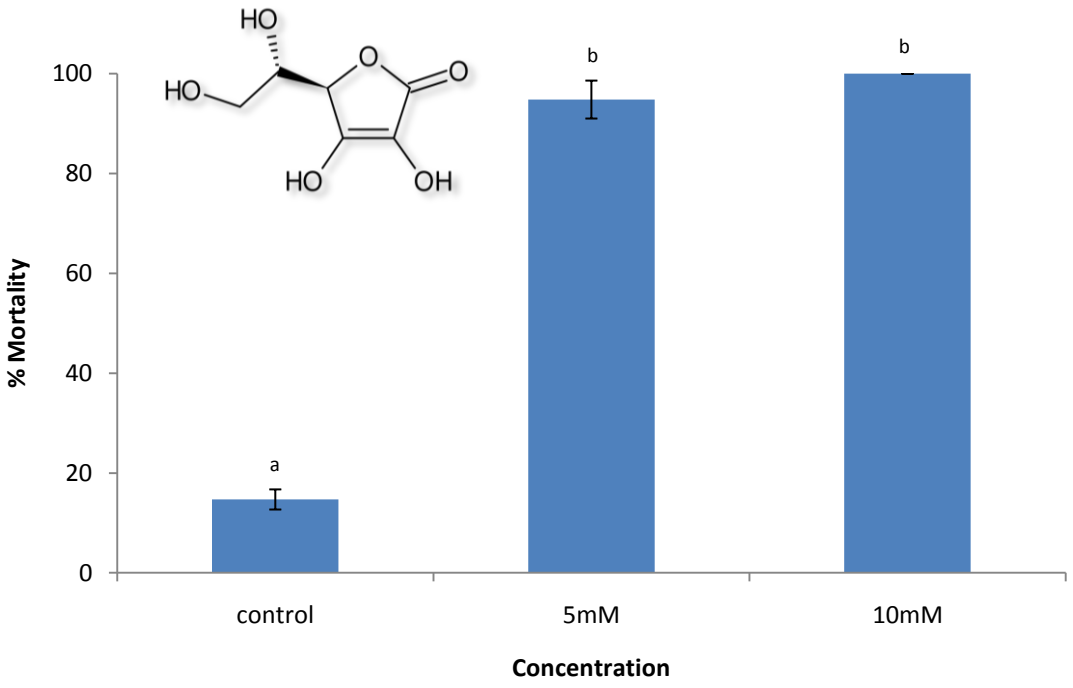

Figure 47. Effect of ascorbic acid on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.2.3 Sodium metabisulphite

Exposure to sodium metabisulphite at all treatment levels also produced high mortality rates (Figure 48). However, note that mortality in the control was quite high (>20%), suggesting other factors may have been involved in the demise of these larvae. Almost 100% of individuals died after exposure to 5 mM $Na_2S_2O_4$.

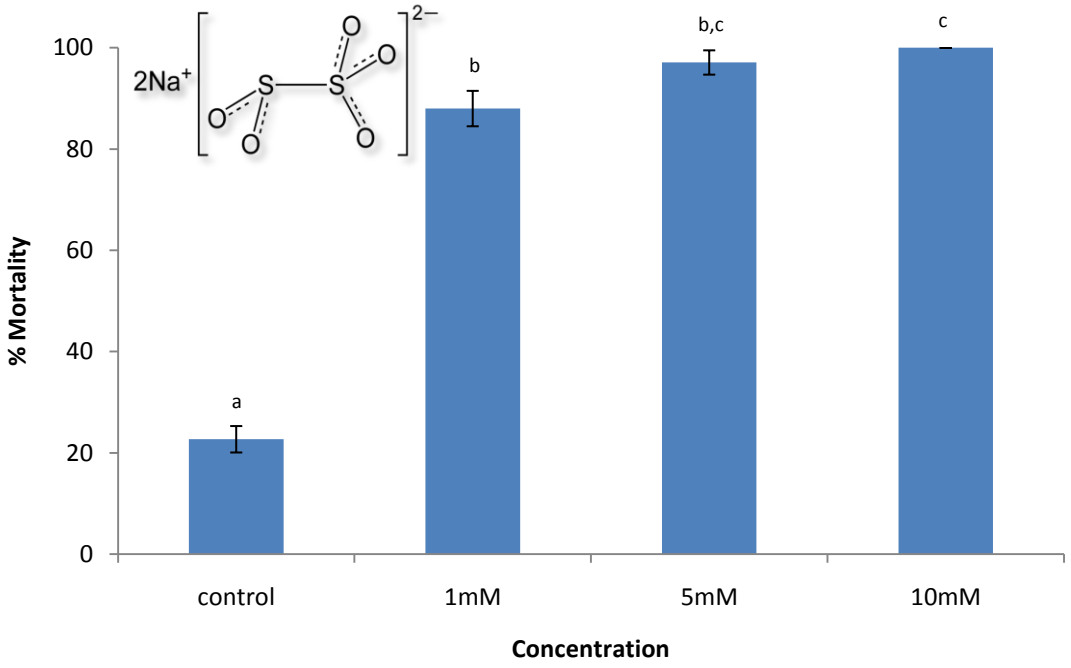

Figure 48. Effect of sodium metabisulphite on larval mortality after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

DISCUSSION

The results of this chapter were surprising. Hydrogen peroxide, an oxidation product of L-DOPA and epinephrine, induced larval settlement at 400 ppm. This suggests that the inductive effects of L-DOPA and epinephrine solutions observed in Chapter 7 may not be due to the native conformity of these molecules. The level of induction caused by hydrogen peroxide was not as great as that previously induced by L-DOPA and epinephrine treatments (see Chapter 7, p164). This may imply that exogenously applied L-DOPA and epinephrine may have a slight ability to induce settlement. However, this additive effect is just as likely to be due to other oxidation products which were not tested in this chapter, such as adrenochrome, dopachrome, or melanin.

The inductive effect of hydrogen peroxide is intriguing. Catecholamines have been suggested to induce settlement and metamorphosis due to the detected increase in endogenous levels of these compounds and presence of adrenogenic receptors around this early developmental stage in marine invertebrate larvae (e.g. Coon & Bonar 1986; Croll et al. 1997; Croll 2006; Wang et al. 2006). Perhaps the signal transduction mechanism of settlement also relies heavily on the oxidative breakdown of catecholamines into other metabolites. This would infer a signalling model which is much more complex than previously thought. Endogenous degradation of catecholamines in biological systems involves the enzyme catechol-O-methyltransferase (COMT), which converts them into metanephrine, normetanephrine or 3-methoxytyramine. These substrates are then further degraded by monoamine oxidases (MAO's) producing hydrogen peroxide (Yasuhara 1993; Nakos & Gossrau 1995). There are two types of monoamine oxidases, MOA-A and MOA-B. The production of hydrogen peroxide is known to further activate the MOA-B enzyme (Conradi et al. 1986). Perhaps there also is an increase in the levels of hydrogen peroxide at the onset of settlement and metamorphosis competency and it is this molecule which plays a key role in the next step of the signal transduction mechanism. How hydrogen peroxide

stimulates settlement is a mystery. However, some suggestions are made here that might offer insight into its importance in biological systems and also may provide a basis for future research.

Hydrogen peroxide is a well known component of living cells. Not only does it play a primary role in oxidative biosynthesis reactions, but there also is mounting evidence that it serves as an important signalling molecule. The discovery of oxidation-dependant steps in signal transduction mechanisms is frequently being exposed. Hydrogen peroxide as a breakdown product of metabolites is constantly being demonstrated to stimulate various cellular responses and activate a number of specific biochemical pathways (Rhee et al. 2005; Stone & Yang 2006). For example, endogenous hydrogen peroxide has been shown to activate protein kinase enzymes (especially tyrosine kinase), which is an important step in the signalling cascade which leads to cell division, cell growth and proliferation, and cellular differentiation and morphogenesis (Zhang et al. 1998; Cho et al. 2004; Stone 2004). Hydrogen peroxide also has an ability to stimulate gene transcription of numerous other proteins and cause various gene inductions (e.g. Fratelli et al. 2005; Bubici et al. 2006; Terada 2006). Hydrogen peroxide is known to deplete intracellular and extracellular nitric oxide levels (Song et al. 2007). This may be important since endogenous nitric oxide has been demonstrated to regulate marine invertebrate metamorphosis. In the gastropod *Crepidula fornicata*, increased nitric oxide inhibits metamorphosis (Pechenik 2007). Conversely, nitric oxide synthase gene expression is reduced after metamorphosis in the gastropod *Ilyanassa obsoleta* and inhibition of nitric oxide synthase inhibits larval metamorphosis (Leise et al. 2001). Hydrogen peroxide also has an effect on voltage-gated potassium ion channels (Wang et al. 1996), an ion which has previously demonstrated an ability to induce larval settlement in *P. canaliculus* (see Chapter 5).

Hydrogen peroxide evidently plays varying roles in biochemical signalling pathways. It is, however, unknown if the hydrogen peroxide induction of settlement observed in this thesis is naturally caused endogenously by the oxidative breakdown of catecholamines or by oxidation of some other internal compound/s. Nonetheless, it

appears that hydrogen peroxide formation from the auto-oxidation of aqueous L-DOPA and epinephrine induces larval settlement in *P. canaliculus*. Therefore, it is apparent that marine invertebrate settlement studies involving L-DOPA or catecholamine treatment solutions should be discussed with great caution unless simultaneously testing a variety of other treatment compounds as controlling factors. Perhaps it is better to treat larvae with substances which regulate endogenous production of L-DOPA and the catecholamines such as enzyme inhibitors, enzyme activators, or precursor molecules. Alternatively, the discovery of a suitable antioxidising agent to incorporate into treatment solutions may resolve the problem of auto-oxidation.

The investigation into the suitability of using ascorbic acid and sodium metabisulphite to limit oxidation of L-DOPA and catecholamine treatment solutions revealed some very surprising results – they both showed an unanticipated ability to induce settlement. They also were very toxic at the concentrations assayed and for these reasons ascorbic acid and sodium metabisulphite would not be good antioxidants to use.

The rational for the induction of settlement caused by ascorbic acid was not an initial principal concern of this chapter. However, the role of ascorbic acid in marine invertebrate larval settlement and metamorphic signalling pathways has not been investigated in the current literature. Therefore, a brief discussion will be provided. There are two possible reasons why ascorbic acid may have induced settlement – firstly, the involvement of this vitamin in catecholamine biosynthesis. During conversion of dopamine to norepinephrine, ascorbic acid is required as a cofactor by the enzyme dopamine β-hydroxylase (Bohlen & Halbach 2006). Perhaps endogenous levels of this cofactor are normally rate limiting for this reaction and exogenous application leads to an increase in enzyme activation. Evidence also has been provided that ascorbic acid increases levels of dopamine and L-DOPA by increasing gene expression three-fold of the enzyme tyrosine hydroxylase, the enzyme involved in the conversion of L-tyrosine to L-DOPA (Seitz et al. 1998). Also, in ventral mesencephalic cells from rat brains, ascorbic acid was found to increase the number of dopamine neurons (Bagga et al. 2008). Consequently, the results of the ascorbic acid trial support the hypothesis of the

involvement of catecholamines as a key step in the signal transduction mechanism of settlement. This involvement was unbeknown before the initiation of these settlement trials. Therefore, it is a paradox that ascorbic acid was tested in order to minimise oxidation of treatment solutions so that L-DOPA and epinephrine could be successfully administered exogenously when in actual fact ascorbic acid can increase endogenous levels of these compounds. There also is evidence that during metamorphosis of amphibians, ascorbic acid levels increase significantly (i.e. Menon & Rozman 2007). However, in their publication it is suggested that levels increase because of oxidative stress within the organisms and that the biological purpose of ascorbic acid for amphibians is simply to act as an endogenous antioxidant – there is no mention of catecholamine biosynthesis. In the giant prawn *Macrobranchium rosenbergii*, endogenous levels of ascorbic acid decrease after metamorphosis has occurred (Dandapat et al. 2003). It was suggested that this may be due to less oxidative stress after metamorphosis – again with no mention of catecholamine biosynthesis. However, could it perhaps also be due to a lesser requirement of catecholamines in signalling pathways after this biological process has occurred?

The high mortality rate induced by ascorbic acid at the concentrations assayed could have been caused by the endogenous build-up of metabolite molecules, such as catecholamines. However, another possibility exists previously not considered or tested for. Lo-Nostro (2004) suggests that ascorbic acid solutions scavenge oxygen and can decrease oxygen levels significantly within 200 minutes (although the concentration of ascorbic acid that does this was not revealed). If this effect was occurring in the treatment solutions, then perhaps mortality was a result of anaerobic conditions. It is suggested that future studies involving ascorbic acid should quantify this effect. Another consideration which should be taken into account when using treatment solutions of ascorbic acid is hydrogen peroxide production. In solutions of this vitamin, reaction with singlet oxygen (i.e. the diamagnetic form of molecular oxygen which is less stable than normal triplet oxygen) can cause degradation leading to a build-up in hydrogen peroxide which may accelerate ascorbic acid oxidation (Galena et al. 2007). Although singlet oxygen can be present in seawater due to photosensitisation, it is

highly unlikely that reaction of this molecule with the ascorbic acid treatments occurred during the trials in the present study. The seawater used for settlement experiments was not exposed to high light levels for at least 24 hours before T_0 and since singlet oxygen only exists for around one hour due to its highly unstable nature, this reactive form of oxygen was unlikely to be present. However, it is pointed out here that any future induction studies involving ascorbic acid, and perhaps any other compounds, should not use freshly collected seawater that has been exposed to high light levels for preparing treatment solutions (i.e. within 1-2 hours of collection). The acute toxicity of $Na_2S_2O_5$ at low concentrations observed in the mortality assays was likely due to the creation of sulphur dioxide, the same toxin produced by breakdown of potassium metabisulphite in Chapter 5.

Sodium metabisulphite showed an ability to induce larval settlement in *P. canaliculus* which was not expected. The role of sodium ions in the signal transduction mechanisms of larval settlement also was not a primary goal of this chapter and will, therefore, not be discussed in detail. However, it is quickly pointed out that sodium ions are very important in the depolarisations of all cell membranes, including neurons, and alterations in the movement of ions across neuronal membranes may trigger or inhibit nerve impulses and a variety of cellular and behavioural responses. Few studies, if any, have investigated the effects of excess sodium ions on marine invertebrate larval settlement. However, there is evidence to suggest they play a key role in chemoreception of some vertebrates. For example, tetrodotoxin is a blocker of voltage-gated sodium channels and is secreted by the epidermis of adult newts. Newts are known to be cannibalistic and it has been demonstrated that olafactory chemoreception of tetrodotoxin by juvenile individuals induces flight and hiding responses indicating it is a cue of predation risk (Zimmer et al. 2006). Perhaps sodium ions also play an important unknown role in the signal transduction mechanisms of settlement in *P. canaliculus* – possibly through binding of external cues, which have the ability to regulate the movement of this ion across cell membranes. Alternatively, perhaps an increase in exogenous sodium ions simply mimics a natural endogenous process (after transportation into extracellular spaces) caused by unknown

internal biochemical activations. For example, in a rotation of events and feedback loops, acetylcholine is known to induce catecholamine production which in turn increases levels of cyclic adenosine monophosphate (c-AMP). Cyclic AMP can lead to a rise in Na^+, enhancing acetylcholine-induced ion fluxes thereby stimulating neuronal depolarisations and nerve impulses and also catecholamine release (Higgins & Berg 1988; Morita et al. 1995). So, it also may be this role of sodium ions in the transduction cascade which caused the increase in settlement observed with exogenous application of sodium metabisulphite. The involvement of c-AMP and its association with other treatment compounds tested in this thesis form the basis for the next chapter.

It is evident from the results obtained in this chapter that exogenously applied treatment solutions of L-DOPA and the three catecholamines may induce larval settlement through oxidative breakdown products, such as hydrogen peroxide. Sumin et al. (2006) has demonstrated that exogenously applied epinephrine increases metamorphosis and alters gene expressions in larvae of the manila clam *Ruditapes philippinarum*. It would be interesting to treat larvae with epinephrine breakdown products (i.e. hydrogen peroxide and adrenocytochrome) and employ differential display reverse transcription PCR to analyse gene expression and determine if results are still the same as those determined in Sumin's study. If they are the same, it would imply that a much more complex signal transduction model exists than the epidermally bound chemoreception of epinephrine hypothesised by these researchers.

In summary: (1) hydrogen peroxide, an oxidation breakdown product of L-DOPA and epinephrine, induces larval settlement in *P. canaliculus*. (2) The mode of hydrogen peroxide action may be through stimulation of protein kinase enzymes, production of various proteins through gene transcription, modulation of endogenous nitric oxide, or regulation of voltage-gated potassium ion channels. (3) Ascorbic acid and sodium metabisulphite are not suitable antioxidant candidates for the purpose of limiting oxidation of treatment solutions. (4) Ascorbic acid induces larval settlement in *P. canaliculus*. (5) Ascorbic acid may induce settlement by activating catecholamine

biosynthesis. (6) Sodium metabisulphite induces larval settlement in *P. canaliculus*. (7) Sodium metabisulphite may induce settlement by mimicking the effect of a natural external inducer and by affecting acetylcholine and c-AMP production. (8) Ascorbic acid may induce mortality due to endogenous synthesis of toxic compounds, or by decreasing oxygen levels in treatment solutions. (9) Sodium metabisulphite likely causes mortality due to sulphur dioxide production in aqueous solutions. (10) The role of catecholamines in larval settlement may be more complex than previously thought. (11) Many researchers performing settlement induction assays need to think more carefully in the future about the treatment selections and experimental designs they employ in order to minimise ambiguous or false interpretations of results.

CHAPTER 9

EFFECT OF CYCLIC ADENOSINE MONOPHOSPHATE ON LARVAL SETTLEMENT

1. INTRODUCTION

Many of the compounds tested in previous chapters of this thesis are known to have the ability to stimulate the biosynthesis of 3'-5'-cyclic adenosine monophosphate (c-AMP). To explore possible links between the compounds previously tested in this thesis and c-AMP involvement, this chapter investigates the effect of c-AMP on larval settlement by exogenous application and endogenous activation.

Cyclic adenosine monophosphate is a second messenger, which is involved in intracellular signal transduction mechanisms. Bioactive molecules, such as hormones, like glucagon and epinephrine cannot cross cell membranes, and for them to induce intracellular biochemical pathways they require a mechanism of transmitting their signal into the cell (Beament et al. 1971). These molecules bind to specific transmembrane G protein-coupled receptors (GPCR's) which in turn activate the intracellular production of c-AMP (Figure 49). There are many types of GPCR's, and each binds to specific ligands ranging from small molecules such as catecholamines, lipids, or neurotransmitters (e.g. acetylcholine and serotonin), to large protein hormones (Beament et al. 1971; Peroutka 1994; Griffin & Ojeda 2004). Attached to the GPCR's are intracellular heterotrimeric G proteins, which when inactive are bound to guanosine diphosphate (GDP). When an external ligand binds to a GPCR it induces a change in its conformity. This change in structure is transmitted to the attached G protein, which converts GDP to guanosine triphosphate (GTP), thereby activating it. After activation of the G protein, a portion of the protein complex called the G_s alpha subunit is released from the receptor, which stimulates the enzyme adenylyl cyclase to convert adenosine monophosphate (AMP) into c-AMP, thereby activating the c-AMP dependant pathway (Peroutka 1994; Goodsell 2004).

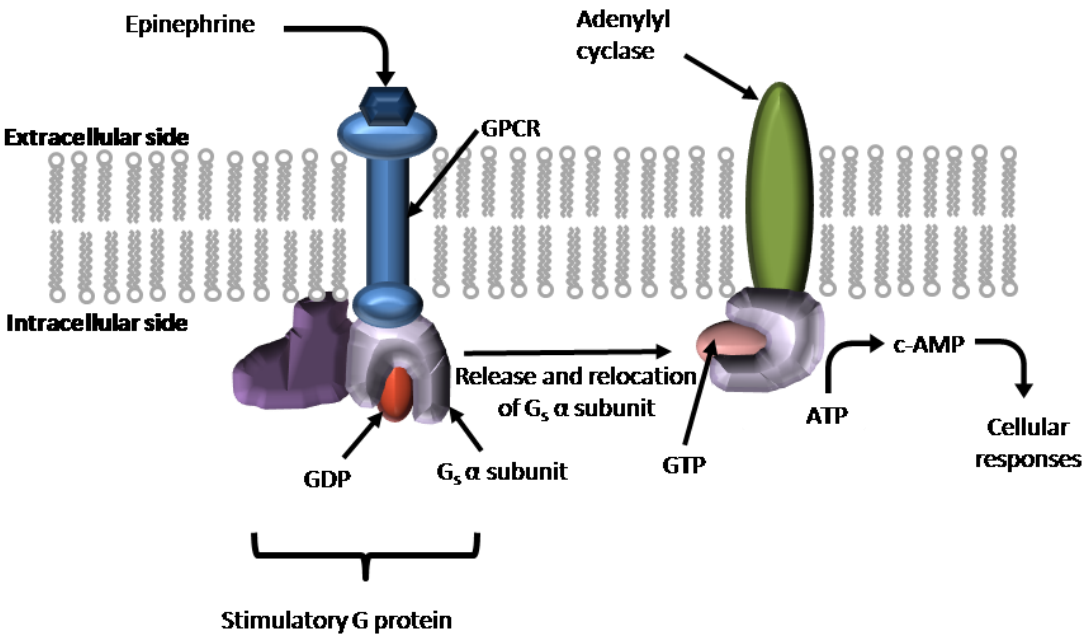

Figure 49. Production of c-AMP mediated by epinephrine activated G protein-coupled receptor.

The c-AMP dependant pathway is necessary for life processes and serves a variety of functions in biological systems. The endogenous increase in levels of c-AMP leads to the activation of: protein kinase A (PKA), cyclic nucleotide-gated ion channels, exchange proteins, enzymes that convert glucogen to glucose, and transcription factors which regulate gene expression. Cyclic AMP also regulates the effects of epinephrine and the passage of Ca^{2+} through ion channels (Walsh & Patten 1994; Griffin & Ojeda 2004; Pfeiffer & Smythies 2006). The decomposition of this secondary messenger is catalysed by the enzyme phosphodiesterase (PDE). A number of molecules (i.e. dibutytyl c-AMP, papaverine, theophylline, caffeine, and 3-Isobutyl-1-methylxanthine [IBMX]) can increase levels of c-AMP by inhibiting this enzyme. Forskolin and choleratoxin also can increase endogenous levels of c-AMP by activating the adenylyl

cyclase enzyme (Parson et al. 1988; Stefanovich & Okyayuz-Baklouti 1988; Matsuo & Iizuka 1990; Tompson 1993; Clare et al. 1995; Vente et al. 1996; Nehlig 2004; Pfeiffer & Smythies 2006).

The involvement of c-AMP in the signal transduction mechanisms of marine invertebrate larval settlement and metamorphosis has been suggested for a number of species (Qian & Pechenick 1998; Zhao et al. 2003; Li 2007; Yu et al. 2008). For example, c-AMP is believed to play a role in transduction of settlement and metamorphic signals in the barnacle *Balanus amphitrite*, since these processes can be induced with IBMX, dibutytyl c-AMP, theophylline, and caffeine (Rittschof et al. 1986; Clare et al. 1995; Clare 1996; Holm et al. 2000; Li 2007). Similarly, exogenous treatment with forskolin and IBMX induces larval settlement and metamorphosis in the polychaete *Phragmatopoma californica* (Jensen & Morse 1990). In addition, Qian and Pechenik (1998) demonstrated that IBMX induces metamorphosis in a different polychaete, *Hydroides elegans.* Li (2007) also established that this phosphodiesterase inhibitor has the ability to induce larval settlement in *H. elegans*. In contrast, Holm et al. (1998) found that an inhibitor, 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone, selective for cAMP-specific phosphodiesterase IV did not induce metamorphosis in *H. elegans*. Activation of protein kinase C (PKC) induces metamorphosis in the barnacle *B. amphitrite* (Yanamoto et al. 1995), the sea urchin *Strongylocentrotus purpuratus* (Amador-Cano et al. 2006), the polychaete *Capitella* sp. (Biggers & Laufer 1999), and the cnidarians *Mitrocomella polydiademata, Cassiopea* spp., *Aurelia aurita, Chrysaora hysoscella,* and *Cyanea lamarckii* (Freeman & Ridgway 1990; Fleck & Bischoff 1992; Siefker et al. 2000). Although PKA production is mediated by c-AMP availability, activation of PKC can stimulate adenylyl cyclase leading to increased formation of intracellular c-AMP (e.g. Chambert-Guerin & Thomopoulos 1987; Nikula & Huhtaniemi 1989; Ransjo 1991; Tachado et al. 1993). Therefore, these findings may also indicate that this secondary messenger is important in larval metamorphosis transduction mechanisms for these organisms.

The involvement of cyclic adenosine monophosphate in larval settlement and metamorphosis also has been suggested for representatives of the Phylum Mollusca. For example, exogenous treatment with IBMX induces settlement in the pearl oysters *Pinctada maxima* (Zhao et al. 2003) and *Pinctada fucata martensii* (Yu et al. 2008), the mussel *Mytilus edulis* (Dobretsov & Qian 2003), and the gastropod *Haliotis rufescens* (Trapido-Rosenthal & Morse 1986b). Treatment with the phosphodiesterase inhibitors caffeine and theophylline also induces metamorphosis in the scallop *Agropecten irradians* (Tao et al. 2003). Conversely, IBMX does not induce larval settlement in the scallop *Chlamys varia* (Mesias-Gansbiller et al. 2008).

In an attempt to identify further components of the signal transduction mechanism of larval settlement in *P. canaliculus*, and to explore possible links between treatments used in previous chapters, exogenously applied 3'-5'-cyclic adenosine monophosphate and the phosphodiesterase inhibitor caffeine were assayed for their settlement inducing activities.

2. METHODS

2.1 Organisms

Larvae for these assays were sourced from Sealord Ltd. and from the Cawthron Institute. Organisms were reared and transported according to the methods outlined in Chapter 3, p60. For details of larval source and age for each settlement assay performed, see Appendix II, p285.

2.2 Settlement assays

Settlement assays were conducted using the same methods and environmental parameters outlined in Chapter 3, p62. Briefly, assays were performed at 17±1°C in sterile polystyrene Petri plates under diffuse light with 20–30 larvae per plate (from a larval solution pre-calibrated to 20–30 larvae/ml). Static conditions were maintained, no food provided, and FSW/treatment solutions were well oxygenated at T_0 but not aerated during the assay. Ten replicates were used for each treatment and control. The number of settled larvae was detected after 48 hour for each Petri plate using the suction-by-pipette technique.

2.2.1 Treatments

3'-5'-cyclic adenosine monophosphate and caffeine were dissolved in 0.45 µm filtered seawater (FSW). Stock solutions of each treatment were prepared immediately prior to all settlement assays. Following serial dilution in FSW, treatment solutions were

prepared as 10X concentrates. Controls consisted of 9 ml FSW and 1 ml larval solution. Treatments consisted of 8 ml FSW, 1ml larval solution, and 1 ml treatment solution. Final exposure concentrations for treatments were: c-AMP; $1x10^{-6}$ and $1x10^{-5}$ M, caffeine; $1x10^{-5}$, $1x10^{-4}$, and $1x10^{-3}$ M.

2.3 Mortality assays

Mortality assays were not performed for these treatments due to time constraints and larval availability. However, some general observations were noted during the settlement assays.

2.4 Statistics

2.4.1 Larval Settlement

Statistical methods used for settlement induction assays were the same as those outlined in the methods section in Chapter 3, p64. Briefly, all percent data were arcsin transformed and analysed using statistical software, Minitab v.15. Where data satisfied the assumptions of parametric analysis, 1-way ANOVA with Tukey's multiple comparisons test was used. Non-parametric data were analysed using the Kruskal-Wallis test, and multiple comparisons made with a specialised Dunn's macro enabled for multiple comparisons of medians. Actual *p*-values for all comparisons can be found in Appendix I, p277.

3.1 RESULTS

3.1 Larval settlement

3.1.1 Cyclic adenosine monophosphate

Treatment with c-AMP for 48 hours induced larval settlement (Figure 50) with significant difference detected among treatments (ANOVA; $F_{2,27}$=4.47; p<0.05). While not as inductive as many of the other compounds tested in this thesis, exogenous application of cyclic adenosine monophosphate at both concentrations revealed very similar capacities to induce larval settlement with around 25% settlement compared to 10% in the control. Compared with the control assay, exposure of larvae to c-AMP at concentrations of $1x10^{-6}$ and $1x10^{-5}$ M resulted in more than a two-fold increase in mean percent settlement. At $1x10^{-6}$ M c-AMP, no statistical difference in settlement was detected compared with the control due to high variance (Tukey test; p>0.05). However, compared to the control assay, significant difference in larval settlements was detected after exposure of larvae to $1x10^{-5}$ M solutions of c-AMP (Tukey test; p<0.05).

A mortality assay was not performed for c-AMP and dead individuals were not enumerated during the settlement assays due to time constraints. However, it was observed during the settlement assay that exposure for 48 hours did not appear to induce acute toxicity effects at either of the concentrations tested. Based on quick visual inspection of velum, gut, and pedal organ, almost 100% survivability was routinely estimated in every Petri plate with individuals appearing healthy.

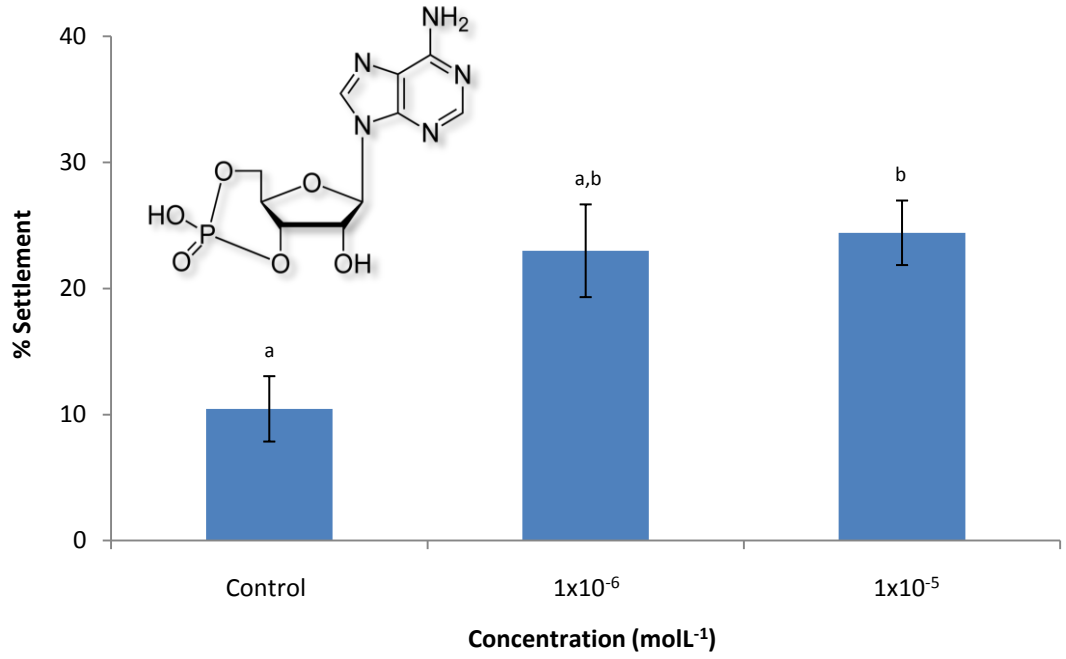

Figure 50. Effect of 3'-5'-cyclic adenosine monophosphate on larval settlement after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

3.1.2 Caffeine

An increase in endogenous c-AMP, stimulated by the phosphodiesterase inhibitor caffeine, also induced settlement (Figure 51) with significant difference detected among treatments (ANOVA; $F_{3,36}$=19.83; p<0.001). Caffeine was approximately twice as active at inducing settlement as exogenous application of c-AMP. Induction was dose-dependent, gradually increasing to the maximum concentration assayed ($1x10^{-3}$ M), which induced almost 50% of larvae to settle. At all treatment level concentrations assayed, significant induction was detected over the control (Tukey test; p<0.01 in each comparison). Again, mortality assays were not performed for this compound. However, it was observed that approximately 50–75% of larvae died after 48 hours exposure to $1x10^{-3}$ M solution of caffeine. At $1x10^{-5}$ M, very few individuals were dead and were comparable to the control.

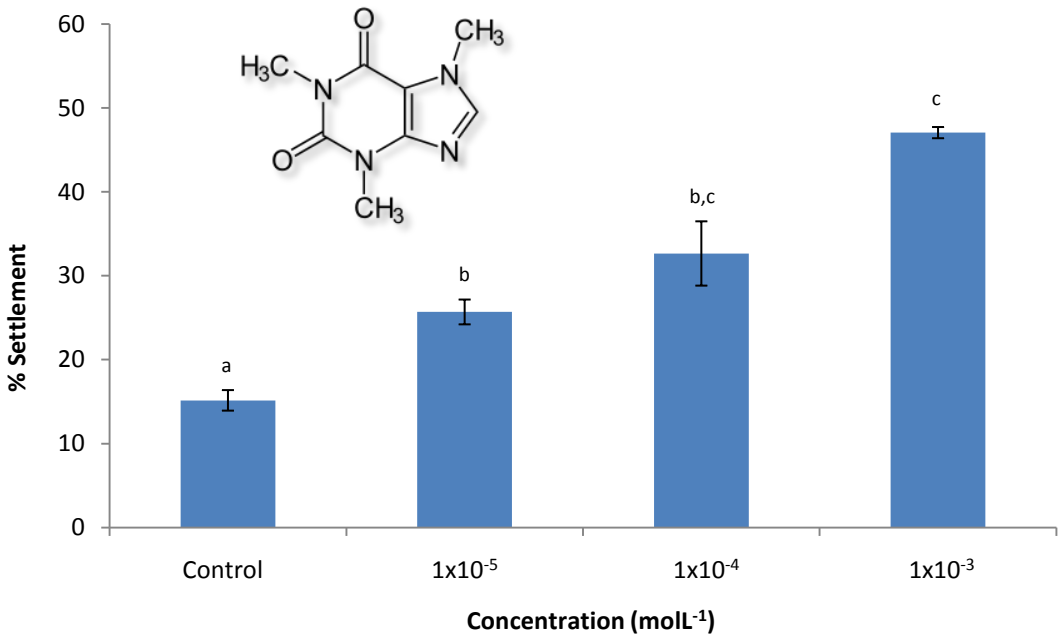

Figure 51. Effect of caffeine on larval settlement after 48 hours exposure. Data plotted are mean ± standard error of 10 replicates. Superscripts above error bars reveal significant differences (Tukey test).

4. DISCUSSION

Induction of larval settlement in *P. canaliculus* has been demonstrated by exogenous treatment with c-AMP and endogenous stimulation of c-AMP by inhibition of intracellular degradation with caffeine. In combination, these results infer that 3'-5'-cyclic adenosine monophosphate is a likely component in the signal transduction mechanism of settlement in this species. The role of c-AMP in the settlement transduction mechanism may be a very important one. This role may provide a link with most of the compounds tested in this thesis, which also have demonstrated an ability to induce settlement. For example, L-Tryptophan can be converted to serotonin (Suni-Ichinose et al. 1992) – serotonin treatment increases levels of c-AMP in the mussels *M. edulis* and *Mytilus galloprovincialis* (Kohler & Lindl 1980; Fabrri & Capuzzo 2006), the scallop *Agropecten purpuratus* (Martinez et al. 1998), and in the gastropods *Aplysia kurodai* and *Aplysia californica* (Sawada et al. 1984; Weiss et al. 1985). L-Phenylalanine is converted to L-Tyrosine which is converted to L-DOPA (Bohlen & Halbach 2006). Exogenous treatment with L-DOPA increases intracellular c-AMP levels in larvae of the scallop *A. irradians* (Tao et al. 2003). L-DOPA can be converted to the first catecholamine dopamine (Squire et al. 2003), which has the ability to stimulate a rise in c-AMP levels in the bivalves *M. edulis* and *A. purpuratus*, and also the crab *Limulus polyphemus* (Kohler & Lindl 1980; Groome & Watson 1990; Martinez et al. 1998). Dopamine can be converted to epinephrine (Bohlen & Halbach 2006). Epinephrine has been demonstrated to have the ability to increase intracellular c-AMP levels in larvae of the scallop *A. irradians* (Tao et al. 2003). Excess potassium ions have also proved effective at increasing endogenous c-AMP concentration in larvae of *A. irradians* (Tao et al. 2003). However, acetylcholine is not known to stimulate c-AMP production, although c-AMP can stimulate acetylcholine release (Briggs et al. 1988).

It was suggested from the results in Chapter 6 that acetylcholine is a component in the signalling pathway of settlement and acts by binding to nicotinic receptors, since the

muscarinic receptor antagonist atropine was demonstrated to have no inhibitory effect on larval settlement. In fact, atropine on its own increased mean settlement rates compared to the control. Shiloh et al. (2000) reveal that activation of muscarinic acetylcholine receptors decreases intracellular c-AMP levels. Therefore, since c-AMP is suggested to play a role in the signal transduction mechanism of settlement, based on the results in this chapter, the conclusion that the nicotinic receptor is responsible for the inductive effects of acetylcholine is further substantiated, and suggests that the muscarinic receptors are not involved at all. Furthermore, if muscarinic receptors were involved, then acetylcholine treatment may decrease c-AMP levels, which would be counterintuitive for the involvement of c-AMP in the signalling pathway. However, the slight inductive effects of atropine suggest that muscarinic receptors may be present in some cells. Conceivably by blocking them an increase in endogenous levels of c-AMP occurs, leading to increased settlement rates. Therefore, the question remains as to why exogenous application of acetylcholine does not inhibit settlement by activating the muscarinic receptors, thereby decreasing levels of intracellular c-AMP? Perhaps because the involvement of c-AMP in the settlement transduction pathway is upstream from that of acetylcholine. Briggs et al. (1988) provide evidence that c-AMP stimulation enhances nicotinic transmission by increasing the evoked release of acetylcholine from presynaptic neurons into the synapse. Morita et al. (1995) also provide evidence that c-AMP enhances acetylcholine induced ion fluxes at nicotinic receptors in postsynaptic neurons, thereby stimulating neuronal depolarisations and nerve impulses. This would mean that exogenous application of acetylcholine in the settlement assays would bypass the signalling cascade prior to intracellular c-AMP production.

Although the results of this chapter indicate that c-AMP is involved in the signal transduction mechanisms of larval settlement in *P. canaliculus* (since exogenous c-AMP and endogenous activation through caffeine induced settlement), there is the possibility that caffeine also stimulated other unrelated biochemical mechanisms. The phosphodiesterase inhibitor caffeine induced approximately double the amount of settlement than that induced by exogenous c-AMP treatment. This additive effect could

suggest that the exogenous c-AMP treatment may not have been as effective at increasing intracellular levels of c-AMP as caffeine. Conversely, it also could infer that caffeine stimulates other biochemical reactions. Further analysis of the literature reveals some unusual observations. IBMX induces settlement and metamorphosis in the polychaete *H. elegans* presumably by increasing endogenous levels of c-AMP (Qian & Pechenik 1998; Li 2007). Holm et al. (1998) also found that IBMX induces metamorphosis in this species. However, 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone, an inhibitor selective for cAMP-specific phosphodiesterase IV, did not induce metamorphosis. Holme et al. (1998) also determined that the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (an activator of protein kinase C) did not stimulate metamorphosis and forskolin (an activator of adenylyl cyclase) which increases c-AMP levels inhibited the inductive response of biofilms. This indicates that IBMX induces by way of alternative mechanisms other than inhibition of c-AMP degradation. Similarly, Pawlik (2000) found that IBMX induced metamorphosis in the polychaete *Phragmatopoma californica*, whereas other c-AMP stimulants, dibutytyl c-AMP and choleratoxin, did not. Although it is possible that polychaetes are unique among marine invertebrates in their responses to phosphodiesterase inhibitors, it is unlikely. These observations suggest that other biochemical mechanisms are being stimulated by IBMX.

By reviewing the recent medical literature of phosphodiesterase inhibitors, some surprising points have been uncovered which may cast doubt on some of the past studies investigating marine invertebrate larval settlement or metamorphosis. Caffeine, like theophylline, is a non-selective and relatively weak PDE inhibitor and also has an ability to block adenosine receptors (Gupta & Gupta 1999; Pfeiffer & Smythies 2006). Caffeine and theophylline have likely been employed as presumptive PDE inhibitors in several thousand scientific papers investigating various biochemical mechanisms in numerous organisms (see Stefanovich & Okyayuz-Baklouti 1988), including those involving the role of c-AMP in larval metamorphosis (e.g. Clare et al. 1995; Tao et al. 2003). In recent years, it has become apparent that the use of these compounds for this purpose may lead to erroneous conclusions since they

are much more potent as adenosine antagonists than PDE inhibitors (Nehlig 2004). IBMX also is sometimes used to increase c-AMP levels in settlement assays and although IBMX is around 10–100 times more potent than caffeine and theophylline as a PDE inhibitor, it also blocks adenosine receptors (Stefanovich & Okyayuz-Baklouti 1988). Therefore, there is the possibility that authors of settlement studies involving only these PDE inhibitors may misinterpret the results leading to the belief that larval settlement or metamorphosis is induced by increases in endogenous cyclic AMP. In these scenarios, it is just as likely that these effects are caused by adenosine antagonisation. It may be interesting to point out that in rat superior cervical ganglion, stimulation of the adenosine receptor by 2-chloroadenosine leads to a decrease in presynaptic acetylcholine release and postganglionic response (Briggs et al. 1988). It could therefore be assumed that antagonisation of this receptor may increase acetylcholine vesicle release. This is an interesting notion and one that has not yet been explored in the literature and could provide a new avenue of research into the mechanisms of larval settlement.

After taking note of these findings in the literature, it seems that the induction of settlement achieved by treatment with caffeine cannot solely be attributed to the intracellular increase in c-AMP levels. However, because exogenous treatment with cyclic adenosine monophosphate induced larval settlement it may be concluded that c-AMP does still play a role in the biochemical signalling pathway of larval settlement. Endogenous modes of amplifying c-AMP levels could be caused by increases of metabolites, such as the catecholamines upstream in the signalling pathway. Alternatively, there is the possibility that increased levels of this second messenger may be caused by exogenous transfer of cyclic adenosine monophosphate from the external environment.

Marine slime moulds such as *Labyrinthula* spp. likely have external c-AMP chemoreceptors for chemotaxis and excrete c-AMP for cell-cell communication akin to their terrestrial counterparts (Armiger 1964; Hanna et al. 1984; O'Day 1990; Louis et al.

1993; Steele et al. 2005). These slime molds commonly live on the surfaces of a variety of marine macroalgae species (e.g. Sakata & Fujisawa 2006) and could be associated with substrates frequently preferred by *P. canaliculus* larvae, such as the red seaweeds *Osmundaria colensoi* and *Gigartina alveata* (Alfaro et al. 2006). Slime molds exude c-AMP for communication at concentrations up to $1x10^{-6}$ M (Nanjundiah 1972; Devreotes 1982), a concentration which showed settlement inductive effects in *P. canaliculus*. This leads to the possibility that marine slime moulds could partially be responsible for primary settlement stage substrate selection in this mussel. In bacteria, endogenous c-AMP is responsible for the production of quorum sensing molecules that regulate cell-cell communication (e.g. Dunny & Winans 1999; Liang et al. 2007). Treatment with c-AMP also has been demonstrated to enhance protease activity in marine bacterial exudates of *Vibrio* spp. and *Pseudomonas* spp. (Albertson et al. 1990). Marine bacteria can regulate endogenous levels of this second messenger by taking up dissolved c-AMP that is naturally distributed in the ocean and increase intracellular concentrations by up to 3µM per hour (Ammerman & Azam 1981, 1982, 1987). It has been suggested that exogenous c-AMP also may be produced as a component of bacterial exudates and be involved as a quorum sensing molecule itself (Chassy et al. 1969; Ammerman & Azam 1981; Duursma & Dawson 1981; Albertson et al. 1990). Exudates of marine bacterial biofilms have been demonstrated to induce larval settlement in *P. canaliculus* (i.e. Ganeesan et al. 2008). Fractionation and identification of the molecular components of these exudates have not yet been determined. However, it is possible that bacterial biofilms associated with macroalgal surfaces secrete c-AMP, modulating substrate preferences of *P. canaliculus* larvae. Furthermore, unicellular marine algae can secrete c-AMP (e.g. Franko 1989) which may hint to algal/invertebrate associations controlled by exogenous release of this compound. These suggestions are purely hypothetical, however, may provide a basis for future research.

In summary: (1) 3'-5'-cyclic adenosine monophosphate is likely involved in the biochemical signalling pathway of larval settlement in *P. canaliculus*. (2) Endogenous increases in the levels of catecholamines through bioconversion of L-Phenylalanine or

L-Tyrosine (by the L-DOPA intermediate) may lead to intracellular synthesis of c-AMP by means of membrane bound G protein-coupled receptors – this may explain why epinephrine and its precursors induced larval settlement. (3) Endogenous increases in serotonin leads to increased c-AMP which may explain why L-Tryptophan induced settlement (conversion to serotonin). (4) Treatment with excess potassium ions increases intracellular c-AMP concentration which may also explain the inductive effects of this ion. (5) c-AMP stimulates acetylcholine release at presynaptic neurons and regulates ion passage in nicotinic receptors – combined with the results of acetylcholine induced settlement and muscarinic receptor antagonisation with atropine in Chapter 6, it is inferred that nicotinic receptors are the primary acetylcholine receptors involved in the signalling mechanism. Also, due to this, acetylcholine involvement may function downstream of c-AMP in the signal cascade. (6) Treatment with the phosphodiesterase inhibitor caffeine may have stimulated other biochemical pathways, such as antagonisation of adenosine receptors thereby increasing acetylcholine release into the synaptic region and amplifying settlement induction. (7) The role of c-AMP in the signalling pathway of settlement and metamorphosis in a variety of marine invertebrate organisms has been claimed by numerous authors because IBMX induces these processes. However, IBMX also stimulates other biochemical mechanisms casting doubt on these assertions. (8) Marine slime molds, bacterial biofilms, and microalgae on macroalgal surfaces may secrete c-AMP for cell-cell communication aiding in the regulation of substrate selection for *P. canaliculus* larva.

CHAPTER 10

SPECIES CLUSTER ANALYSIS OF SETTLEMENT RESPONSES

1. INTRODUCTION

In this chapter a database is constructed from the literature containing the settlement responses of various marine invertebrate species to some of the compounds tested in this thesis. As an exploratory investigation, multivariate statistical analysis is performed to cluster species based on behavioural response, to identify any relationships among the species.

Taxonomic problems involving the clustering of species have intrigued chemically-orientated biologists for many years (Throckmorton 1968). In fact, an interest in the correlation between traditional morphological classifications and chemical compositions of organisms can be traced as early as 1699 (Fairbrothers 1968). However, it was not until much later in 1867 that a field of science called chemotaxonomy (also known as chemosystematics or biochemical taxonomy) first emerged with the discovery that potassium hydroxide and calcium hypochlorite applied to the thallus of lichens produced specific colour forming reactions depending on the species (Lamb 1951). This basic form of analytical chemistry remains relevant and is still used today by modern lichenologists (e.g. Jayalal et al. 2007). Chemotaxonomy is based on the chemical characteristics of organisms and the classification of these organisms according to demonstratable differences or similarities in their biochemical compositions (Frisvad et al. 1998).

Living organisms produce numerous types of natural products, and the biosynthetic pathways responsible for the manufacture of these compounds often differ from one taxonomic group to another. In many cases, the distribution of these molecules and their biosynthetic pathways correspond well with the more traditional methods of taxonomic classification, such as morphological characteristics (e.g. Hooper et al. 1992; Stadler et al. 2004). However, occasionally, mapping of these compounds among species also have

contradicted existing hypotheses of classification (e.g. Brenner et al. 1993; Castellanos et al. 2003), and in these instances it may become necessary to re-examine the problem. On a more positive note, chemical data have supplied information in circumstances where other forms of data are insufficiently discriminatory (e.g. Flagel et al. 2008). Chemotaxonomy is now widely utilised as a component within the general field of taxonomy, in conjunction with morphological and genetic approaches.

In previous chapters it was demonstrated that *P. canaliculus* larvae can be induced to settle after exposure to: L-Tryptophan (precursor of the neurotransmitter serotonin, and the hormone and antioxidant melatonin), excess potassium ions (regulator of membrane polarisation and nerve impulses), acetylcholine (a neurotransmitter), the amino acids L-Phenylalanine, and L-Tryptophan (precursors to catecholamines, thyroid hormones, morphine, and melanin), L-DOPA (a catecholamine precursor), epinephrine (a catecholamine, neurotransmitter and hormone), hydrogen peroxide (an oxidation product of L-DOPA and the catecholamines and a second messenger), ascorbic acid (an enzyme cofactor, electron donor, and antioxidant), cyclic adenosine monophosphate (a second messenger for numerous biochemical pathways), and caffeine (a phosphodiesterase inhibitor and adenosine receptor antagonist). These inductive responses offer insights into the signal transduction mechanism of settlement and provide information as to the identification of key metabolites within this biochemical pathway. From these results it is suggested that each of the chemical species mentioned above are involved in the transmittance of a settlement cue, exogenous or endogenous, resulting in the movement of larvae from a pelagic environment to a benthic one, and subsequent production of mucoid threads for the purpose of attachment to a substratum.

The settlement responses of various marine invertebrate larvae to exogenously applied molecules are diverse, and have routinely been demonstrated to be species-specific. This would infer that the signalling pathways involved are different in some respects across taxa. However, some similarities in these responses do exist among and within marine

invertebrate taxa. This leads to the hypothesis that perhaps these behavioural responses can be utilised to explore relationships among species in terms of the stimulants which demonstrate a capacity to regulate the signal transduction mechanisms of settlement. Such an approach to species clustering does not involve direct analytical measurement of the endogenous biochemical compositions of organisms. Therefore, this technique is not synonymous with chemotaxonomy as it is currently defined. Grouping marine invertebrate species by behavioural responses to exogenously applied compounds, which have the ability to regulate neuronal and cellular processes, involves a cross-discipline approach incorporating principles in neurobiology, biochemistry, chemotaxonomy, ethology, and pharmacology. Such an approach is novel and one which has not yet been explored in the literature. A comparative meta-analysis of particular behavioural stimulation in response to neuroactive compounds across marine invertebrate taxa could be utilised as a new division of chemotaxonomy to identify currently unknown relationships and cluster species accordingly. In this thesis, due to the fields that this approach encompasses, this technique has been coined 'neuroethological taxonomy'. This should not be confused with a sub-field of taxonomy that has recently been termed neurotaxonomy (i.e. Christophe et al. 2002). Neurotaxonomy involves clustering of species based on similarities or differences in the anatomy of the nervous system among taxa, thereby further expanding upon traditional approaches involving morphological characteristics. Neuroethological taxonomy refers to the clustering of species based on similarities in the neuronal circuits and biochemical signalling pathways of specific behaviours, such as larval settlement. However, it would be feasible to apply such a technique to other natural behaviours (also termed intrinsic behaviors or innate behaviors), such as navigation (e.g. through chemotaxis), locomotion, or predator avoidance.

It is clearly stated, and should be recognised, that this investigation into an innovative form of clustering is not by any means an attempt to reclassify marine invertebrate species, nor is it an attempt to explain ancestral associations from an evolutionary perspective. Such interpretations may challenge a wide spectrum of current philosophies and is outside of the scope of this thesis. The approach investigated here is simply an exploratory one to

determine if similarities or differences exist between taxa, which may have been overlooked by some of the more traditional methods. The suggestion of neuroethological taxonomy as a scientific discipline will require vast amounts of further research to determine if it has any applications. However, this chapter explores the birth of such a concept and takes the first steps into initial data analysis of behavioural responses, in this case settlement, to a range of pharmacologically active compounds to investigate neuroethological associations.

2. METHODS

2.1 Database construction

The larval settlement induction responses of *P. canaliculus* to the compounds used in this thesis were compared to those observed in other marine invertebrate species from the literature using multivariate statistical analysis. A species database was constructed including the presence or absence of inductive settlement responses to the 16 compounds tested in this thesis.

The studies cited in the database were very carefully chosen from a thorough review of the literature. The terms settlement, attachment, and metamorphosis often are not defined clearly by authors, and care must be taken when reviewing results from studies investigating marine invertebrate larval settlement. For example, larvae may 'settle' out of the water column to a benthic habitat, but not physically attach themselves to a substrate – this may be termed settlement (e.g. Yu et al. 2007; Buchanan 1998). Settlement also can be perceived as the combination of this movement along with a physical attachment to a substrate, as in this current study. Some authors consider settlement to be the combination of larval attachment and metamorphosis (e.g. Roberts et al. 2002). Furthermore, some authors consider larval settlement and attachment as the initial stage of metamorphosis (see Hadfield & Paul 2001), while others regard settlement and attachment as a behaviour observed prior to metamorphosis (e.g. Fitt et al. 1990). In essence, only studies which clearly defined settlement behaviour as being the same response used in this thesis (firm attachment to the substratum) were incorporated in the database.

From the initial 37 invertebrate species incorporated in the database, nine species had been tested for settlement responses to seven of the 16 compounds used in this study. Although more than seven compounds were investigated for their ability to induce settlement in *P. canaliculus* larvae, the statistical analysis of the data was restricted to the involvement of only seven compounds. The number of compounds included in the analysis was limited to those seven based on the availability of data in the literature. The dataset is relatively small because many of the compounds tested in this study have not been tested for settlement response behavior in other species. The seven compounds used for the analysis were; epinephrine, L-DOPA, acetylcholine, serotonin or its precursor L-Tryptophan, potassium chloride, γ-amino butyric acid (GABA), and the non-selective phosphodiesterase inhibitors caffeine or IBMX . The constructed database is shown overleaf in Table 14.

Table 14. Species database of settlement inductive effects. Shaded boxes represent settlement responses that are known to result from treatment with various compounds where: 1 = induction, 0 = no effect, -1 = inhibition, 1/0 = conflicting data. Green shading represent responses of the nine species which are included in the cluster analysis to seven of the compounds tested in this thesis. Compounds in the row heading are: 1 = epinephrine, 2 = L-DOPA, 3 = acetylcholine, 4 = potassium chloride, 5 = GABA, 6 = serotonin or precursor, 7 = non-selective phosphodiesterase inhibitor (caffeine/IBMX), 8 = norepinephrine, 9 = dopamine, 10 = calcium.

Phylum	Class	Family	Genus/Species	1	2	3	4	5	6	7	8	9	10
Mollusca	Bivalvia	Mytilidae	*Mytilus edulis*	1	1	1	0	0	1	1	-	-	-
			Mytilus galloprovincialis	1	1	1	0	1	1	1	-	-	-
			Perna canaliculus	1	1	1	1	0	1	1	-	-	-
			Perna viridis	0	-	-	1	1	-	-	-	-	0
		Ostreidae	*Crassostrea gigas*	0	1	1	0	0	0	1	1/0	0	-
			Crassostrea virginica	-	1	-	-	-	-	-	-	-	-
			Ostrea edulis	1	-	-	-	1	-	-	-	-	-
		Pectinidae	*Argopecten irradians irradians*	-	-	-	1	-	-	-	-	-	-
			Argopecten irradians concentricus	-	-	-	1	-	-	-	-	-	-
			Argopecten purpuratus	1	-	-	1	-	-	-	-	-	-
			Chlamys hastata	-	-	-	-	0	-	-	-	0	-
			Chlamys nobilis	-	-	-	1	-	-	-	-	-	-
			Chlamys varia	1	-	-	-	1	-	0	-	-	-
			Pecten maximus	1	1	-	-	-	-	-	-	-	
		Pteriidae	*Pinctada fucata martensii*	0	0	1	1	1	1	1	-	0	1
			Pinctada margaritifera	0	-	-	-	1	-	-	-	-	-
			Pinctada maxima	0	0	1	1	1	1	1	-	0	1
		Veneridae	*Ruditapes philippinarum*	0	1	1	1	1	1	1	1	-	-
			Venerupis pullastra	1	-	-	-	1	-	-	-	-	-
	Gastropoda	Haliotidae	*Haliotis asinina*	-	-	-	1	1	-	-	-	-	-
			Haliotis discus hannoi	-	-	-	1	1	-	-	-	-	-
			Haliotis diversicolor diversicolor	-	-	-	1	1	-	-	-	-	-
			Haliotis diversicolor supertexta	-	-	-	1	1	-	-	-	-	-
			Haliotis iris	-	-	-	-	1	-	-	-	-	-
			Haliotis rufenscens	0	0	0	1	1	0	1	0	-	0
			Haliotis virginea	-	-	-	-	1	-	-	-	-	-
		Calyptraeidae	*Crepidula fornicata*	-	-	-	1	0	-	-	-	-	-
		Cuthonidae	*Phestilla sibogae*	0	0	1	1	0	0	1	0	0	-
		Nassariidae	*Ilyanassa obsolete*	-	-	-	-	0	1	-	-	1	-
		Astraeinae	*Astraea undosa*	-	-	-	1	-	-	-	-	-	
		Muricidae	*Chorus giganteus*	-	-	-	1	-	-	-	-	-	-
Arthropoda	Maxillopoda	Balanidae	*Balanus amphitrite*	0	1/0	1	-	0	1	-	0	1/0	-
			Balanus improvisus	-	-	-	-	-	-1	-	-	1	-
			Balanus balanoides	-	-	-	-	-	-	1	-	-	-
Annelida	Polychaeta	Sabellariidae	*Phragmatopoma californica*	0	1	-	-	0	-	1	0	0	-
			Hydroides elegans	-	-	-	1	1	-	1	-	-	-
Bryozoa	Gymnolaemata	Bugulidae	*Bugula neritina*	-	0	0	1	1	0	-	-	0	0

2.2 Statistics

The Plymouth Routines in Multivariate Ecological Research statistical software package PRIMER-E developed by Clarke & Warwick (2001), using principles outlined by Field et al. (1982), was used to analyse the data. To identify how the taxa relate to each other in terms of different receptor and compound involvement in the signal transduction mechanism for larval settlement, an average linkage hierarchical agglomerative cluster analysis using Euclidian distance measures was performed. This clustered the taxa based on the similarity (Bray Curtis Index) of settlement response (presence/absence of induction) to each compound, indicating similarities between species of the signal transduction mechanisms of larval settlement, and is represented by a dendrogram.

Further analysis was performed employing multidimensional scaling (MDS) using Euclidian distance to: provide a spatial representation of the data, facilitate interpretation, reveal relationships, and give statistical fit of the data to the analysis. Results gained from the cluster analysis allowed the taxa to be plotted on the MDS and grouped according to similarity. The 2-D MDS plot created represents the data at a level of 80% similarity. Although the level of similarity chosen to represent multivariate data in many biological studies is often 50–70% (for examples see: Dwivedi et al. 2001; Haynes et al. 2007; Montesanto & Panayotidis 2000; Harriott et al. 1999), the 80% level of similarity was chosen since most species (with the exception of *Haliotis* sp. And *Phestilla* sp. as out-groups) are within the class Bivalvia and it was assumed they would likely cluster together at lower percentages. Also, a more conservative estimate is provided at an 80% similarity level.

3. RESULTS

The hierarchical cluster analysis of the settlement responses is represented by a dendrogram (Figure 52). All species analysed can be grouped together at around 53% similarity. The mussels *Mytilus galloprovincialis*, *Mytilus edulis*, and *Perna canaliculus* are approximately 87% similar in inductive responses with both the *Mytilus* species being separated from one another at around 91%. The pearl oysters *Pinctada maxima* and *Pinctada fucata martensii* are indistinguishable at any level and are approximately 91% similar to the clam *Ruditapes philippinarum*. This level of similarity (91%) is as close as *M. galloprovincialis* and *M. edulis* are to one another.

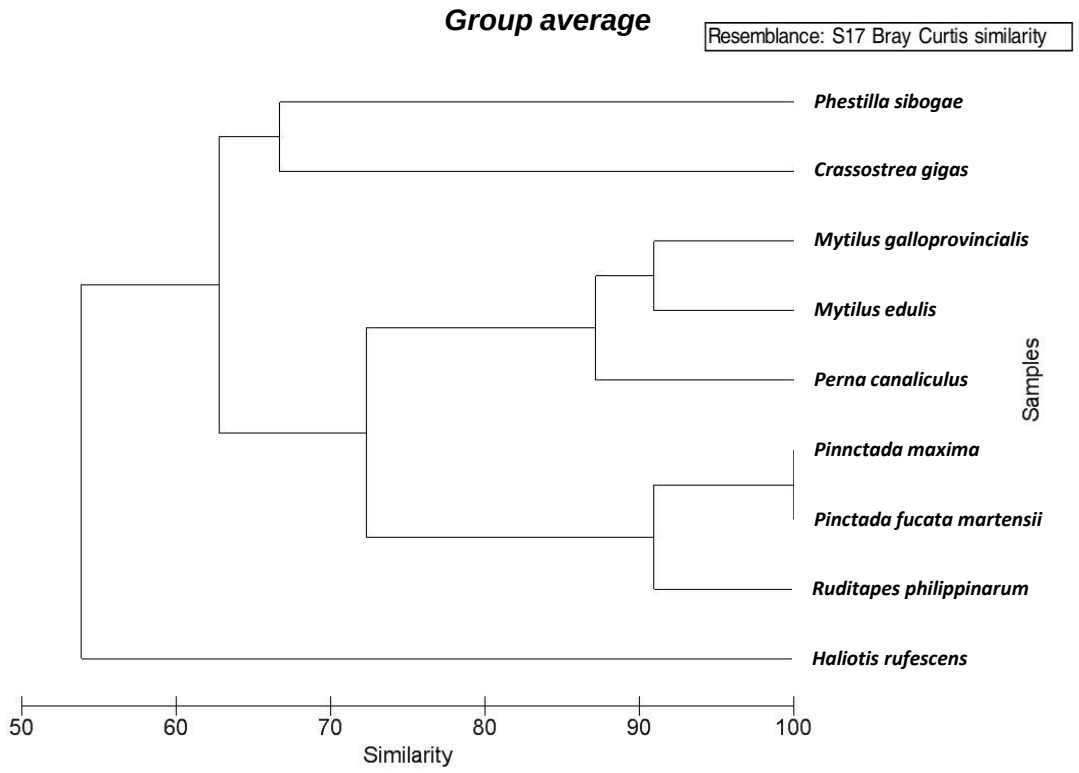

Figure 52. Species cluster analysis dendrogram based on inductive effects of selected neuroactive compounds.

The true oyster, *Crassostrea gigas*, is relatively distant in settlement responses compared to the other bivalve species. As expected, the gastropod *Haliotis rufescens*, chosen as the out-group, was the most distant. The only other gastropod incorporated into the analysis, *Phestilla sibogae*, was separated from all other bivalves at 67% similarity. However, *P. sibogae* appeared to be more similar to the bivalves in settlement responses than to *H. rufescens*.

Displayed on an MDS plot at an 80% similarity level, relationships can be easier visualised (Figure 53). Five clusters were produced from the nine species incorporated into the analysis. The mussels cluster together in one group as do the *Pinctada* oyster species with *R. philipinarum*. At this level, both gastropods and *C. gigas* are quite separated from each other and from all other bivalve species.

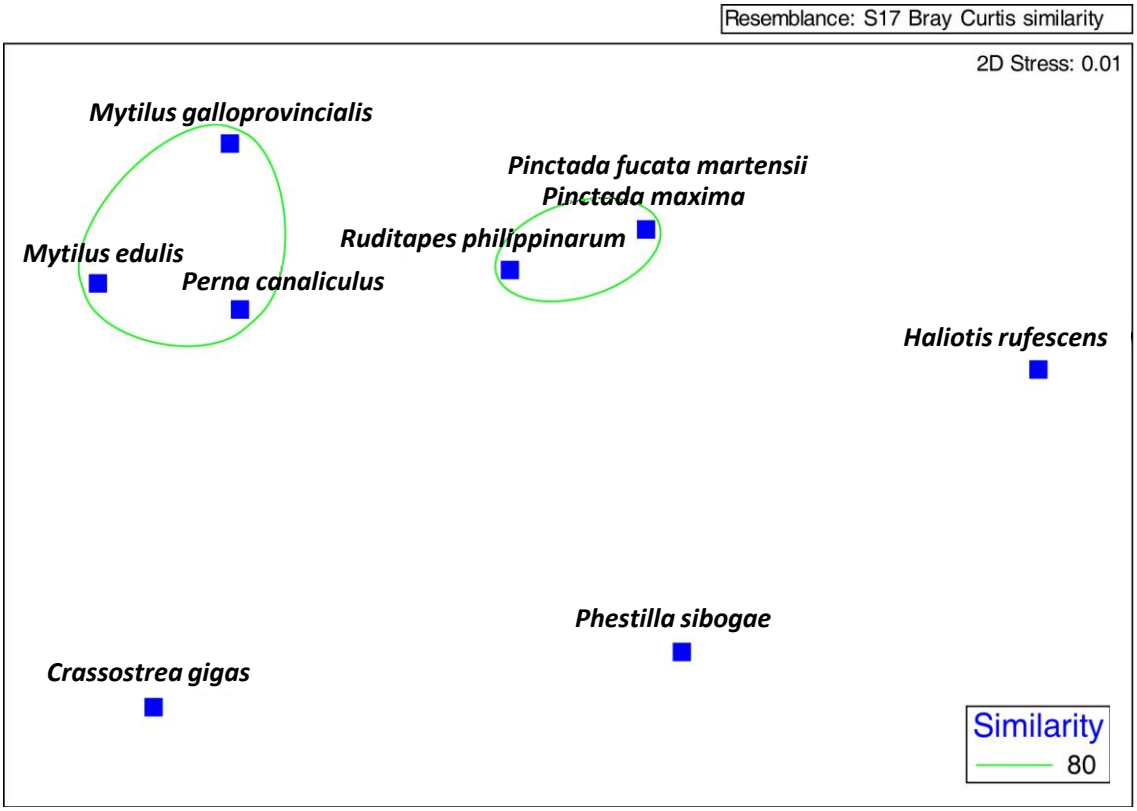

Figure 53. MDS plot of species response to selected neuroactive compounds at 80% similarity.

4. DISCUSSION

In this chapter, the meta-analysis of settlement responses across taxa to pharmacologically active compounds was explored to determine possible unknown relationships among the species analysed and to investigate a novel approach of clustering taxa. Neuroethological taxonomy is the grouping of species based on metabolite involvement in the biochemical signalling pathways that regulate particular behaviours or life changing processes, such as settlement or metamorphosis. This approach is very different from traditional chemotaxonomy. Chemotaxonomic grouping of taxa is based on similarities or differences in the identity of metabolites produced by organisms regardless of their involvement in signalling pathways. A neuroethological approach involves compounds that may be shared among all taxa, whereas chemotaxonomic relationships are determined by differences in the chemical composition of organisms. Therefore, for traditional chemotaxonomy to have application there must be differences in biochemical profiles among taxa. Such a prerequisite may provide an opportunity for important relationships to be overlooked. For example, in a hypothetical scenario, if organisms A, B, and C were all to have the same biochemical profile of key metabolites, traditional chemotaxonomic clustering may group these organisms together. However, among those three species perhaps the endogenous uses of particular shared metabolites in biochemical processes are different. By specifying the process (e.g. signal transduction mechanism of settlement behaviour), dissimilarities in the involvement of those compounds would infer that those organisms are different in some way. It is not impossible that such information could provide implications for determining evolutionary ancestry since ultimately these signalling pathways and their components must be genetically coded for. Correlation of the results gained from a neuroethological approach with evolutionary trends is outside of the scope of this thesis. However, a possible restriction to using this technique to divulge evolutionary implications should be pointed out. The level of conservation such neuroethological characters have over time is currently unknown. How conserved biological features are can be dependent on the involvement of convergent evolution among the taxa of interest. Convergent evolution is the acquisition of similar biological

traits in unrelated lineages, and can be problematic for cladistic studies (Kitazoe et al. 2004). In the past, the role of convergent evolution in the regulation of sub-cellular features (e.g. organelles or other cellular components which may control biochemical processes) has received relatively little attention compared to genetic or morphological characteristics (Elde et al. 2007). It was formerly thought that shared sub-cellular features among taxa were exclusively due to descent from a common ancestor already possessing such characteristics (Elde et al. 2007). However, there is recent evidence to suggest that convergent evolution may be a factor in the adaption of sub-cellular components including secretory organelles involved in vesicle release of neurotransmitters (e.g. Elde et al. 2005; Perry et al. 2006; Elde et al. 2007). If true, this may be a concern for the development of neuroethological taxonomy as a discipline although certainly no more so than for morphological approaches. Nevertheless, a neuroethological approach may still have important applications in taxonomic studies since no clustering technique presently used is without restrictions (including neuroethological taxonomy), and each approach may reveal different aspects of the wider story. This concept is currently in its infancy and much research will have to be conducted to expand it further and to theorise upon evolutionary implications.

The multivariate cluster analysis of marine invertebrate settlement responses from the literature to pharmacological compounds produced some noteworthy results. Had this neuroethological approach shown clustering that did not confer at all with some of the more traditional techniques then it may be argued that this method would unlikely reveal relationships of any value, and have limited application. However, there were striking similarities in the dendrogram created and those produced using morphological and genetic characteristics. The marine mussel clustering of *M. edulis*, *M. galloprovincialis*, and *P. canaliculus* is indistinguishable from the clustering generally agreed upon by modern taxonomists. The true oyster *C. gigas* was separated from all other species, and the separation of the two gastropod species from each other and the remaining bivalves also correlate well with the general molluscan phylogeny model. These features of the dendrogram may suggest that the way in which marine invertebrate larvae respond to

pharmacologically active compounds can be a useful characteristic for identifying important associations among taxa.

Some of the results revealed relationships that are not generally accepted in the taxonomic field. The close clustering of *R. philippinarum* with the two *Pinctada* pearl oyster species demonstrates a similarity in the biochemical pathways involved in larval settlement. However, the two genera that these three species belong to fall under two different orders (i.e. Pteriomorpha and Heterodonta). This clustering cannot be explained by genetic or morphological characteristics. Perhaps this grouping may have uncovered similarities in their biochemistry that are presently unknown. The gastropod *P. sibogae*, like other Opisthobranchs, are considered to have highly evolved nervous systems and unusual neuroethological mechanisms compared with many other molluscan species (Widdows 2001; Mikkelson 2002). While these organisms may be more advanced than the other species analysed, the present investigation demonstrates that components in the signalling pathways of larval settlement is shared to some extent with bivalves and may be more similar to bivalves than to the gastropod *H. rufescens*. However at this stage, the full extent of these similarities and differences remains a mystery due to the low number of compounds incorporated into the analysis.

Oysters are some of the most well known organisms of all marine invertebrate species; however knowledge of their phylogeny and systematics is rudimentary (Lepegue & Boudry 2006). Within the genus *Crassostrea*, significant debate surrounding speciation has existed for many years (Varela et al. 2007). A series of recent publications employing new mitochondrial and nuclear DNA markers have provided fresh evidence that may grant solutions to this quandary (e.g. Cordes et al. 2008, Reece et al. 2008). However, there remains debate over the systematics of the pearl oysters, *Pinctada* genus. Although they are not true oysters, such as *Crassostrea* spp., the question of their most closely related clade is a hot topic. It is currently unknown if pearl oysters are more closely related to true oysters (Ostreidae) or to representatives of the Family Pinnidae, which includes the horse mussel (Southgate & Lucas 2008). In this thesis the pearl oysters

P. fucata martensii and *P. maxima* were only 67% similar to *C. gigas*. Perhaps a comparative neuroethological study of those species involved in the dispute may reveal relationships that could aid in solving the problem. However, at the present time the inductive settlement response data is not available for all of those species. New studies would therefore have to be carried out to gain information for such an analysis.

There is certainly room for new approaches to be incorporated alongside existing taxonomic techniques. Although every taxonomic approach currently used to cluster taxa have their strengths, each technique also is flawed in some way. The traits selected by morphological taxonomists to distinguish speciation and to group higher taxa categories are controversial in many cases (Krishnankutty & Chandrasekaran 2008). Reproducible and quantitative techniques considering chromosomal (Ranganath & Ramachandran 1987), molecular (Padmesh et al. 1999), and behavioural (Ambrose 1987) criteria have often revealed inconsistencies in species resolution by the morphological approach. Speciation can occur in the absence of visible variations in morphology (Templeton 1981). An example of this would be in the cryptic marine mussel genus *Brachidontes*, where some phenotypes are indistinguishable (Terranova et al. 2007). In contrast, morphological variations in many taxa often are not correlated with speciation. An example of this would be the intraspecific variations in morphology observed within species of Echinoderms (e.g. Rahman & Uehara 2004). Phenotype variations within species are common for marine invertebrates and can be dependent on nutritional condition, habitat, gonad maturity, and genetic factors (e.g. Andrews 1987; Johnson et al. 1993; Grabowsky 1994). Homoplasy (i.e. convergent and parallel evolution) is thought to mediate the grouping of taxa when determining phylogenies based on morphological features, more so than molecular data (e.g. Siblet & Ahlquist 1987; Sytsma et al. 1991; Hedges & Sibley 1994; Hedges & Maxson 1996; Givnish & Sytsma 1997). Phenotype characters may interact more directly and frequently with the environment making them less selectively neutral than molecular characters (Wiens et al. 2003). Molecular taxonomy based on genetic information is regarded by some as a superior technique for grouping taxa and contestably considered a better method than

morphological taxonomy (e.g. Tanksley 1983; Crawford 1998; Herbert et al. 2003; Tautz et al. 2003; Blaxter 2004). However, genetic approaches also have their limitations.

One of the main problems associated with phylogenies constructed from genetic data is that the genes selected for the analysis only represent a fraction of the genome. Therefore, portions which may code for very important taxonomic associations are overlooked when only using particular sequences. The selection of sequences also can influence the relationships observed (Herniou et al. 2001; Khiripet 2005; Chan et al. 2006). Although using the whole genome may be arguably better than using a singular component of it, there also are problems connected with such an approach. Firstly, the whole genome has not been sequenced for many species and, therefore, such an analysis becomes impossible for comparing those taxa. Secondly, there are believed to be many sequences within the genome that either do not code for any apparent traits or are not as conserved as other sequences. Incorporation of these sequences into cladistic studies creates noise that may override any associations formed among the useful portions (e.g. Montague & Hutchison 2000; Herniou et al. 2001; Henz et al. 2005). However, new evidence uncovering the purpose of these 'non-coding' sequences, and increased knowledge about the levels of conservation in particular genetic fragments, suggest that the use of 'non-coding' sequences in taxonomic studies may have significant applications. Whole genome analysis in phylogenetic studies is becoming more widely accepted (Creer 2007).

Chemotaxonomy as a discipline (i.e. the biological classification based on similarities of particular metabolite structures among taxa) also may have weaknesses. Exogenous uptake and bioaccumulation of compounds from the environment may interfere with the assumed endogenous biochemical profiles of marine organisms. In red seaweeds, adjacent species of *Microcladia* and *Plocamium*, belonging to the orders Ceramiales and Plocamiales, respectively, can concentrate the same terpenoids from seawater (Naylor et al. 1983). In another study involving red seaweeds, the same halogenated monoterpenes were found in *Plocamium cartilagineum* and *Schottera*

nicaeensis, also belonging to different orders (i.e. Plocamiales and Gigartinales respectively; Rivera et al. 1987). However, it has been demonstrated that different populations of *P. cartilagineum* contain diverse terpenoid chemotypes (San-Martin & Rovirosa 1986), and it was suggested by Cole and Sheath (1990) that the differences were due the absorption of these compounds from the external environment. Therefore, any chemotaxonomic associations formed among taxa of particular marine macroalgae based on terpenoid chemistry may not actually be true. In the case of seaweeds, since chemotaxonomy assumes terpenoids are specific to different orders, and are produced internally, this highlights one of the problems associated with such an approach. It is not impossible that marine invertebrates also uptake various compounds from the environment which could lead to the misinterpretation of chemotaxonomic data for these organisms. Although, as long as the compounds are carefully selected to reveal associations without such possible interferences, chemotaxonomy can be a useful approach. As a benefit for the neuroethological approach developed in this thesis, consideration does not have to be given to the possibility of exogenous uptake of metabolites. The behavioural responses to pharmacologically active compounds are not affected by earlier bioaccumulation of various molecules from seawater. On a more positive note for chemotaxonomy, it has been suggested that the analysis of proteins rather than other metabolites can be useful indicators of genetic relationships (Kikuchi & Tamiya 1987; Moriera et al. 1995). It is well established that protein coding sequences in DNA are more conserved than other fragments (Kang 2002). The structures of many proteins, being less subject to natural selection and more closely controlled by the genes, are more conserved over time than anatomical characteristics (e.g. Schmelz 1996). Therefore, analysis of these structures can enable chemotaxonomy to have advantages over morphology-based techniques. Since proteins (i.e. receptors) play a significant role in signalling pathways, perhaps this also works in the favour of a neuroethological approach.

Certainly, the fundamental reasons for the differences in the way marine invertebrates respond to their natural environment through chemoreception, and the biochemical mechanisms involved in their behaviours, must be controlled by evolutionary processes.

To resolve questions around evolutionary implications of the novel form of taxa clustering developed in this thesis, an extensive cross disciplinary investigation involving specialists in neurobiology, biochemistry, genetics, evolution, and ecology would be necessary.

The separations of species performed by traditional taxonomic techniques undoubtedly are just in many cases. However, there may be deeper underlying roots which link organisms together that have not currently been elucidated. There is an urgent need for compilation and incorporation of non-morphological features into taxonomic studies. Fundamental processes that may account for contrasting molecular and morphological datasets (e.g. convergent evolution, natural selection, morphological and ecological plasticity, cryptic taxa, and gene transfer) will only be discerned if there are many sources of data (i.e. morphological, genetic, behavioural, ecological, physiological, cytogenic, and biochemical) to position irregularities into context (Lipscomb et al. 2003; Krishnankutty & Chandrasekaran 2008). This collaboration will reveal a multi-dimensional concept of species which will undoubtedly be more accurate than current species discriminations. Molecular-only methods may be an effective means for assessing biodiversity by non-taxonomists but in isolation the results are not very meaningful (Limbscombe et al. 2003). Providing a 'total evidence' approach by combining disciplines, the full story of evolution can be unravelled and a complete picture brought into focus. All sources of data can be united to help describe and explain the diversity of life. Neuroethological taxonomy could be utilised as a building block within this framework and offer important insights into the evolution of marine invertebrate taxa.

In summary: (1) the larval responses of marine invertebrate species to pharmacologically active compounds reveal important information about the biochemical pathways involved in settlement behaviour. (2) These responses may be useful for determining presently unknown associations among taxa. (3) The neuroethological approach to species clustering in the current study produced results that confer with morphological and genetic techniques in some cases (e.g. marine mussel clustering and separation of

gastropods from bivalves), but some associations did not agree with traditional taxonomic grouping (e.g. similarities between *Pinctada* spp. and *R. philippinarum*). (4) The clustering approach developed in this thesis may have identified a currently unknown relationship between *Pinctada* spp. and *R. philippinarum*. (5) All current taxonomic approaches are flawed in some way, and there is the possibility that convergent evolution may influence neuroethological clustering to a degree, as is the case with morphological based techniques. (6) The biochemical pathways involved in behaviour modulation (e.g. larval settlement) are most likely controlled by genetics, and differences in behavioural responses to exogenous compounds may indicate genetic variations. Hence, the neuroethologies of organisms may have implications for evolutionary studies. (7) Genetic taxonomy depends on selection of particular DNA sequences, and important characteristics may be overlooked using such methods. Whereas differing behavioural responses to pharmacologically active compounds suggests genetic variation and does not depend on the selection of specific sequences. Therefore, a neuroethological approach to species clustering may be less prone to errors in some cases. (8) New forms of cladistic approaches are required to determine species diversity, and incorporation of neuroethological taxonomy may provide a partial solution for developing a framework for multi-dimensional species clustering.

CHAPTER 11

EFFECTS OF CHARGED SURFACES & ACETYLCHOLINE ON SPAT ATTRACTION, ATTACHMENT & RETENTION

1. INTRODUCTION

This chapter details the investigation into the effects of charged surfaces on *Perna canaliculus* spat attachment, and retention. Also, the effect of exogenously applied acetylcholine on mussel spat retention and the effect of surface bound acetylcholine on spat attraction are examined.

The inspiration for this chapter manifested after a provisional patent was granted halfway though this thesis for the use of acetylcholine in aquaculture (Young et al. 2008). The patent was filed specifically detailing the use of acetylcholine for enhancing larval settlement within confined hatchery systems. Because part of the patenting process involved the identification of other possible applications, it was conceived that the technology may be of use in other areas, or stages, of the mussel culturing practice. Two possible applications for using acetylcholine were considered which may improve the sustainability of this industry.

(1) Enhancing wild spat collection techniques.
(2) Enhancing mussel spat retention on growing, or holding ropes.

The mussel aquaculture industry in New Zealand relies heavily on obtaining juvenile mussel spat from natural stocks and re-settling them onto ropes for on-growing (Alfaro & Jeffs 2002). Mussel spat are found densely attached to drifting macroalgae which are regularly washed up on the foreshore (Alfaro & Jeffs, 2002; Alfaro et al., 2004). The event occurs solely at west coast locations in the northern region of the North Island of New Zealand (Ninety Mile Beach). This collection depends on local environmental conditions, leading to seasonal variations in the supply of mussel spat.

Collection of 'beach cast spat' is also often unreliable – instances have occurred in the past where no spat has been washed up for a whole season, causing dire economical consequences for the mussel farmers. Therefore, considerable commercial efforts have been made to aquire juvenile mussels by other techniques. One such method is the use of 'spat catching ropes'. Catching drifting larvae, or very small spat, from the water column has demonstrated to be successful with at least one New Zealand company (Kaitaia Spat Ltd.) specialising in such collection. By deploying synthetic polypropylene or natural coir (coconut husk) ropes, very young pelagic mussels moving through the water column tend to freely attach when they come into contact with the substrates. The ropes generally are placed at specified locations where high numbers of larvae/spat are known to occur, at paticular times of the year. These ropes are left in the water column for around 4 weeks, during which time larvae hopefully attach. Although successful, there may be scope to improve this catching technique by increasing the numbers of larvae/spat that attach to a given area of substrate. It is possible that this could be achieved by providing different substrates to those currently used (i.e. polypropylene and coir rope). For example, attachments may be increased by utilising substrates with attachment enhancing stuctural properties (e.g. highly filamentous structure or increased porosities). Alteration of chemical surface properties (e.g. hydrophobicity and surface charge) may also be useful for modulating attachments of the young mussels, potentially promoting adhesion. Altering these properties could enhance spat collection. It is also possible that incorporation of a chemical attractant into spat catch ropes could persuade more individuals to contact the substrate, therefore enhancing attachments and spat collections.

Once spat are collected and harvested, they are brought back to a land-based holding facility for ongrowing until they are a suitable size for re-seeding and distribution to individual farms. During this holding period between capture and distribution, significant numbers of juvenile mussels are lost through poor retention (C. Hensley, director, Kaitaia Spat Ltd, November 6, 2008). There is a significant oppourtunity to enhance the retention of spat on growing ropes. One possibility would be to determine if

the properties of the rope substrates have an effect on spat retention. By improving these properties, spat retention may be enhanced. Alternatively, since mussels are retained in enclosed systems at this holding stage, there is the possibility that exogenously applied compounds to the water could cause spat retention enhancing effects.

To investigate some of the aspects outlined, which may improve current mussel industry processes and techniques, the potential use of acetylcholine was studied. It is possible that acetylcholine may have applications as a chemical attractant if bound to a substrate, such as a synthetic rope. This may enhance pelagic spat catchments. During this investigation, a method of binding acetylcholine to a surface was required. Acetylcholine, being a positively charged quaternary ammonium compound, can be retained on negatively charged surfaces, such as ethylvinylbenzene/divinylbenzene copolymers containing sulphonated side groups (Dionex 1991; Dionex 2003). Such polymers are commonly used for chromatographic separations of acetylcholine in mixed solutions. Therefore, to provide a substrate for testing the effects of ionically bound acetylcholine on spat attraction, acetylcholine was bound to a cation exchange resin with suitable surface chemistry. Acetylcholine also may have applications as a water treatment additive in enclosed systems to improve mussel retention in land-based operations. Because acetylcholine previously demonstrated an ability to affect *P. canaliculus* larvae (i.e. increasing settlements, see Chapter 6), it was considered possible that acetylcholine may also interact biochemically with the older mussel spat and produce retention enhancing effects. In order to investigate this, the effect of dissolved acetylcholine on spat retention was determined with mussel spat attached to glass substrates and rope substrates.

The effects of some physicochemical properties of marine surfaces on biofouling has been extensively reasearched in the literature. For example, hydrophyllic surfaces are known to promote colonisations of marine bacteria (Dexter et al. 1975), and promote the adhesive attachments of mussels (Aldred et al. 2006; Petrone et al. 2008b), and

barnacles (Rittschof & Costlow 1989). However, little research, if any, has has been conducted on the effects of negatively, or positively, charged surfaces on bivalve attachments and retentions to substrata. Therefore, a significant opportunity is presented to do so. It is possible that spat prefer to attach, and remain, on substrates with specific surface charge characteristics. If true, numbers of wild spat collected may be increased, and the retention of mussels in land-based operations enhanced. Furthermore, identifying substrates which improve attachments may assist in other areas of the mussel industry. Beach cast spat, collected from Ninety Mile Beach, is distibuted to mussel farms for re-seeding onto grow-ropes. During this process, the seaweed to which the spat are attached to is introduced to the ropes, then surrounded by a thin mesh stocking. Once these ropes are deployed in the ocean, the stocking and macroalgae disintegrate – the spat then attach directly to the ropes. Providing an attractive rope surface for the young mussels during this process may increase the numbers of spat which attach, thereby minimising spat losses which commonly occur. The effect of surface charge on mussel spat attachment and retention was investigated in this thesis using two synthetic nylon polymers that were highly hydrophyllic and porous (to promote mussel spat attachments and enhance the strength of the adhesive bonding), but containing oppositely charged pole characteristics.

2. METHODS

2.1 Organisms

Mussel spat ranging from 0.5 to 5.0 mm in diameter were collected from Ninety Mile Beach, northern New Zealand, during July 2008. Spat were found attached to clumps of drifting macroalgae washed up on the surfzone. During transport to the AUT aquaculture facility in Auckland, seaweed clumps were placed in bags and kept cool and moist. Upon arrival the spat, still attached to the macroalgae, was placed in an aerated 150L tank with a re-circulating water system incorporating a bio-filter, chiller unit, and protein skimmer. During the holding period, flow rates were set to approximately 2Lmin^{-1} and water temperature was maintained at 17±1ºC. Organisms were fed a mixed microalgal diet of *Pavlova lutherii* and *Isochrysis galbana*, which were cultured as previously described in Chapter 2 (p45), but on a larger scale in 25 L containers. Daily food rations were provided to allow *ad libitum* feeding for 4–6 hours, during which time the re-circulating water was turned off. Calculations to determine accurate feeding rations were difficult to achieve since unknown quantities of spat were obtained, and accurate dry tissue mass of such small spat could not be resolved. Excess food rations were not deemed problematic due to the re-circulating water system being turned back on after the set feeding period. Also, it was assumed that a limited food supply would have been supplemented by the large quantities of macroalgae present, undoubtedly providing a source of nutrition from bacteria and microalgae growing and being released into the water from their surfaces.

To obtain healthy motile individuals for all assays, rather than manually stripping the macroalgae of spat, clumps were taken and placed in 5 L beakers containing fresh seawater and gently agitated and stirred for 2–3 minutes. After 24 hours, individuals that detached from the macroalgae, and moved vertically up the sides of the beakers to the water/air interface, were collected. This procedure maintained that only live healthy

individuals, capable of attachment and translocation, were selected. Using a double stacked sieve apparatus immersed in seawater, spat were filtered with fresh seawater and only those between 1–3 mm in diameter used for all assays.

2.2 Spat attachment assays: Charged surfaces

The effect of charged surfaces on spat attachment rates were determined using two types of substrates – modified nylon-6,6 membranes and styrene-based resin beads. The selections of these materials were chosen since each type can be sourced containing strong positive or negative surface charges. Although the resin beads and the membranes have very different physical and chemical characteristics, the charged surface subtypes of each are very similar in physical structure and also contain particular similarities in chemical composition. One limitation of these attachment studies is that no 'neutrally charged' subtype of the resin beads, or the membranes, could be sourced to provide a control substrate. Although options were available to obtain neutral surfaces, these surfaces did not have the same physical characteristics and would therefore not serve as controls.

2.2.1 Biodyne® Nylon Transfer Membranes

Biodyne® Nylon Transfer Membranes, commonly used for DNA separations, were selected since they provide the unique physicochemical characteristics required for this study. A variety of these membranes, composed of modified nylon-6,6, are manufactured each with specific chemical properties which determine their charge characteristics. The negatively charged, or anionic, membrane selected for this study contains a high density of strongly anionic carboxyl functional groups. The positively charged, or cationic, membrane selected contains a high density of strongly cationic quaternary ammonium functional groups. Both membranes are highly hydrophilic,

identical in porosity (0.45 µm), and indistinguishable in physical structure, providing a uniform smooth flat surface which spat can easily attach to.

Preparation of the membranes to provide a firm surface that could be handled and immersed in water was required. This was done by cutting 2.5x7.6 cm pieces of nylon transfer membrane and attaching them to glass microscope slides along the edges with water resistant adhesive tape. Slides were placed membrane-side-up in a 40x30x7 cm tray and covered with approximately 1 cm of FSW. Twenty individual spat were placed on each slide and allowed 2 hours to attach. The effects of surface charge on spat attachment using nylon transfer membranes was not determined for longer exposure periods due to the limited amount of substrate obtained. After 2 hours, slides were then carefully removed from the tray and slowly dipped twice vertically in a 2 L beaker containing FSW. After this process, only those individuals that were maintained on the filter membranes were considered firmly attached. Ten replicates were performed for each treatment. Attachment assays were conducted at 17±1°C under ambient light in static conditions.

2.2.2 Ion Exchange Resins

Anion and cation ion exchange resins, commonly used for column chromatography, were selected since they provided similar charge characteristics as the nylon transfer membranes, but different physical structure (microbeads). A large variety of resins are available with varying physicochemical properties. The negatively charged product chosen, Amberlite IR-120 (Na), is a strongly acidic cation exchange resin composed of styrene divinylbenzene copolymer with sulphonated functional groups. The positively charged product chosen, Amberlite IR-400 (Cl), is a strongly basic anion exchange resin also composed of styrene divinylbenzene copolymer, but with quaternary ammonium functional groups. These two products were selected from an extensive list of possible resins since they are very similar in all aspects other than surface charge (i.e. porosity, particle size, wettability, and exchange capacities), see Table 15.

Table 15. Properties of ion exchange resins selected for spat attachment assay (modified from Sigma-Aldrich 2008).

Exchanger	Product (Amberlite)	% cross linking	Matrix	Bead size wet (US mesh)	Ionic form	Total Exchange Capacity		pH range
						meq/ml	meq/g	
Cation	IR-120 (Na)	8	Gel	16–50	Na^+	1.9	4.4	0–14
Anion	IRA-400 (Cl)	8	Gel	16–50	Cl^-	1.4	3.8	0–14

Ion exchange resins were reconstituted according to the manufacturer's specifications. Briefly, the resins were washed through a 250 ml glass chromatography column with 10X volume of 8% HCl (cation exchange resin) or 4% NaOH (anion exchange resin) at a flow rate of 2 ml/min. Resins were rinsed with 10X volume of deionised (DI) water, or until pH was equal to 5.5 (the pH of the DI water used for washing ion exchange resins was predetermined as being 5.5 using the calomel electrode available – low ionic strength and electrostatic interference may have caused this value to be lower than expected). Due to the possibility that the reconstituted resins may indirectly cause adverse effects to the organisms by sequestering anions and cations from the experimental medium (desalination), resin surfaces were equilibrated in seawater. The charged beads were placed in two 2 L beakers with FSW and stirred continuously with a magnetic stirrer bar. The FSW was replaced every 10 minutes for 30 minutes.

Multi-welled concave ceramic spot plates were positioned in 40x30x7 cm trays and covered with 1 cm of FSW. Approximately 1 g of ion exchange resin treatments were carefully added by pipette to each well and three spat individuals placed on top of the resin. After 2, 24, and 48 hours spat were carefully removed with a pair of tweezers and the presence or absence of resin attached to the mucus adhesive recorded. Twelve replicates were performed for each treatment.

2.3 Spat attraction assay: Bound acetylcholine

To determine the attraction capability of acetylcholine, the compound was immobilised and bound, through ionic bonding, to a cation exchange resin (Amberlite IR-120 (Na)). The resin was reconstituted as previously described and placed in 500ml of freshly prepared $1x10^{-2}$ M solution of acetylcholine chloride in DI H_2O. The resin was stirred continuously with a magnetic stirring bar and the acetylcholine chloride solution renewed every 15 minutes for 2 hours. The acetylcholine solution was decanted and washed twice with 1 L of FSW to prevent addition of DI H_2O into the experimental medium and equilibrate the surfaces of the exchange resin. Controls consisted of reconstituted cation exchange resin with no bound acetylcholine. Multi-welled concave ceramic spot plates were placed in 40x30x7 cm trays and covered with 1 cm of FSW. Approximately 1 g of treatment or control resins were placed strategically, arranged in various positions on the spot plates. Utilising a square section of each spot plate consisting of 9 wells for each replicate, all possible location combinations of treatment and control resins were determined (Figure 54). Designs 1, 2, and 3 were configured as choice experiments, where mussel spat had three choices in each design. Spat had the option of moving towards control resins, moving towards treatment resins, or moving towards neither. In design 4, control and treatment resins were separated and placed into two individuals ceramic spot plates. Spat only had 2 choices – moving towards the respective resin or not. Ten individuals were placed in every centre well. Six replicates were performed for each design combination.

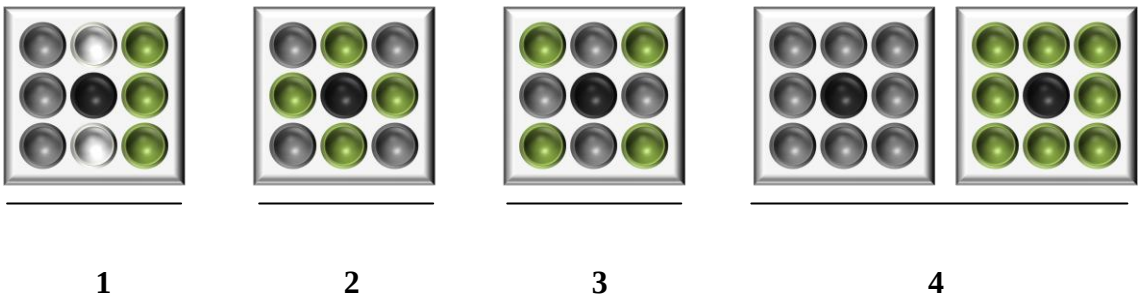

Figure 54. Combination designs of treatment wells for determining attraction of spat to acetylcholine. Coloured wells represent anion exchange resin with bound acetylcholine (green), or control resin with no acetylcholine (grey). White wells are empty. Middle black wells represent placement of spat. Designs 1, 2 & 3 are choice experiments, whereas locations of control and treatment resins in design 4 are independent of each other.

After 2, 24, and 48 hours, the number of spat which had relocated from the centre well into any treatment wells were counted. Spat which had either moved out of the experimental area or to the region between treatment wells were not considered attracted by either treatment. All attraction assays were conducted at 17±1°C under ambient light in static conditions.

2.4 Spat retention assays

To investigate ecological implications and possible commercial applications, the effect of charged surfaces on spat retention was determined. Also, due to a provisional patent being granted, i.e. Young et al. (2008), for the use of acetylcholine in aquaculture to induce larval settlement, the effect of exogenously applied acetylcholine on spat attraction and retention was investigated to extend the range of viable commercial applications of this technology. In the acetylcholine assays, two substrates were used, glass and coir rope. Since the spat retention experiments were partially designed to examine possible solutions to the commercial problem of poor spat retention on growing ropes, it was important that the experimental medium provided an environment that did not encourage optimal retention. For this reason, all assays were conducted without aeration or periodical water changes. Mussel spat also were not fed over the duration of the trials.

2.4.1 Charged surfaces

Biodyne® Nylon Transfer Membranes were used to determine the effect of charged surfaces on the retention of spat after a firm attachment had been established. Surfaces were prepared on microscope slides as previously described in the attachment study on p242. Slides were placed membrane-side-up in a 40x30x7 cm tray and covered with around 1 cm of FSW. Approximately 30–40 spat were placed on each treatment surface

and allowed 24 hours to attach (the extended time period was necessary due to short time exposure effects of negative surface charge on spat attachment – see Results section, p250. Also, the higher number of individuals placed on the membrane treatment surfaces were higher than the 20–30 used in other retention experiments (i.e. using glass substrates) because of their highly mobile nature – during the 24 hours, some mussel spat tended to relocate away from the treatment surfaces. After 24 hours, slides were removed from the trays and suspended vertically in separate 1 L beakers filled with FSW. A proportion of attached spat were often observed to detach from the substrates within the first couple of hours of suspension – possibly due to the disturbance created by relocation of the slides and/or sensing a change in plane through the statocyst organ. Because of this initial induced stress, individuals were given four hours to equilibrate before T_0 was recorded. At this time, the total numbers of spat remaining on each treatment surface were considered as 100% retained. Spat attached to nylon transfer membranes were counted every 24 hours for 5 days. Ten replicates were performed for each treatment surface, with assays being conducted at 17±1°C under ambient light.

2.4.2 Acetylcholine

2.4.2.1 Glass substrate

Microscope slides were used for the substrate in this experiment. Slides were placed in a 40x30x7 cm tray and covered with approximately 1 cm of FSW. Around 20 to 30 spat were placed on each slide and were given 4 hours to attach. After this period, slides were suspended vertically in 1 L beakers containing a control medium (FSW), or acetylcholine treatment solutions. Treatment solutions were prepared at concentrations of $1x10^{-5}$ M and $1x10^{-4}$ M in FSW. Due to the toxic effect of acetylcholine at higher concentrations (see Results section, p258), $1x10^{-4}$ M was the highest concentration assayed. Individuals were given 4 hours to equilibrate before T_0 was recorded. Mussel spat retention on the glass surfaces were quantified every 12 hours, for 60 hours. Ten replicates were performed for every control and treatment level. Assays were conducted at 17±1°C under ambient light.

2.4.2.2 Coir substrate

Due to the results of the effects of exogenously applied acetylcholine on spat retention when attached to glass surfaces (see Results section, p254), the assay was repeated using a filamentous surface that is commonly used within the industry, coir. Approximately 20–30 spat were placed into medium sized glass test tubes containing FSW. Pieces of 5 mm diameter coir rope were cut into 15cm lengths and inserted. The tubes were inverted and rotated a few times to distribute the spat along the length of the coir and left for 24 hours in a slanted position to allow spat to attach. After 24 hours, the coir substrates with attached spat were suspended vertically in 1 L beakers containing FSW and given 4 hours to equilibrate before T_0 was recorded. A $1x10^{-4}$ M acetylcholine solution was prepared in FSW to serve as the treatment medium. The control medium consisted of FSW only. Spat retention was recorded every 24 hours for 168 hours. Nine replicates were performed for every control and treatment, with assays being conducted at 17±1°C under ambient light.

2.5 Acetylcholine toxicology

The acute toxicity of acetylcholine on *P. canaliculus* spat was investigated to determine maximal concentrations that may be used for possible aquaculture applications without short-term lethal effects. After a pilot study to determine the approximate range of concentrations required, $1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$, and $1x10^{-2}$ M acetylcholine chloride treatment solutions were prepared in FSW. The Control medium consisted of FSW only. Around 30–40 individuals were placed in 100 ml beakers, and 80 ml of control or treatment solution was added. Using the standard exposure time of 72 hours for determining lethal concentration values (LC_x), beakers were left to stand under static and ambient light conditions at 17±1°C for the full duration of the study. Organisms were not fed during the assays. Three replicates were performed for the control, and for each treatment level. Beakers were checked routinely and any dead individuals removed. At the end of the exposure period, individuals were placed in a large tray,

submerged with FSW, and left to stand for 30 minutes. Mussel spat were carefully observed for a further 30 minutes while determining mortality rates. Any individual which exhibited constant shell gaping and resisted manual closure were considered dead. Any individual that did not display evidence of shell gaping movements, relocation, attachment, or substrate searching behaviours within the 30 minute observation period were considered dead.

2.6 Statistics

To compare treatment effects in this chapter, 2-sample t-tests were used for parametric data. In this case, normality and homogeneity of variance was tested according to the methods outlined for ANOVA analysis in Chapter 3, p64. Data that were non-parametric was analysed using the Mann-Whitney test. All data were arcsin transformed, and all analyses were performed using statistical software, Minitab v.15. Spat toxicology in response to acetylcholine was determined by probit analysis. Using specialised software, EPA Probit Analysis Program Used for Calculating LC/EC Values Version 1.5 (see the Methods section in Chapter 4 for further information), estimated LC values were calculated.

3. RESULTS

3.1 Spat attachment: Charged surfaces

Using Biodyne® nylon transfer membranes and ion exchange resins, the effect of substrate surface charge was investigated on the attachment of mussel spat (Table 16). After placing spat on the membranes for 2 hours, a significant difference in attachment was detected between the negatively and positively charged surfaces (t-test: $p<0.01$), with 10% and 66% attachment being observed respectively. Attachment of mussel spat to negatively and positively charged ion exchange resins, also revealed differences between treatments after 2 hours exposure (Mann-Whitney; $U_{12,12}=108$; $p<0.05$). After 24 hours, attachment on the cationic surface increased from approximately 10% to 70%, and the difference between treatments were more significant (Mann-Whitney; $U_{12,12}=144$; $p<0.001$). Significant difference in mussel attachment between resin treatments were still apparent after 48 hours exposure (Mann-Whitney; $U_{12,12}=144$; $p<0.001$). No attachment of spat to the negatively charged ion exchange resin was observed during the experiment. The combined results of this investigation reveal that juvenile *P. canaliculus* mussels preferentially attach to positively charged substrates.

Table 16. Effect of surface charge on mussel spat attachment.

Treatment Substrate	Exposure (hrs)	Negative surface mean ± S.E. (%)	Positive surface mean ± S.E. (%)	*p*-value
Nylon Transfer Membranes	2	10.0 ± 3.0	66.0 ± 4.9	0.001[a]
Ion Exchange Resins	2	0.0	11.1 ± 4.7	0.039[b]
	24	0.0	72.2 ± 6.9	0.000[b]
	48	0.0	66.7± 7.1	0.000[b]

[a] T-test statistic.
[b] Mann-Whitney statistics.

3.2 Spat attraction: Bound acetylcholine

Attractions of mussel spat to negatively charged ion exchange resins (Amberlite IR-120(Na)), with or without bound acetylcholine are shown in Table 17. Details of experimental designs can be found in the Methods section, p245. In five cases out of eight, the mean percent of spat which had moved towards the resin with bound acetylcholine was slightly higher than that which moved towards the control treatment (resin with no bound acetylcholine). However, due to the low numbers of spat which were attracted to either treatment, combined with the high variation, Mann-Whitney analyses detected no statistical differences between spat attractions to either treatments, in any of the designs, at either of the exposure times allowed (i.e. 24 and 48 hours).

Table 17. Attraction of mussel spat to acetylcholine ionically bound to anion exchange resin after 24 and 48 hour exposures.

Design[a]	Exposure (hrs)	Control mean ± S.D. (%)	Treatment mean ± S.D. (%)	*p*-value[b]
1	48	0.0	1.7 ± 4.1	0.700
	72	1.7 ± 4.1	3.3 ± 5.2	0.700
2	48	0.0	0.0	1.000
	72	0.0	1.7 ± 4.1	0.700
3	48	0.0	0.0	1.000
	72	0.0	0.0	1.000
4	48	6.7 ± 12.1	10.0 ± 7.1	0.329
	72	8.3 ± 13.3	24.0 ± 16.7	0.177

[a] See Figure 54 in the Methods section for details of design combinations of treatment wells.
[b] Mann-Whitney statistics.

3.3 Spat retention

3.3.1 Charged surfaces

From the results of the attachment study on charged nylon transfer membranes, it was apparent that after 2 hours, only around 10% of mussel spat attached (see Results section p250). To investigate the effects of surface charge on spat retention, obtaining positively and negatively charged membranes with sufficient attached spat was essential. Although it was determined that spat do not attach frequently to anionic membranes during short exposures (i.e. 2 hours), pilot work showed that given 24 hours, spat will attach. However, for this to occur, increased numbers of spat were initially required to be placed on treatment surfaces (i.e. 30–40). Unfortunately, the numbers of spat attached after 24 hours in this investigation could not be incorporated into the results of the attachment assays since mussel spat were highly mobile during the 24 hours. It was impossible to accurately quantify attachment *vs*. non-attachment for each replicate surface with the spat freely moving around.

Once membrane substrates were obtained after 24 hours, with approximately equal numbers of attached spat, the effects of surface charge on mussel spat retention was investigated (Figure 55). The results reveal little difference in spat retention between treatments over a period of five days. A gradual decrease in retention was observed over time for both treatments. Observations were made every 24 hours during the experiment. However, at no time was the number of retained mussels on either treatment surface significantly different from the other surface (see Table 18 for results of statistical analyses). At the end of the trial, approximately 72% of mussels remained attached to the positively charged membranes, and around 66% remained attached to the negatively charged membranes.

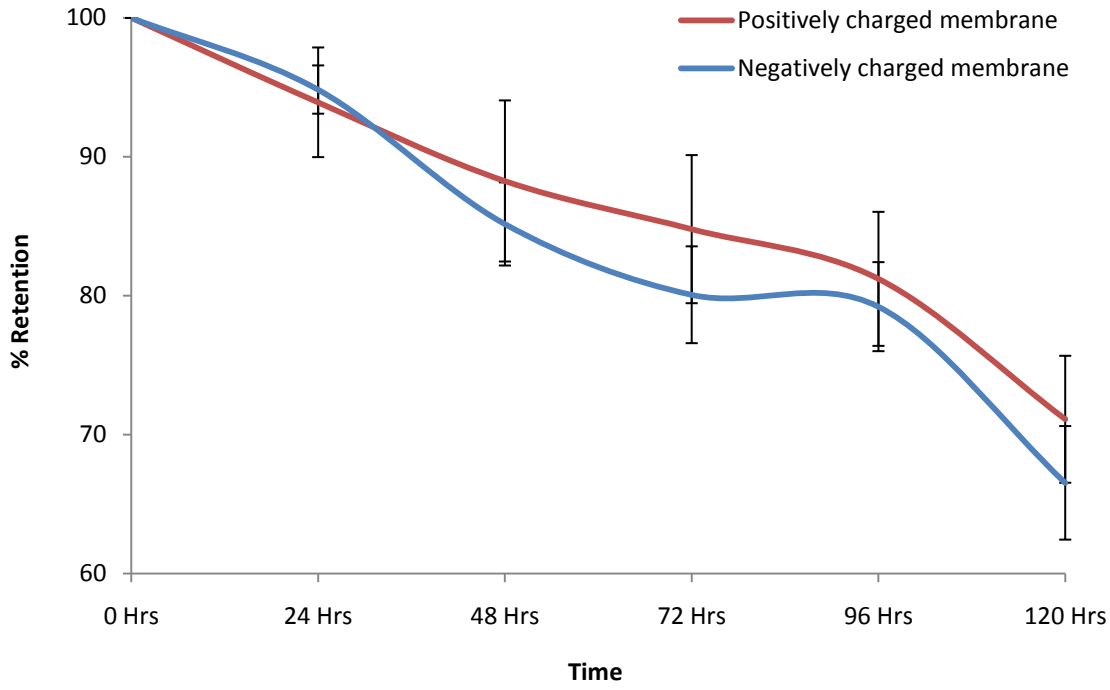

Figure 55. Effect of charged surfaces on spat retention using Biodyne ® Nylon Transfer Membranes.

Table 18. Effect of charged surfaces on *P. canaliculus* spat retention. Summary of statistics (arcsin transformed data; 2-sample t-tests).

Time (hrs)	Negative surface mean ± S.D. (arcsin)	Positive surface mean ± S.D. (arcsin)	Pooled DF	*t*-value	*p*-value
24	81.58 ± 13.06	80.03 ± 9.23	18	0.31	0.764
48	68.97 ± 9.59	75.307 ± 16.33	18	1.06	0.304
72	64.82 ± 7.85	70.12 ± 14.14	18	1.04	0.314
96	63.36 ± 6.86	65.24 ± 9.95	18	0.49	0.628
120	57.92 ± 7.79	58.08 ± 9.34	18	0.82	0.422

3.3.2 Acetylcholine

3.3.2.1 Glass substrates

Mussel spat attached to glass microscope slides were assayed to determine the effects of exogenously applied acetylcholine, at a concentration of $1x10^{-5}$ M, on spat retention over 60 hours (Figure 56). In this experiment, a large decrease in spat retention was observed after 12 hours in both the control and treatment assays. These declines in retention slowed dramatically after 12 hours of exposure, very gradually declining thereafter until completion of the trial. After every 12 hour observation, mean percent retention was higher when bathed in acetylcholine treatment solutions than in FSW. However, at only two time periods (12 & 24 hours) was spat retention significantly higher in the treatment than in the control assays (see Table 19 for results of statistical analyses).

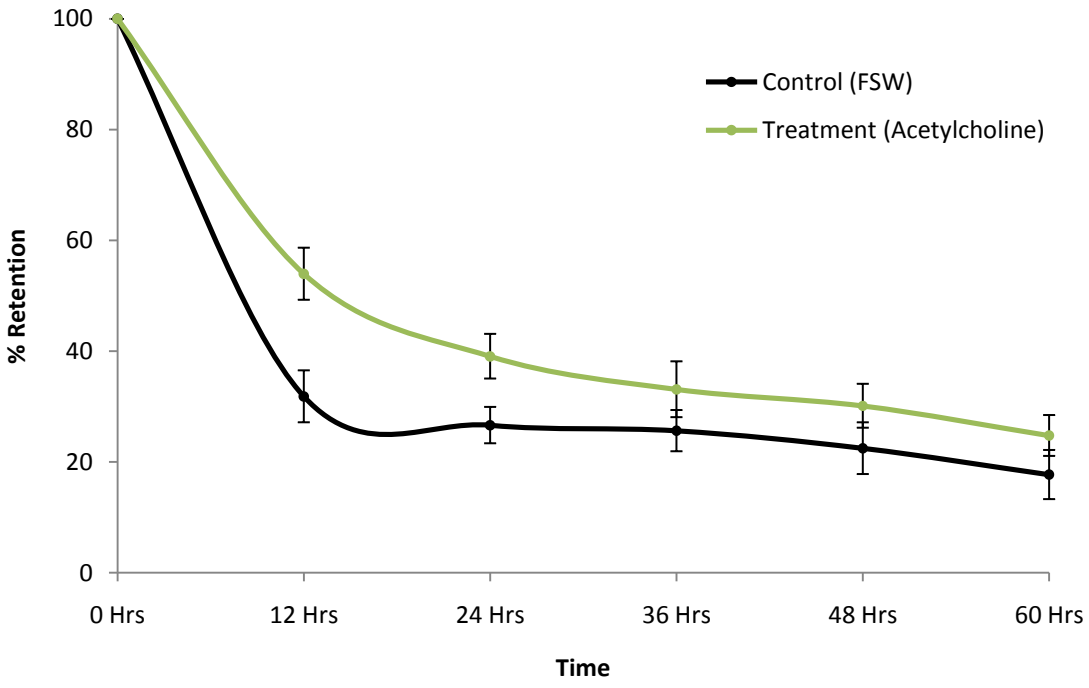

Figure 56. Effect of acetylcholine ($1x10^{-5}$ M) on retention of *P. canaliculus* spat attached to glass substrates. Data plotted are mean ± standard error of 10 replicates.

Table 19. Effect of acetylcholine ($1x10^{-5}$ M) on retention of *P. canaliculus* spat attached to glass substrates**.** Summary of statistics (arcsin transformed data; 2-sample t-tests).

Time (hrs)	Control mean ± S.D. (arcsin)	Treatment mean ± S.D. (arcsin)	Pooled DF	*t*-value	*p*-value
12	34.02 ± 6.98	47.32 ± 6.12	18	3.21	0.013
24	30.92 ± 4.66	38.60 ± 5.35	18	2.42	0.042
36	30.20 ± 5.39	34.94 ± 6.76	18	1.23	0.255
48	27.84 ± 7.07	33.10 ± 5.53	18	1.31	0.226
60	24.04 ± 8.18	29.64 ± 5.28	18	1.29	0.234

At a higher concentration of acetylcholine, $1x10^{-4}$ M (the optimal concentration demonstrated to induce larval settlement – see Chapter 6, Results section, p144), spat retention is considerably improved over control assays (Figure 57). During the 60 hour trial, a gradual decrease in mean percent retention was observed in both the control and treatment assays. At completion of the experiment, approximately 70% of mussels remained attached to the glass substrates when bathed in the acetylcholine treatment. In the control medium (FSW), around 35% of mussels remained attached – half of the proportion in the treatment assays. Variation in mean percent spat retention was generally less in the acetylcholine treatments than that of the control assays. Statistical analyses reveal that significant differences in mussel retention occurs between the controls and treatments at four out of the five 12 hourly observations (i.e. no difference detected after 24 hours), throughout the trial (Table 20).

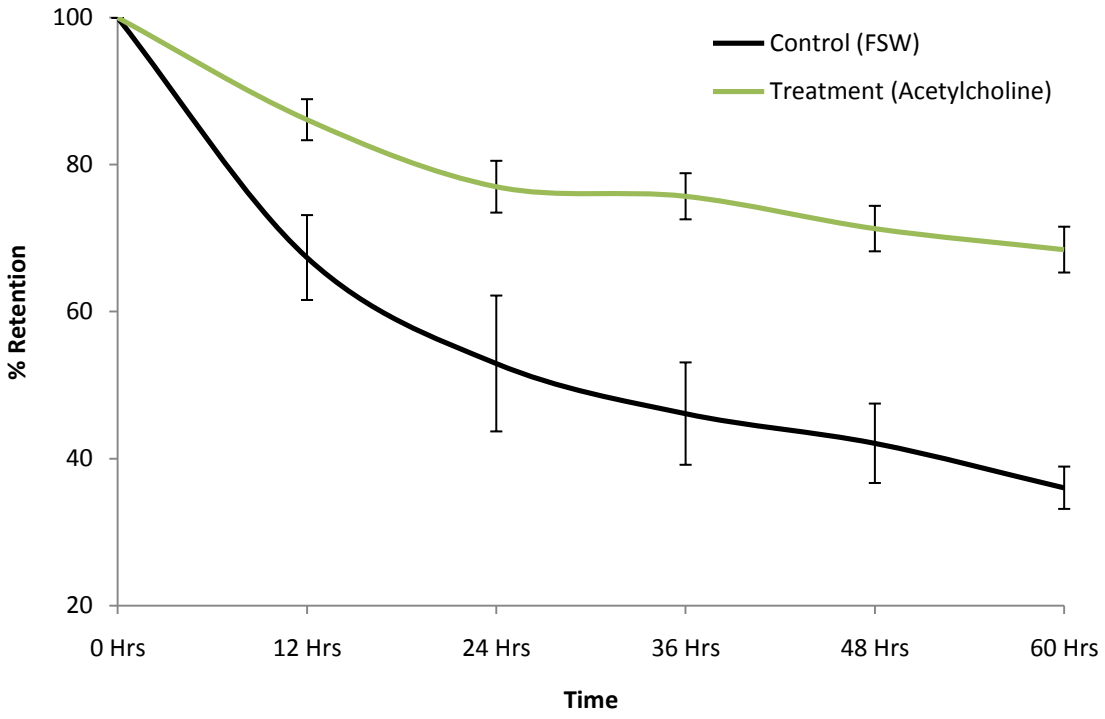

Figure 57. Effect of acetylcholine ($1x10^{-4}$ M) on retention of *P. canaliculus* spat attached to glass substrates. Data plotted are mean ± standard error of 10 replicates.

Table 20. Effect of acetylcholine ($1x10^{-4}$ M) on retention of *P. canaliculus* spat attached to glass substrates**.** Summary of statistics (arcsin transformed data; 2-sample t-tests).

Time (hrs)	Control mean ± S.D. (arcsin)	Treatment mean ± S.D. (arcsin)	Pooled DF	*t*-value	*p*-value
12	55.66 ± 8.74	68.56 ± 5.39	18	2.81	0.023
24	47.32 ± 13.26	61.64 ± 2.52	18	2.22	0.057
36	42.82 ± 9.18	60.66 ± 4.81	18	3.85	0.005
48	40.38 ± 7.04	57.72 ± 4.43	18	4.66	0.002
60	36.84 ± 3.87	55.92 ± 4.34	18	7.34	0.000

3.3.2.1 Coir rope substrates

Over seven days, exogenously applied acetylcholine at a concentration of $1x10^{-4}$ M, showed no ability to improve the retention of mussel spat attached to coir rope compared to the control assays (Figure 58). The proportion of mussel spat attached to the coir substrates decreased almost linearly from 100% to approximately 50%, and 57%, in control and treatment assays respectively. At every 24 hour observation, mean percent spat retention in control and treatment assays were similar, with no statistical differences being detected at any stage during the experiment (

Table 21).

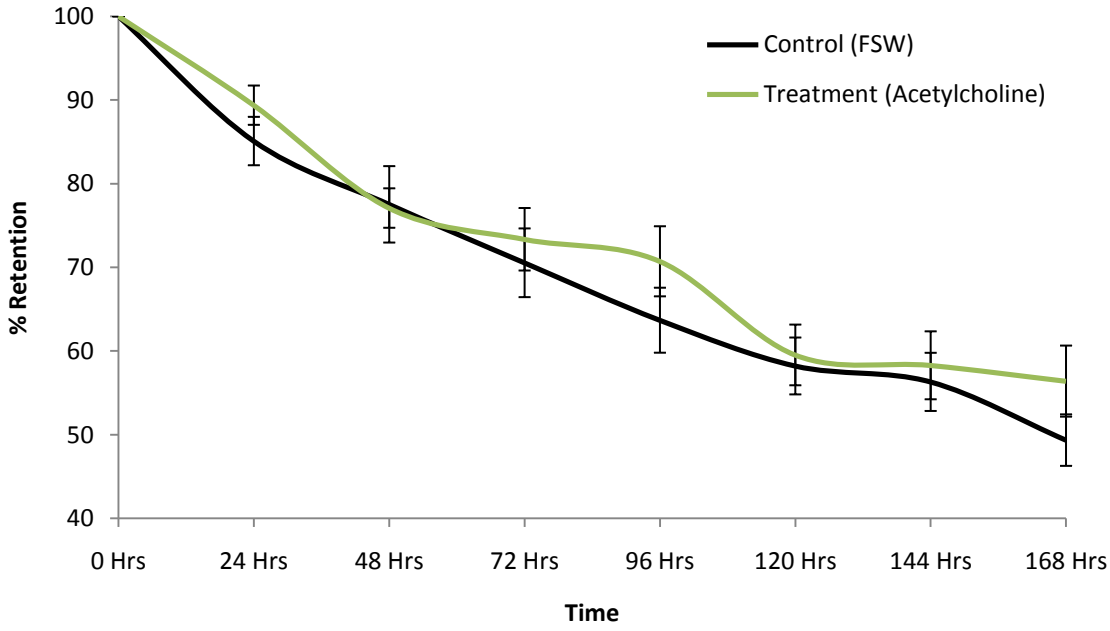

Figure 58. Effect of acetylcholine ($1x10^{-4}$ M) on retention of *P. canaliculus* spat attached to coir rope substrates. Data plotted are mean ± standard error of 10 replicates.

Table 21. Effect of acetylcholine ($1x10^{-4}$ M) on retention of *P. canaliculus* spat attached to coir rope substrates**.** Summary of statistics (arcsin transformed data; 2-sample t-tests).

Time (hrs)	Control mean ± S.D. (arcsin)	Treatment mean ± S.D. (arcsin)	Pooled DF	*t*-value	*p*-value
24	68.86 ± 9.42	72.49 ± 8.32	16	0.87	0.399
48	62.47 ± 9.18	61.63 ± 4.87	16	0.24	0.812
72	57.44 ± 7.60	59.35 ± 7.30	16	0.54	0.595
96	53.05 ± 6.85	57.71 ± 8.07	16	1.32	0.206
120	49.78 ± 5.97	50.60 ± 6.40	16	0.28	0.782
144	48.68 ± 6.10	49.88 ± 7.16	16	0.38	0.707
168	44.63 ± 5.35	48.79 ± 7.48	16	1.36	0.194

3.4 Acetylcholine toxicology

To determine the acute toxicity effects of acetylcholine, a mortality assay was performed. Mussel spat were exposed to four concentrations of acetylcholine ($1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$, and $1x10^{-2}$ M) for 72 hours. The results of the mortality experiments are shown in

Table 22. At an acetylcholine concentration of $1x10^{-5}$ M, 13% of spat died, slightly less than the 13.4% observed in the control assay. Therefore, acetylcholine is not acutely toxic at $1x10^{-5}$ M. However, at $1x10^{-4}$ M, percent mortality increased to 19.2%, demonstrating toxic effects. At an acetylcholine concentration of $1x10^{-2}$ M, maximum 100% spat mortality was observed.

Table 22. Effect of acetylcholine on mussel spat mortality after 72 hour exposures.

Concentration ($molL^{-1}$)	Total spat exposed (n)[a]	Total spat responding (n)[a]	Mean % mortality	S.D.
Control	112	15	13.39	0.21
$1x10^{-5}$	115	15	13.04	3.31
$1x10^{-4}$	99	19	19.19	5.94
$1x10^{-3}$	101	78	77.22	9.47
$1x10^{-2}$	112	112	100	0.00

[a] Total number of mussel spat over three replicates.

From the lethal concentration values calculated by probit analysis (Table 23), it is estimated that 1% of the spat population would die after exposure to acetylcholine at $5.0x10^{-5}$ M. Exposure of mussel spat to $1x10^{-4}$ M acetylcholine solution (the concentration which was previously found to be effective at increasing spat retention on glass substrates – see Results section, p255), is estimated to kill 5% of the population.

Table 23. Estimated toxicological parameters for acetylcholine: lethal concentration values and their associated 95% confidence intervals (italics).

Treatment	LC_1 (mol L^{-1})	LC_5 (mol L^{-1})	LC_{50} (mol L^{-1})	LC_{99} (mol L^{-1})
Acetylcholine	$5.0x10^{-5}$	$1.0x10^{-4}$	$5.2x10^{-4}$	$5.3x10^{-3}$
	$<1.0x10^{-6} – 1.5x10^{-4}$	*$1.0x10^{-4} – 2.4x10^{-4}$*	*$1.9x10^{-4} – 8.0x10^{-4}$*	*$2.6x10^{-3} – 5.5x10^{-2}$*

4. DISCUSSION

It is well established that surface properties such as the roughness of substrates, have an effect on the adhesion of marine organisms to substrata (e.g. Brewer 1984; Walker 1987). Surface wettability (hydrophobicity or surface energy) also has been demonstrated to factor in modulating attachments of marine organisms to surfaces. Marine invertebrates generally adhere much more strongly to substrata with hydrophilic, rather than hydrophobic, surface characteristics (Grenon & Walker 1981; Flammang & Walker 1997; Waite 2002). For example, hydrophilic surfaces are known to promote the adhesion of mussels (Aldred et al. 2006; Petrone et al. 2008b), and barnacles (Rittschof & Costlow 1989). This relationship with surface wettability can be explained by the fact that marine bio-adhesives are rich in charged and polar residues (Flammang 2003). These residues are likely involved in adhesive interactions with hydrophilic surfaces through hydrogen and ionic bonding (Waite 1987). Although the wettability of surfaces is related to surface charge, there is not a directly proportional relationship between hydrophobicity and electric charge. For example, it is possible to have two separate surfaces, one negatively charged, and one positively charged, that are both hydrophilic, or both hydrophobic. Little research, if any, has been conducted on the specific effect of surface charge and its ability to modulate attachments, or colonisations, to surfaces by marine invertebrates. However, the chemical characteristics of marine bio-adhesives strongly hint that surface charge would have an effect on these attachments. For example, adhesive mucous-like materials secreted by echinoderms, contain significant amounts of acidic protein residues (negatively charged), as well as acidic carbohydrate moieties (Flammang et al. 2005). Furthermore, recent characterisation of secreted mucous from *P. canaliculus* larvae reveal that the adhesive contains acidic carboxylate and sulphate moieties (Petrone et al. 2008a). The presence of such constituents in marine bio-adhesives infer that these

secretions may bind more strongly to surfaces that are positively charged rather than negatively charged, through ionic bonding.

Most natural marine surfaces typically carry a net negative charge (Wnek & Bowlin 2008). In adult mussels, where attachment to a substrate is solely regulated by byssal threads, it has been suggested that the high numbers of basic residues in mussel adhesive proteins, formed at the byssal plaques, promote the attachment of mussels to negatively charged surfaces (Wnek & Bowlin 2008). However, in *P. canaliculus* mussel larvae, the pedal organ which secretes L-DOPA residues for the production of byssal threads is not developed until after the primary settlement process (Petrone et al. 2008b). These larvae rely on the secretion of filamentous mucoid material to adhere to surfaces. Because of the negatively charged characteristics of the secreted material, it is likely that *P. canaliculus* larvae would find it difficult to attach to highly negatively charged surfaces, unlike its adult form by use of byssal threads. Perhaps this may partially explain why very young mussels rarely undergo primary settlement on substrates where adult mussel beds occur (see Alfaro 2006a). It was observed in this thesis that *P. canaliculus* spat (1–3 mm diameter), although they have the ability to produce byssal threads, also rely on an initial secretion of a mucus-like exudate (similar in appearance to that secreted by larvae) to form temporary bonds to a substrate, before subsequent byssal thread production occurs. From these observations, it appeared that the mucoid attachment lasted up to a matter of hours before byssal thread secretion commenced. Therefore, mucoid material is inferred to play a role in initial attachment of mussel spat to substrates. Since larval secretions were found to contain negatively charged moieties (Petrone et al. 2008a), it is plausible that the adhesive secreted by mussel spat also has negatively charged characteristics. This would suggest that attachment of mussel spat to negatively charged surfaces would be difficult to accomplish, and easier to achieve on substrates with positive surface charge characteristics.

In this thesis, the effect of surface charge on the ability of mussel spat to attain a firm attachment to a substrate was investigated using two types of charged surfaces, Biodyne® Nylon Transfer Membranes, and Amberlite ion exchange resin beads. The results showed that mussel spat generally had a much greater affinity to attach to the positively charged surfaces of both types of materials. On the transfer membranes, 10% of spat attached to the negatively charged surface whereas 66% of spat attached to the positively charged surface. On beds of ion exchange resins, exposures of 2, 24, and 48 hours resulted in 0% attachment of spat to negatively charged beads. Exposure of spat to positively charged beads resulted in 11%, 72%, and 66% attachments after 2, 24, and 48 hours respectively. The differences observed in the attachment abilities between the two types of materials (i.e. membranes *vs*. beads) may be explained by their physical structure. Firstly, the nylon transfer membranes provided a smooth flat surface for the spat to attach to. Spat were observed to move around and probe the surfaces of the membranes with their pedal organs before secretion of the mucous adhesive. Perhaps the sensing of firmness and uniform structure of this substrate by the organisms promoted adhesive secretions on both of the charged membrane surfaces within 2 hours. The 10% of attachment on the negatively charged membranes could have been due to the formation of byssal threads during the 2 hours provided. In the case of the ion exchange resins, only 11% attachment to the positively charged beads was observed after 2 hours. This low value compared to that achieved on the positively charged membranes (66%), could have been due to the unstable nature of the beads. Although spat were observed to initially probe the resins with their pedal organs, these organs were retracted within approximately 2–5 minutes. Spat were not able to move around the substrates since traction could not be achieved on the small beads. This may have caused the spat to limit the production of mucous adhesive since the substrate likely would have been deemed unsuitable for habitation. Furthermore, if the ion exchange resins were considered unsuitable because of their unstable nature, perhaps any subsequent byssal threads were not formed. This may explain why 0% of spat attached to the negatively charged beads at any of the exposure times (i.e. 2, 24, and 48 hours). Although not quantified due to problems with spat mobility, it was observed that spat could form strong attachments to negatively charged membranes when allowed 24 hours to occur. After initial mucous adhesion, the production of byssus threads,

containing basic residues, might account for this. The results of these experiments on surface charge *vs*. mussel spat attachment not only support the hypothesis that spat have a greater affinity to attach to positively charged substrates, but also infer that the initial mucoid material produced by spat as an adhesive, before byssal thread production, is similar to that of larvae. This adhesive likely contains high proportions of negatively charged moieties.

It is possible that the reduced attachment of *P. canaliculus* spat on negatively charged surfaces was partially due to effects other than the chemical composition of the secreted mucous adhesive. Whether surface charge characteristics of substrata can be sensed by the juvenile mussels is uncertain. However, it is possible that spat can distinguish between opposing surface charges and display a preference for a particular pole. The ability of some organisms to sense and respond to electrical gradients (electrotaxis) is well known. For example, electrotaxis has been demonstrated in bacteria (Rajnicek et al. 1994), fungi (Gow 1994), amoeba (Koroda et al. 2000), and in nematodes (Gabel et al. 2007). Perhaps mussel spat too can sense electrical gradients, and in their case, a negative surface charge may limit the production of mucous adhesive materials due to the sensing of an unsuitable substrate. Such a response, combined with ionic repulsion of the spat adhesive with a negatively charged surface, may explain the results of the attachment assays in the present study. This role of electrotaxis or electroreception is highly speculative since no studies were incorporated into this thesis to determine such effects. However, future investigations into surface charge reception may lead to new hypotheses being formed about how marine organisms select substrata, and why colonisations on specific marine surfaces occur.

Because mussel spat tend to attach to positively charged surfaces, there may be applications for improving techniques in aquaculture. Rope substrates could be synthesised from materials such as nylon or polypropylene, with the addition of positively charged side groups grafted onto their polymeric carbon backbone (e.g. carboxyl, quaternary ammonium, or quaternised pyridyl groups). Alternatively, entirely

new polymers could be synthesised from specific monomer constituents to provide a rope with positively charged surface chemistry. Increasing attachment rates during juvenile seeding onto nursery ropes, or reseeding onto grow-out ropes, would be economically beneficial for the mussel industry.

The effect of surface charge on larval attachment (settlement) was not investigated in this thesis due to limited substrate material, larvae, and time. However, it is hypothesised that larvae of *P. canaliculus* would behave in much the same manner as the spat did, preferring to attach to positively charged surfaces. Therefore, it is advised that such an investigation is performed in the future. Significant improvements to aquacultural techniques may be developed by determining larval responses to charged surfaces. For example, 'spat catching ropes' with positively charged surfaces could have the potential to substantially increase the amount of larvae (or very young spat) caught from the water column. Substrates currently used for catching larvae primarily consist of either coir rope, or synthetic polypropylene ('Christmas tree rope'). Polypropylene is a neutral substrate, generally having no surface charge due to its basic hydrocarbon structure. On the other hand, coir is a negatively charged substrate containing a high content of anionic lignin (Lam et al. 2001; Namasivayam 2001; Suksabye 2007). It has been determined that coir 'spat catching ropes' are effective at catching larvae due to their highly filamentous structure (Mason & Drinkwater 1981), and they are suggested to be more effective than polypropylene ropes for catching these young mussels (A.C. Alfaro, senior lecturer, Auckland University of Technology, personal communication, September 3, 2008). Because negatively charged substrates such as coir are likely to partially inhibit the attachments of mussel larvae, this may indicate that the physical structure is more important than chemical composition. However, there may be applications in modifying coir substrates to provide positively charged surfaces to enhance the catchment rates currently experienced.

The primary interest of this chapter was to identify factors which may be manipulated to enhance techniques in aquaculture. However, the determined effects of surface charge on spat attachment also may have applications in antifouling technologies. Biofouling is a major problem in marine industries. An estimated $25 billion (NZD) per annum is

spent worldwide in an effort to control fouling of organisms to aquatic surfaces (Azis et al. 2000). Materials with charged surfaces (e.g. polymer plastics or paints), could aid in combating this problem.

The effect of surface charge on the retention of mussel spat attached to Biodyne® Nylon Transfer Membranes was investigated to determine if this physicochemical property can be utilised to enhance retention. The results of this investigation revealed that surface charge characteristics had little effect on spat retention over the five days assayed. Although initial attachment of mussel spat to substrates appear to be regulated by the secretion of mucoid adhesive, prolonged exposure to surfaces result in byssal thread attachments. It is suggested that the similarity in spat retention between the two charged surfaces were due to these byssal attachments, which easily bond to negatively charged surfaces (i.e. Wnek & Bowlin 2008). It is therefore unlikely that providing substrates (i.e. grow ropes) with a negative surface charge would have any application for enhancing the retention of *P. canaliculus* mussels in aquacultural activities.

Exogenously applied acetylcholine was tested for its ability to increase retention of *P. canaliculus* spat on glass substrates. After 60 hours, the results revealed that a concentration of $1x10^{-5}$ M acetylcholine did not significantly enhance retention. Conversely, attached spat bathed in a $1x10^{-4}$ M solution of acetylcholine resulted in an almost 100% increase in mean percent retention compared to control assays (FSW). However, since it is not feasible to use glass as a substrate in the aquaculture industry, the assay was repeated using coir rope substrates. After prolonged exposure to acetylcholine at a concentration of $1x10^{-4}$ M for seven days, there was no significant difference in mean percent mussel spat retention compared to the control assay. The combined results suggest that acetylcholine does in some way have an effect on spat retention. However, this effect can be overridden by the physical structure of the substrate. The enhancement of spat retention by acetylcholine treatment is not an additive effect to the high retention observed on suitably filamentous substrates (e.g. coir). The mechanism of acetylcholine action that enhances the retention on glass

substrates is unknown. The mechanisms of action could involve interaction of the compound on the nervous system of the organisms, affecting internal biochemistry. Byssal threads contain L-DOPA residues (Saby & Loung 1998; Vreeland et al. 1998; Zhao et al. 2006) and there is the possibility that acetylcholine treatment could lead to an increase in L-DOPA production and secretion by some unknown signaling pathway. Alternatively, acetylcholine could be involved in the manufacture of byssal thread constituent compounds other than L-DOPA. In contrast, acetylcholine may interact externally with the secreted byssal filaments which provide the attachment to the substrate. Expanding on these possibilities is too speculative since nothing is known about the involvement of acetylcholine in byssal thread production, or the role of acetylcholine in the neuroethology of substrate preference for juvenile mussel spat, or any other marine invertebrate species. The results of these experiments suggest that acetylcholine would unlikely have any application for increasing mussel spat retention in aquacultural activities. Even in an enclosed hatchery system where exogenously applied acetylcholine could be provided, the use of substrates with enhanced physical structures could potentially be a better alternative. Furthermore, from the 72 hour toxicology study, the concentration of acetylcholine which showed positive effects on mussel spat retention when using glass substrates ($1x10^{-4}$ M), was estimated to kill 5% of the organisms. This toxicity effect would obviously be undesirable for aquacultural practices had acetylcholine been shown to enhance retention on coir substrates.

The effect of acetylcholine bound to ion exchange resins was investigated for its ability to attract *P. canaliculus* spat. Exogenously applied acetylcholine has previously demonstrated an ability to increase larval settlement (see Chapter 6), and has demonstrated some ability to modulate spat attachments in this chapter (i.e. retention on glass substrates). However, for ocean based applications in aquaculture, exogenously applying acetylcholine solutions are not a feasible method of delivering this compound due to the problem of dilution. The development of a substrate to which spat (or larvae) are attracted to would be advantageous to the mussel aquaculture industry, possibly having applications for 'catching' wild spat from the environment. The results of the investigation into the attraction of spat to acetylcholine-bound surfaces revealed that

mussel spat were not attracted to such surfaces – this was not especially surprising. Firstly, for acetylcholine to bind to protein receptors and have an effect on the biochemistry of an organism (chemoreception), it must be a free molecule. Secondly, even if an acetylcholine concentration gradient of free molecules existed around the resin beads, the concentration would be very low.

In summary, acetylcholine ionically bound to substrates does not attract mussel spat, and is unlikely to attract larvae. To explain this, acetylcholine needs to be a free molecule for receptor binding (internal or external), and it also is possible that chemotactic response of *P. canaliculus* spat to acetylcholine simply does not occur. Therefore, surface bound acetylcholine would not have any applications in aquaculture for attracting *P. canaliculus* juveniles. Exogenously applied acetylcholine in an enclosed system increases spat retention when attached to glass substrates, but not to coir substrates which are presently used in the industry. This implies that the physical structure of substrata can override the positive retention effects of acetylcholine. Therefore, exogenously applied acetylcholine probably has no application for increasing mussel spat retention since such effects could be achieved by enhancing the physical structures of grow ropes. The effect of surface charge on spat retention revealed no differences between positively and negatively charged surfaces. The reason why there was no difference in retention between positively and negatively charged surfaces was likely due to byssal thread attachments, which can attach easily to negatively charged surfaces. There does not appear to be any potential aquacultural applications for incorporating charged surfaces into rope substrates to enhance long-term spat retention. Mussel spat showed considerable preference for attaching to positively charged surfaces during short-term exposures. This is likely due to initial mucoid adhesion to substrata before subsequent byssal threads are produced and attached. The mucoid adhesive likely contains negatively charged moieties, promoting ionic bonding to cationic surfaces. The low attachments of spat observed on negatively charged surfaces could have been due to the ionic repulsion of mucous adhesive, detection of electric charge through external receptors and subsequent limitation of adhesive secretion, or a combination of the two effects. There may be considerable applications for using charged surfaces in marine industries, such as avoiding biofouling. The design and use of charged surfaces (e.g. ropes) could be useful for enhancing juvenile mussel transfers (reseeding) from one

substrate to another – positively charged surfaces may increase initial attachments and speed up the attachment process. There also may be considerable application of using positively charged rope substrates for enhancing *P. canaliculus* larval and spat catchments from the wild.

CHAPTER 12

GENERAL DISCUSSION & RECOMMENDATIONS

1. DISCUSSION

The investigations undertaken in this thesis have identified, for the first time, 15 compounds which have the ability to induce larval settlement in *P. canaliculus*. These results have provided important insights into the biochemistry of larval settlement of this endemic species of mussel. It is suggested that potassium ions, acetylcholine, epinephrine, L-DOPA, and cyclic adenosine monophosphate likely play key roles in the signaling pathways involved in the neuroethology of *P. canaliculus* larval settlement. The order in which these compounds act cannot be deduced, since neuronal circuits are complex, and rarely are linear. However, there is evidence to suggest that the involvement of acetylcholine is downstream in the signaling pathway from the involvement of cyclic adenosine monophosphate. It is suggested that the inductive effects of these compounds act at intermediary sites in the nervous system, downstream from external chemoreceptors. This inductive effect on settlement can be metaphorically considered similar to the 'jump starting' of a car without a battery. This thesis also has identified problems associated with the treatments used in settlement assays, such as oxidative breakdown of treatment compounds. This factor questions the reliability of results published by some authors. The identification of pharmacologically active compounds which have the ability to induce larval settlement may be of great importance to the aquaculture industry. These compounds could be used as routine, inexpensive, and effective treatments for the induction of synchronous settlement in hatchery production of larvae, enhancing economical viability of the industry. However, further research would have to be performed on many of the compounds identified to mitigate adverse toxicity effects.

The novel form of clustering species developed in this thesis, based on the neuroethologies of marine invertebrates, may have uncovered a technique to elucidate important relationships that are currently not known. It is possible that this approach may have evolutionary implications.

Finally, of most importance, the results of this thesis have provided a significant baseline for further studies to investigate larval settlement in the green-lipped mussel. This has led to the identification of over 50 new areas of study to be investigated in *P. canaliculus* (many of which apply to other species). From these areas, in excess of 100 individual experiments have been identified which would greatly enhance our understanding of the developmental biology, biochemistry, neuroethology, and pharmacology of this endemic New Zealand species. These areas are presented as recommendations for future research.

2. RECOMMENDATIONS

Chapter 2:

- Investigate the temporal variation in the reproductive cycle of *Perna canaliculus* at sites in the Firth of Thames, Coromandel.
- Investigate small scale spatial variation (tidal *vs*. sub-tidal) in the reproductive cycle of *P. canaliculus* at sites in the Firth of Thames, Coromandel.

Chapter 3:

- Determine daily rates of larval settlements in hatchery reared *P. canaliculus* larvae from day 1 post-fertilisation until 100% settlement is observed.
- Investigate the mode of larval detachments from substrata, and determine how often this may occur before a final attachment is established.
- Investigate timing of velum loss after the settlement process occurs.
- Investigate the effect of substrate plane on larval settlement (horizontal *vs*. vertical).
- Investigate the mechanisms behind the apparent attraction of larvae to one another. Do larvae secrete compounds which act as attractants through chemotaxis? Perform attraction studies and identify any inducing structures in secreted mucoid material.
- Determine the rates of larval settlement observed in the laboratory under moderate to high water flows in settlement pre-competent larvae.

Chapter 4:

- Re-investigate the larval settlement induction ability of GABA in *Mytilus galloprovincialis*. Are the results concordant with Garcia-Lavandeira et al.'s 2005 study?
- Re-test the effects of L-Tryptophan on larval settlement to determine if inductive effects are observed using non-acutely toxic concentrations.
- Use isotope labelled L-Tryptophan to determine if endogenous serotonin can be biosynthesised from external sources of the precursor amino acid.
- Determine the effects of exogenously applied serotonin on larval settlement in *P. canaliculus*.
- Determine the effects of serotonin receptor agonists and antagonists on larval settlement in *P. canaliculus*, these might include: the specific receptor agonists ergotamine, aripirazole, sumatripan, tryptamine, psilocybin, lysercid acid, and mescaline, and the specific receptor antagonists propanolol, ritanserin, fluoxetine, and L-Lysine.
- Investigate the role of serotonin biosynthesis in larval settlement of *P. canaliculus*. From day one post-fertilisation until metamorphosis is complete, analyse endogenous levels of: L-Tryptophan, tryptophan hydroxylase, and serotonin.
- Determine the distribution of serotonin-containing cells throughout larval development using immunohistochemical techniques.

Chapter 5:

- Investigate the effects of exogenously applied potassium ions on the endogenous production and/or release of particular metabolites that are now known to modulate larval settlement in *P. canaliculus*, such as: serotonin, acetylcholine, epinephrine, L-DOPA, L-Tyrosine, cyclic adenosine monophosphate, and hydrogen peroxide. Other endogenous compounds to be analysed could include tryptophan hydroxylase, phenylalanine hydroxylase, tyrosine hydroxylase,

DOPA decarboxylase, norepinephrine, phenethanolamine N-methyltransferase, protein kinase A, and choline esterase among others.

- Investigate the role of chloride ions in larval settlement of *P. canaliculus* by designing experiments with carefully selected treatments containing the chloride ion.
- Investigate the role of other ions on larval settlement of *P. canaliculus* (e.g. sodium, lithium, magnesium, calcium, fluoride, bromide).

Chapter 6:

- Determine the effects of nicotinic receptor antagonists, such as alpha bungarotoxin, on larval settlement of *P. canaliculus*. This may substantiate the claims made in this thesis.
- Determine the effects of specific acetylcholine receptor sub-types to further elucidate the receptors involved in acetylcholine induced settlements (see Appendix IV, p293).
- Analyses endogenous levels of cyclic adenosine monophosphate, L-DOPA, dopamine, norephinephrine, epinephrine, serotonin, glycine, aspartate, and glutamate in response to exogenous treatment with acetylcholine.
- Determine if exogenous treatment of larvae with acetylcholine alters gene expression.
- Determine the effect of exogenously applied acetylcholine on velar ciliary action.
- Determine the effect of exogenously applied acetylcholine on exploratory behaviour of substrata by larvae, using their pedal organs.
- Investigate the effect of acetylcholine on mucoid secretions of larvae.

Chapter 7:

- Investigate the role of catecholamine biosynthesis in larval settlement of *P. canaliculus*. From day one post-fertilisation until metamorphosis is complete, analyse endogenous levels of: L-Phenylalanine, phenylalanine hydroxylase, L-Tyrosine, tyrosine hydroxylase, L-DOPA, DOPA decarboxylase, dopamine, dopamine β-hydroxylase, norepinephrine, phenethanolamine N-methyltransferase, and epinephrine.
- Determine the effects of dopamine, and norepinephrine on larval settlement in *P. canaliculus*.
- Use isotope labelled precursors to determine catecholamine biosynthesis rates (i.e. labelled L-Phenylalanine, L-Tyrosine, L-DOPA, dopamine)
- Determine if exogenous treatment of larvae with L-Phenylalanine, L-Tyrosine, L-DOPA, dopamine, norepinephrine, and epinephrine alter gene expression in larvae of *P. canaliculus*.
- Determine the effects of morphine on larval settlement in *P. canaliculus*.
- Investigate the role of L-DOPA and dopamine conversion to morphine in settlement of *P. canaliculus*. This could be achieved using radio isotope labelled treatments.
- Determine if exogenous treatment of larvae with morphine affects velar ciliary activity.
- Investigate the role of thyroid hormone (e.g. thyroxine) biosynthesis in larval settlement of *P. canaliculus.*

Chapter 8:

- Investigate the role of endogenous catecholamine degradation in larval settlement of *P. canaliculus*. From day one post-fertilisation until metamorphosis is complete, analyse endogenous levels of: adrenochrome, dopachrome, melanin, metanephrine, normetanephrine, 3-methoxytyramine, CMOT, MAO-A, MAO-B, hydrogen peroxide, and ascorbic acid.

- Determine the effects of adrenochrome, dopachrome, melanin, metanephrine, normetanephrine, 3-methoxytyramine on larval settlement in *P. canaliculus*.
- Determine a suitable antioxidant which can be incorporated into L-DOPA and catecholamine treatment solutions to prevent oxidation, and has no inadvertent settlement inducing ability, or toxicity effects.

Chapter 9:

- From day one post-fertilisation until metamorphosis is complete, analyse endogenous levels of cyclic adenosine monophosphate, adenylyl cyclase, protein kinase A, and protein kinase C.
- Using specific c-AMP selective phosphodiesterase inhibitors as treatments to increase intracellular c-AMP levels determine larval settlement rates in *P. canaliculus*. Inhibitors could include: 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Holme et al. 1998), methyl 2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate dihydrochloride (Tatsuzo et al. 2001), and 4-(3-cyclopentyloxy-4-methoxy-phenyl)pyrrolidin-2-one (e.g. Kato et al. 1995).
- Use other compounds which increase intracellular c-AMP levels by stimulating adenylyl cyclase, such as forskolin and choleratoxin, and determine larval settlement rates in *P. canaliculus*.
- Determine if treatments with compounds which increase intracellular c-AMP levels alter gene expression in *P. canaliculus* larvae.
- Investigate the effect of adenosine receptor antagonisation in larval settlement.
- Determine if adenosine receptor antagonisation increases endogenous levels of acetylcholine in this species.
- Possibly re-examine other species in the literature which have claimed to be induced to settle and undergo metamorphosis by increases in endogenous c-AMP via IBMX treatment. Employ c-AMP selective PDE inhibitors, forskolin, and choleratoxin for this purpose.

- Determine if those marine bacteria species isolated by Ganesan et al. (2008) which have the ability to induce settlement in *P. canaliculus* secrete extracellular c-AMP.
- Determine if those bacteria secrete exudates which have the ability to increase endogenous levels of c-AMP in *P. canaliculus* larvae, and other metabolites (or their derivatives) assayed in this thesis i.e. acetylcholine, L-Tryptophan, serotonin, L-Phenylalanine, L-Tyrosine, L-DOPA, dopamine, norepinephrine, epinephrine, morphine, hydrogen peroxide, and ascorbic acid.

Chapter 10:

- Increase the dataset constructed in this thesis to include more marine invertebrate species, and more settlement inductive compounds. Do this by extensively reviewing the literature.
- Determine the effects of other pharmacologically active compounds which have been demonstrated to induce larval settlement in various species in the literature on *P. canaliculus* – incorporate the results into the dataset.
- Endeavor to expand the dataset by conducting new settlement studies to fill in the larval response gaps which currently limit the cluster analysis (performing these experiments on species other than *P. canaliculus*).
- Construct a new dataset from larval metamorphosis responses to pharmacologically active compounds from the literature and re-run cluster analysis.

Chapter 11:

- Determine the charge characteristics of larval and spat mucoid exudates (possibly through titration, dye assays, electrophoresis, or chromatography).
- Determine the effects of substrate wettability on *P. canaliculus* mussel spat attachments to substrata.

- Re-investigate the effects of charged surfaces on mussel attachments using Biodyne ® filter membranes – with the inclusion of a neutrally charged membrane.
- Determine the effect of charged surfaces to the attachments strength of mussel spat to substrata.
- Determine if *P. canaliculus* mussel spat respond to charged surfaces over electrical gradients (i.e. electrotaxis).
- Investigate the modification of rope substrates to provide positively charged surfaces or surfaces with low hydrophobicities. Such surfaces may enhance capture of wild larvae on spat catching ropes.

APPENDIX I

TABLES OF LARVAL SETTLEMENT STATISTICS

Age effects on larval settlement at 24 & 48 hour incubations (Sealord Ltd.)

Comparisons	Incubation (hrs)	Model	*p*-value
18DO vs. 21DO vs. 23DO	24	1-way ANOVA	0.037
18DO vs. 21DO	24	Tukey's	0.348
18DO vs. 23DO	24	Tukey's	0.396
21DO vs. 23DO	24	Tukey's	0.028
18DO vs. 21DO vs. 23DO	48	1-way ANOVA	0.302
18DO vs. 21DO	48	Tukey's	0.360
18DO vs. 23DO	48	Tukey's	0.369
21DO vs. 23DO	48	Tukey's	0.999

Batch effects on larval settlement at 24 & 48 hour incubations (Sealord Ltd.)

Comparisons	Incubation (hrs)	Model	*p*-value
1 vs. 2 vs. 3	24	1-way ANOVA	0.017
1 vs. 2	24	Tukey's	0.049
1 vs. 3	24	Tukey's	0.936
2 vs. 3	24	Tukey's	0.023
1 vs. 2 vs. 3	48	Kruskal Wallis	0.577
1 vs. 2	48	Dunn's	0.540
1 vs. 3	48	Dunn's	0.705
2 vs. 3	48	Dunn's	0.288

Batch effects on larval settlement at 48 hour incubations (Cawthron Institute)

Comparisons	Incubation (hrs)	Model	*p*-value
1 vs. 2 vs. 3	24	1-way ANOVA	0.217
1 vs. 2	24	Tukey’s	0.459
1 vs. 3	24	Tukey's	0.189
2 vs. 3	24	Tukey's	0.746

Hatchery effects on larval settlement

Comparisons	Model	*p*-value
Sealord vs. Cawthron	2-sample t-test	0.042

Effect of GABA on larval settlement (48hrs exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.003	-	-
Control vs. 10^{-5}M	-	0.382	0.267
Control vs. 10^{-4}M	-	0.008	0.004
Control vs. 10^{-3}M	-	0.007	0.004
10^{-5}M vs. 10^{-4}M	-	0.210	-
10^{-5}M vs. 10^{-3}M	-	0.202	-
10^{-4}M vs. 10^{-3}M	-	1.000	-

Effect of L-Tryptophan on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.000	-	-
Control vs. 10^{-5}M	-	0.000	0.000
Control vs. 10^{-4}M	-	0.000	0.000
Control vs. 10^{-3}M	-	0.000	0.000
10^{-5}M vs. 10^{-4}M	-	0.053	-
10^{-5}M vs. 10^{-3}M	-	0.986	-
10^{-4}M vs. 10^{-3}M	-	0.112	-

Effect of KCl on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 20mM vs. 40mM vs. 60mM	0.002	-	-
Control vs. 20mM	-	0.520	0.390
Control vs. 40mM	-	0.315	0.215
Control vs. 60mM	-	0.032	0.019
20mM vs. 40mM	-	0.027	-
20mM vs. 60mM	-	0.002	-
40mM vs. 60mM	-	0.578	-

KCl Effect on Settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 5mM vs. 10mM vs. 15mM vs. 20mM	0.000	-	-
Control vs. 5mM	-	0.005	0.002
Control vs. 10mM	-	0.000	0.000
Control vs. 15mM	-	0.003	0.001
Control vs. 20mM		0.638	0.455
5mM vs. 10mM	-	0.133	-
5mM vs. 15mM	-	0.999	-
5mM vs. 20mM	-	0.100	-
5mM vs. 20mM	-	0.100	-
10mM vs. 15mM	-	0.192	-
10mM vs. 20mM	-	0.000	-
15mM vs. 20mM	-	0.067	-

Effect of K_2SO_4 on larval settlement (48hrs Exposure): $[K^+]$

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 5mM vs. 10mM vs. 15mM vs. 20mM vs. 40mM	0.000	-	-
Control vs. 5mM	-	0.575	0.354
Control vs. 10mM	-	0.038	0.015
Control vs. 15mM	-	0.014	0.006
Control vs. 20mM	-	0.991	0.964
Control vs. 40mM	-	0.907	0.762
5mM vs. 10mM	-	0.626	-
5mM vs. 15mM	-	0.373	-
5mM vs. 20mM	-	0.892	-
5mM vs. 40mM	-	0.119	-
10mM vs. 15mM	-	0.998	-
10mM vs. 20mM	-	0.128	-
10mM vs. 40mM	-	0.004	-
15mM vs. 20mM	-	0.054	-
15mM vs. 40mM	-	0.001	-
20mM vs. 40mM	-	0.601	-

Effect of $K_2S_2O_5$ on larval settlement (48hrs Exposure): $[K^+]$

Treatment	*p*-values (Kruskal-Wallis; Dunns post-hoc)				
Control	1.000				
5mM	0.129	1.000			
10mM	0.229	0.012	1.000		
15mM	0.108	0.003	0.716	1.000	
20mM	0.064	0.002	0.561	0.827	1.000

Effect of acetylcholine on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.000	-	-
Control vs. 10^{-5}M	-	0.001	0.001
Control vs. 10^{-4}M	-	0.000	0.000
Control vs. 10^{-3}M	-	0.000	0.000
10^{-5}M vs. 10^{-4}M	-	0.002	-
10^{-5}M vs. 10^{-3}M	-	0.385	-
10^{-4}M vs. 10^{-3}M	-	0.095	-

Effect of epinephrine on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.000	-	-
Control vs. 10^{-5}M	-	0.000	0.000
Control vs. 10^{-4}M	-	1.000	1.000
Control vs. 10^{-3}M	-	0.000	0.000
10^{-5}M vs. 10^{-4}M	-	0.000	-
10^{-5}M vs. 10^{-3}M	-	0.000	-
10^{-4}M vs. 10^{-3}M	-	0.000	-

Effect of L-DOPA on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.000	-	-
Control vs. 10^{-5}M	-	0.000	0.000
Control vs. 10^{-4}M	-	0.343	0.233
Control vs. 10^{-3}M	-	0.000	0.000
10^{-5}M vs. 10^{-4}M	-	0.000	-
10^{-5}M vs. 10^{-3}M	-	0.000	-
10^{-4}M vs. 10^{-3}M	-	0.000	-

Effect of L-Tyrosine on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.000	-	-
Control vs. 10^{-5}M	-	0.000	0.000
Control vs. 10^{-4}M	-	0.000	0.000
Control vs. 10^{-3}M	-	0.000	0.000
10^{-5}M vs. 10^{-4}M	-	0.000	-
10^{-5}M vs. 10^{-3}M	-	0.000	-
10^{-4}M vs. 10^{-3}M	-	0.950	-

Effect of L-Phenylalanine on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.000	-	-
Control vs. 10^{-5}M	-	0.000	0.000
Control vs. 10^{-4}M	-	0.000	0.000
Control vs. 10^{-3}M	-	0.000	0.000
10^{-5}M vs. 10^{-4}M	-	0.007	-
10^{-5}M vs. 10^{-3}M	-	0.910	-
10^{-4}M vs. 10^{-3}M	-	0.038	-

Effect of H_2O_2 on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 800mM vs. 400mM	0.024	-	-
Control vs. 800mM	-	0.469	0.399
Control vs. 400mM	-	0.019	0.014
800mM vs. 400mM	-	0.178	-

Effect of ascorbic acid on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 5mM vs. 10mM vs. 15mM	0.000	-	-
Control vs. 5mM	-	0.000	0.000
Control vs. 10mM	-	0.001	0.001
Control vs. 15mM	-	0.010	0.006
5mM vs. 10mM	-	0.051	-
5mM vs. 15mM	-	0.007	-
10mM vs. 15mM	-	0.808	-

Effect of $Na_2S_2O_5$ on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 1mM vs. 5mM vs. 10mM vs. 15mM vs. 20mM	0.000	-	-
Control vs. 1mM		0.9979	0.9909
Control vs. 5mM	-	0.002	0.001
Control vs. 10mM	-	0.000	0.000
Control vs. 15mM	-	0.003	0.001
Control vs. 20mM	-	0.000	0.000
1mM vs. 5mM	-	0.007	-
1mM vs. 10mM	-	0.000	-
1mM vs. 15mM	-	0.001	-
1mM vs. 20mM	-	0.000	-
5mM vs. 10mM	-	0.000	-
5mM vs. 15mM	-	0.000	-
5mM vs. 20mM	-	0.000	
10mM vs. 15mM	-	0.791	
10mM vs. 20mM	-	0.224	
15mM vs. 20mM	-	0.010	

Effect of c-AMP on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. $1x10^{-6}$M vs.$1x10^{-5}$M	0.030	-	-
Control vs. $1x10^{-6}$M	-	0.068	0.051
Control vs. $1x10^{-5}$M	-	0.040	0.029
1x10-6M vs.$1x10^{-5}$M	-	0.958	-

Effect of caffeine acid on larval settlement (48hrs Exposure)

Comparisons	ANOVA Statistic	Tukey *p*-value	Dunnet *p*-value
Control vs. 10^{-5}M vs. 10^{-4}M vs. 10^{-3}M	0.000	-	-
Control vs. 10^{-5}M	-	0.001	0.001
Control vs. 10^{-4}M	-	0.000	0.000
Control vs. 10^{-3}M	-	0.000	0.000
10^{-5}M vs. 10^{-4}M	-	0.800	-
10^{-5}M vs. 10^{-3}M	-	0.006	-
10^{-4}M vs. 10^{-3}M	-	0.059	-

APPENDIX II

LARVAL POST-FERTILISATION AGE & LARVAL SOURCE FOR SETTLEMENT ASSAYS

Age and source of larvae for settlement assays

Settlement assay	Larval age (days)	Source (Hatchery)
GABA	22	Sealord Ltd.
L-Tryptophan	19	Cawthron Institute
Potassium chloride (high conc.)	22	Sealord Ltd
Potassium chloride (low conc.)	19	Cawthron Institute
Potassium sulphate	19	Cawthron Institute
Potassium metabisulphite	19	Cawthron Institute
Acetylcholine	19	Sealord Ltd
Atropine & acetylcholine	22	Sealord Ltd
Epinephrine	19	Sealord Ltd
L-DOPA	19	Cawthron Institute
L-Tyrosine	19	Cawthron Institute
L-Phenylalanine	19	Cawthron Institute
Hydrogen peroxide	19	Cawthron Institute
Ascorbic acid	19	Cawthron Institute
Sodium metabisulphite	19	Cawthron Institute
Cyclic adenosine monophosphate	19	Cawthron Institute
Caffeine	19	Sealord Ltd

APPENDIX III

EPA PROBIT ANALYSIS OUTPUTS FOR COMPUTING LETHAL CONCENTRATION VALUES

Gamma-Aminobutyric Acid

Probit Analysis

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

GAMMA-AMINOBUTYRIC ACID

Conc. $\mu molL^{-1}$	Observed Proportion Responding	Proportion Adjusted for Controls	Predicted Proportion Responding
Control	0.0200	0.0000	0.0186
10	0.0420	0.0238	0.0304
100	0.4130	0.4019	0.3610
1000	0.7870	0.7830	0.8777
10000	1.0000	1.0000	0.9475
100000	1.0000	1.0000	0.9964

Chi - Square for Heterogeneity (calculated) = 147.591
Chi - Square for Heterogeneity(
(tabular value at 0.05 level) = 7.815

Mu = 0.234135
Sigma = 0.658109

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	4.644230	0.245729	(3.862322, 5.426138)
Slope	1.519506	0.286588	(0.607583, 2.431428)
Spontaneous Response Rate	0.018634	0.027973	(-0.070375, 0.107643)

Estimated LC/EC Values and Confidence Limits

Exposure Point	Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	**5.0**	**0.0**	**029.9**
LC/EC 5.00	14.2	0.1	060.3
LC/EC 10.00	24.6	0.3	088.8
LC/EC 15.00	35.7	0.8	116.5
LC/EC 50.00	**171.4**	**27.9**	**458.4**
LC/EC 85.00	824.5	310.6	5578.3
LC/EC 90.00	1195.5	448.1	12352.6
LC/EC 95.00	2073.3	719.7	42985.0
LC/EC 99.00	**5822.3**	**1565.1**	**49847.4**

L-Tryptophan

Probit Analysis

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

L-TRYPTOPHAN

Conc. $\mu molL^{-1}$	Observed Proportion Responding	Proportion Adjusted for Controls	Predicted Proportion Responding
Control	0.0700	0.0000	0.0700
10	0.7420	0.7204	0.7193
100	0.9740	0.9720	0.9735
10000	1.0000	1.0000	0.9995

Chi - Square for Heterogeneity (calculated) = 0.0551
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 3.8410

Mu = -0.428747
Sigma = 0.738148

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	5.580842	0.044574	(5.493477, 5.668206)
Slope	1.354741	0.089289	(1.179736, 1.529747)
Spontaneous Response Rate	0.070029	0.008070	(0.054212, 0.085846)

Estimated LC/EC Values and Confidence Limits

Exposure Point	Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	**0.070**	**0.030**	**0.140**
LC/EC 5.00	0.230	0.120	0.380
LC/EC 10.00	0.420	0.240	0.660
LC/EC 15.00	0.640	0.390	0.950
LC/EC 50.00	**3.730**	**2.860**	**4.610**
LC/EC 85.00	21.690	18.990	24.990
LC/EC 90.00	32.910	28.370	39.030
LC/EC 95.00	61.010	50.300	77.300
LC/EC 99.00	**194.270**	**143.090**	**286.540**

Potassium Chloride

Probit Analysis

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

Potassium Chloride

Conc.(mM)	Observed Proportion Responding	Proportion Responding Adjusted for Controls	Predicted Proportion Responding
Control	0.0610	0.0000	0.0767
20.0000	0.3570	0.3036	0.1952
40.0000	0.5060	0.4650	0.6732
60.0000	0.9430	0.9383	0.8876
80.0000	1.0000	1.0000	0.9605

Chi - Square for Heterogeneity (calculated) = 289.371
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 5.991

Mu = 1.498731
Sigma = 0.230187

****
* NOTE *
* Slope not significantly different from zero. *
* LC/EC fiducial limits cannot be computed. *
****

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	-1.510930	2.698023	(-13.120522, 10.098662)
Slope	4.344296	1.662418	(-2.809089, 11.497680)
Spontaneous Response Rate	0.076654	0.100534	(-0.355944, 0.509252)

Estimated LC/EC Values and Confidence Limits

Point	Exposure Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	**9.189**		
LC/EC 5.00	13.185		
LC/EC 10.00	15.985		
LC/EC 15.00	18.204		
LC/EC 50.00	**31.531**		
LC/EC 85.00	54.613		
LC/EC 90.00	62.193		
LC/EC 95.00	75.399		
LC/EC 99.00	**108.197**		

Acetylcholine Chloride

Probit Analysis

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

ACETYLCHOLINE CHLORIDE

Conc. $\mu molL^{-1}$	Observed Proportion Responding	Proportion Adjusted for Controls	Predicted Proportion Responding
Control	0.0113	0.0000	0.0129
10	0.0141	0.0137	0.0200
100	0.0206	0.0883	0.0787
1000	0.7790	0.7462	0.7559
10000	1.0000	1.0000	0.9974

Chi - Square for Heterogeneity (calculated) = 4.314
Chi - Square for Heterogeneity(
(tabular value at 0.05 level) = 5.991

Mu = 0.671025
Sigma = 0.474585

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	3.586080	0.097782	(3.394428, 3.777732)
Slope	2.107105	0.101640	(1.907891, 2.306319)
Spontaneous Response Rate	0.129088	0.007424	(0.114537, 0.143639)

Estimated LC/EC Values and Confidence Limits

Exposure Point	Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	**36.9**	**26.3**	**48.9**
LC/EC 5.00	77.7	59.6	97.1
LC/EC 10.00	115.6	92.1	140.0
LC/EC 15.00	151.1	123.4	179.4
LC/EC 50.00	**468.8**	**418.4**	**520.1**
LC/EC 85.00	1455.1	1310.7	1632.3
LC/EC 90.00	1902.2	1692.3	2170.9
LC/EC 95.00	2829.2	2458.2	3330.1
LC/EC 99.00	**5957.3**	**4906.8**	**7496.7**

L-DOPA

Probit Analysis

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

L-DOPA

Conc. $\mu molL^{-1}$	Observed Proportion Responding	Proportion Adjusted for Controls	Predicted Proportion Responding
Control	0.1360	0.0000	0.1367
10	0.2420	0.1220	0.1203
100	0.8760	0.8564	0.8582
1000	1.0000	1.0000	0.9995

Chi - Square for Heterogeneity (calculated) = 0.428
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 3.841

Mu = -0.477444
Sigma = 0.445313

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	6.072152	0.058426	(5.957637, 6.186666)
Slope	2.245609	0.101584	(2.046504, 2.444715)
Spontaneous Response Rate	0.136653	0.010816	(0.115453, 0.157852)

Estimated LC/EC Values and Confidence Limits

Exposure Point	Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	**3.1**	**2.2**	**4.0**
LC/EC 5.00	6.2	4.8	7.6
LC/EC 10.00	9.0	7.2	10.8
LC/EC 15.00	11.5	9.5	13.6
LC/EC 50.00	**33.3**	**29.6**	**37.1**
LC/EC 85.00	96.4	87.1	107.5
LC/EC 90.00	124.0	111.0	139.9
LC/EC 95.00	179.9	158.3	208.0
LC/EC 99.00	**361.8**	**304.8**	**441.7**

Epinephrine

Probit Analysis

EPA PROBIT ANALYSIS PROGRAM
USED FOR CALCULATING LC/EC VALUES
Version 1.5

EPINEPHRINE

Conc. µmolL^{-1}	Observed Proportion Responding	Proportion Adjusted for Controls	Predicted Proportion Responding
Control	0.0830	0.0000	0.0894
10	0.1890	0.1094	0.0916
100	0.7210	0.6936	0.7168
1000	1.0000	1.0000	0.9934

Chi - Square for Heterogeneity (calculated) = 10.219
Chi - Square for Heterogeneity
(tabular value at 0.05 level) = 3.841

Mu = -0.301063
Sigma = 0.525150

****
* NOTE *
* Slope not significantly different from zero. *
* LC/EC fiducial limits cannot be computed. *
****

Parameter	Estimate	Std. Err.	95% Confidence Limits
Intercept	5.573289	0.139522	(3.800518, 7.346061)
Slope	1.904217	0.256633	(-1.356568, 5.165002)
Spontaneous Response Rate	0.089381	0.028158	(-0.268389, 0.447151)

Estimated LC/EC Values and Confidence Limits

Exposure Point	Conc.	95% Confidence Limits Lower	Upper
LC/EC 1.00	**3.0**		
LC/EC 5.00	6.8		
LC/EC 10.00	10.6		
LC/EC 15.00	14.3		
LC/EC 50.00	**50.0**		
LC/EC 85.00	175.1		
LC/EC 90.00	235.5		
LC/EC 95.00	365.4		
LC/EC 99.00	**832.9**		

APPENDIX IV

SPECIFIC ACETYLCHOLINE RECEPTOR EFFECTORS

Nicotinic acetylcholine receptor effectors.

Receptor type	Effect	Agonists	Antagonists
Muscle type: $(\alpha1)_2\beta1\delta\varepsilon$ or $(\alpha1)_2\beta1\delta\gamma$	EPSP, mainly by increased Na^+ and K^+ permeability	acetylcholine carbachol suxamethonium	α-bungarotoxin α-conotoxin tubocurarine pancuronium
Ganglion type: $(\alpha3)_2(\beta4)_3$	EPSP, mainly by increased Na^+ and K^+ permeability	acetylcholine carbachol nicotine epibatidine dimethylphenylpiperazinium varenicline	α-bungarotoxin mecamylamine trimetaphan hexamethonium bupropion dextromethorphan ibogaine 18-methoxycoronaridine
CNS type: $(\alpha4)_2(\beta2)_3$	Post- and presynaptic excitation, mainly by increased Na^+ and K^+ permeability	nicotine epibatidine acetylcholine cytisine	mecamylamine methylcaconitine α-conotoxin
CNS type: $(\alpha7)_5$	Post- and presynaptic excitation, mainly by increased Ca^{2+} permeability	epibatidine dimethylphenylpiperazinium	mecamylamine α-bungarotoxin

Muscarinic acetylcholine receptor effectors.

Receptor Type	Function	Effectors	Agonists	Antagonists
M_1	EPSP in autonomic ganglia. Secretion from salivary glands and stomach	G_q (G_i) (G_s): Slow EPSP. ↓ K^+ conductance	acetylcholine oxotremorine carbachol McNA343	atropine scopolamine dicycloverine tolterodine oxybutynin ipratropium mamba toxin MT7 pirenzepine telenzepine
M_2	Slow heart rate, reduces contractile forces of atrium, reduces conduction velocity of AV node. In CNS: homotropic inhibition	G_i ↑ K^+ conductance ↓ Ca^{2+} conductance	acetylcholine methacholine carbachol oxotremorine	atropine dicycloverine tolterodine oxybutynin ipratropium methoctramine tripitamine gallamine
M_3	Smooth muscle contraction, increased endocrine and exocrine gland secretions, e.g. salivary glands and stomach	G_q	acetylcholine bethanechol carbachol oxotremorine pilocarpine	atropine dicycloverine tolterodine oxybutynin ipratropium darifenacin tiotropium
M_4	Enhanced locomotion In CNS	G_i ↑ K^+ conductance ↓ Ca^{2+} conductance	acetylcholine carbachol oxotremorine	atropine dicycloverine tolterodine oxybutynin ipratropium mamba toxin MT3
M_5	In CNS	G_q	acetylcholine carbachol oxotremorine	atropine dicycloverine tolterodine oxybutynin ipratropium

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