DOCTOR OF PHILOSOPHY

Induction of metamorphosis and seed attachment in hatchery production of the king scallop Pecten maximus (L.) and the blue mussel Mytilus edulis (L.)

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Induction of metamorphosis and seed attachment in hatchery production of the king scallop *Pecten maximus* (L.) and the blue mussel *Mytilus edulis* (L.)

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Thesis Summary

This thesis assessed the ability to control metamorphosis in the commercially important king scallop, *Pecten maximus* and blue mussel, *Mytilus edulis* using chemical inducers. It also investigated the influence of environmental factors on attachment and dispersal of their motile post-larvae. The aim was to increase our understanding of bivalve biology and behaviour, and provide useful tools for aquaculture.

The influence of potassium chloride (KCl), ammonium chloride (NH_4Cl), acetylcholine chloride, γ-aminobutyric acid (GABA), L-3,4-dihydroxyphenylalanine (L-DOPA) and epinephrine was assessed on larval *P. maximus* metamorphosis and mortality over a range of concentrations. 20mM KCl and 10^{-6}M L-DOPA induced development 208% and 128% higher, respectively, than untreated controls after 7 days. The KCl treatment was toxic, reducing survival by 33%, whilst the L-DOPA treatment significantly increased survival by 49%.

The influence of L-DOPA, epinephrine, KCl and NH_4Cl over a range of concentrations and exposure periods was assessed on *M. edulis* larval metamorphosis, mortality and growth. No chemical provided a lasting improvement in metamorphosis compared to a control. KCl induced a slight improvement in growth at concentrations of $1.3x10^{-3}$M and $1.3x10^{-2}$M after exposure for 24 and 48 hours, without compromising survival.

A series of experiments assessed the impact of substrate, attachment period, food availability, water agitation, seed density and pedal activity on attachment and retention of *M. edulis* seed. Substrate type and length of attachment period affected attachment. Feeding seed increased detachment, although water agitation increased retention. Detachment and dispersal was proportional to seed density, and dependent upon substrate type, with loss higher from glass than from wool materials. Seed crawling activity could also be used as a predictive test of future seed mortality.

The impact of substrate type, substrate pre-conditioning, length of attachment period, feeding ration, agitation and illumination were assessed on the attachment of juvenile *P. maximus* scallops. Substrate type, pre-conditioning and feeding ration was also assessed on juvenile detachment over increasing water velocities. Recommendable parameters for maximising attachment and retention in water velocities up to 12.6±0.2cm $second^{-1}$ include utilising a slate substrate, pre-conditioned for

1 week, feeding a diet of at least 0.025g microalgae.g^{-1} juveniles.$week^{-1}$, and allowing to attach for at least 24 hours.

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Chapter 1

General Introduction

Introduction

The production of bivalves for human consumption is an important industry in Europe. In the last decade Europe produced between 819,500 and 1,041,949 tons of bivalves annually (FAO, 2014). However, unpredictable supplies of wild seed and adults have led to continued interest in the development of bivalve hatcheries to safeguard cultivation and natural fisheries whilst enhancing production capabilities. Hatchery culture enables the careful control of the culture environment, and animal husbandry through the establishment of suitable facilities and rearing protocols (Utting and Spencer 1991; Millican, 1997; Helm and Bourne, 2004). Many forms of aquaculture globally are still dependent upon harvesting wild resources, either through the collection of seed, broodstock or food for cultured animals; however, more countries are now adopting hatchery culture for seed production (Davenport *et al*., 2009). For molluscs, crustaceans and finfish, hatchery culture has enabled the development and application of many advantageous techniques, including broodstock domestication, selective breeding, genetic manipulation and the culture of specific pathogen free animals in order to improve production (Wickins and Lee, 2002; Nell, 2002; Beaumont and Hoare, 2003; Dunham, 2012).

Domestication of broodstock and selective breeding have been applied to unlock beneficial inheritable traits including body shape and antibody production in carp (*Cyprinus carpio*), growth rate in tilapia (*Oreochromis niloticus*), growth in oysters (*Argopecten irradians, Tiostrea lutaria* and *Ostrea edulis*), clams (*Mercenaria mercenaria*), crayfish (*Cherax tenuimanus* and *C. quadricarinatum*), growth and disease resistance in shrimp (*Litopenaeus vannamei*), and larval prawn tolerance to fresh water (Macr*obrachium nipponense*) and tolerance to cold environments (*M. rosenbergii*) (Beaumont and Hoare, 2003). Inbreeding, cross-breeding and hybridisation has also been attempted to promote beneficial characteristics (Wickins and Lee, 2002; Beaumont and Hoare, 2003; Dunham, 2012). Whilst not guaranteed to be successful in marine animals, evidence for superior fitness of cross-bred or hybrid offspring is common in agricultural crops and animals (Beaumont and Hoare, 2003). In fish inter-specific crossbreeding and hybridisation has been seen to improve growth, survival, produce monosex cultures and sterile animals, improve flesh quality, improve disease resistance and improve environmental tolerances in some species, but success

varies (Beaumont and Hoare, 2003; Dunham, 2012). In oysters of the genus *Crassostrea* attempts at hybridisation between species have been unsuccessful and it is therefore not an avenue to pursue (Beaumont and Hoare, 2003). Consideration is given to the amount of genetic diversity within the initial population and the extent which this can be exploited, with genetic techniques being applied to establish when new parent stock should be introduced (Wickins and Lee, 2002; Beaumont and Hoare, 2003). Hatchery culture has also allowed for the development of direct genetic manipulation of molluscs, crustaceans and food fish species by disrupting the normal post-fertilisation mitosis and meiotic divisions to create polyploids, gynogens and androgens through chemical, temperature and pressure shocks (Wickins and Lee, 2002; Beaumont and Hoare, 2003; Dunham, 2012). The benefits of ploidy manipulation is to create sterile and monosex cultures, with prolonged flesh quality and after sexual maturity, improved growth (Wickins and Lee, 2002; Beaumont and Hoare, 2003; Dunham, 2012). Importantly the sterility of triploids makes it feasible to culture non-native exotic species or genetically limited hatchery stock which might cause adverse environmental harm if they escaped into the wild (Beaumont and Hoare, 2003). Whilst triploid induction can be highly effective, the development of tetraploid broodstock is favoured, since they will produce diploid gametes which when combined with a haploid gamete, from a normal diploid, produce 100% triploids (Beaumont and Hoare, 2003). Additionally hatcheries have paved the way for the implementation of transgenesis techniques involving the transfer of beneficial genes, such as growth hormone genes and antifreeze protein genes, from one species to another (Beaumont and Hoare, 2003). Utilising microinjection of DNA material, electroporation, sperm-mediate transfer, viral vectors, lipofection and biolistic methods it is possible to transplant genes to improve growth rate, colour, disease resistance and cold resistance (Wickins and Lee, 2002; Beaumont and Hoare, 2003; Dunham, 2012). Hatchery production has also provided another means of controlling disease, with facilities producing shrimp that are designated as specific pathogen free. These incorporate primary, secondary and even tertiary quarantine facilities and procedures, operate under strict biological isolation and have rigorous screening for pathogens to provide high-health offspring for sale (Wickins and Lee, 2002). Research and development is continuing to refine and improve hatchery technology making it more efficient and profitable.

The aim of this thesis was to address major challenges continuing to face bivalve hatchery culture efforts for the commercially important king scallop (*Pecten maximus*) and blue mussel (*Mytilus edulis*). Whilst hatchery culture techniques for bivalve molluscs are well documented (Loosanoff and Davis 1963; Utting and Spencer 1991; Millican 1997; Helm and Bourne 2004; Laing *et al*. 2004), optimisation of culture methods is required to ensure the high densities and fast growth rates

necessary for commercial production. A number of major challenges remain however to be overcome. In particular difficulties in the culture of bivalves have often been associated with settlement and metamorphosis, the period during which mature larvae undertake exploratory behaviour as they search for a suitable substratum, before undergoing permanent morphological changes allowing them to adapt to their new benthic habitat (Bayne, 1965; Pawlik, 1990; Lutz and Kennish, 1992; Gosling, 2003). Furthermore many bivalve species display motile behaviour facilitating secondary dispersal post-metamorphosis (Sigurdsson *et al*., 1976; Sörlin, 1988; Beukema and de Vlas, 1989; Roper *et al*., 1995; Baker and Mann, 1997). This behaviour represents an important tool in the selection of a suitable habitat, however from an aquaculture perspective it represents a risk to the effective management of a valuable and finite resource if the animal can be lost from production systems.

This thesis investigated the ability to control the timing of metamorphosis in the commercially important king scallop (*Pecten maximus*) and blue mussel (*Mytilus edulis*) using exogenous chemical inducers, and investigated the behaviour of motile post-larvae and the influence of exogenous factors on attachment and dispersal. The intention was to further our understanding of bivalve biology and behaviour, and provide useful tools, techniques and strategies designed to benefit commercial culture efforts.

This chapter provides a review of chemical control of settlement and metamorphosis in bivalves, and post-larvae motile behaviour and factors affecting attachment and retention.

The king scallop (*Pecten maximus*) and the blue mussel (*Mytilus edulis*)

The king scallop *P. maximus* (Figure 1.1) and the blue mussel *M. edulis* (Figure 1.2) are both commercially important bivalves within Europe. However, production continues to face a number of challenges.

P. maximus is a large scallop, growing up to 150mm in shell height, which belongs to the Pectinidae family of bivalves. Commonly known as the king scallop or great Atlantic scallop, it is characterised by unequal shell valves, the lower right being deeply convex and slightly overlapping the upper valve which is flat. The valves vary in colour and patterning, typically off-white to yellow to light-brown, but have 15 to 16 broad, radiating ribs and numerous fine concentric lines known as striae (*Ansell et al*., 1991; Gosling, 2003). There are also two distinctive ears or auricles which project on either side

of the umbo, which vary in size and shape (Gosling, 2003). It is generally found recessed into fine sediment, often just below the surface, with its left valve upper most towards the surface (Ansell *et al*., 1991; Hayward and Ryland, 2000; Bergh and Strand, 2001; Gosling, 2003). It is found from Norway to the Atlantic coast of Spain and West Africa (Hayward and Ryland, 2000). During its early life small *P. maximus* are able to produce byssus threads, much like mussels, allowing it to attached to surfaces (Hayward and Ryland, 2000), however as adults they are unattached allowing them to rapidly swim or jump away if disturbed by potential predators (review by Ansell *et al*., 1991; Hayward and Ryland, 2000).

P. maximus is a high value species with production reaching 63,776 tons in 2011 (FAO, 2014). However, production is almost completely reliant on natural fisheries, typically using dredges to collect recessed animals (Ansell *et al*., 1991; Spencer, 2002; Gosling, 2003), which in 2011 provided 99.9% of the total production (FAO, 2014). Important commercial fisheries are located in France and the United Kingdom, supplemented with small fisheries located in the Isle of Man, the Channel Islands, Ireland, Spain, Belgium, the Netherlands and Norway (Spencer, 2002; FAO, 2014). Traditionally *P. maximus* has been collected from natural beds using dredges deployed from fishing vessels, with an attached toothed bar to lift recessed animals from the seabed, examples of which include the spring loaded Newhaven dredge and the Baird dredge (Chapman *et al*., 1977; Ansell *et al*., 1991). Some attempts to cultivate *P. maximus* in the 1960s and 1970s relied upon the collection of naturally settled juvenile spat which were on-grown to a marketable size using suspended pearl and lantern nets, ear-hanging, and bottom culture (Millican, 1997; Spencer, 2002).

Figure 1.1: The king scallop *Pecten maximus.*

The blue mussel *M. edulis* is a pyriform shaped bivalve belonging to the Mytilidae family, along with the closely related Mediterranean mussel *M. galloprovincialis* (Gosling, 1992; Hayward and Ryland, 2000). Commonly ranging in size from 50 to 100mm, its shell is light blue or purple in colour and possesses prominent raised umbonal ridges curving posteriorly and ventrally. Shell colour darkens with age and contrasts with the mantle edge which is typically yellow-brown (Gosling, 1992; Hayward and Ryland, 2000). A characteristic of mussels in general, *M. edulis* attaches using byssus threads to benthic surfaces, typically in dense beds on both natural and artificial surfaces, ranging from the upper shore to the shallow sublittoral (Hayward and Ryland, 2000; Spencer, 2002; Gosling, 2003). *M. edulis* has an extremely wide distribution, ensured in part by their high fecundity and pelagic larval phase, which encompasses both boreal and temperate waters in the northern hemisphere, ranging from southern France north to Russia and Iceland and parts of the eastern coast of North America, and also the southern hemisphere, being found in Argentina and Chile, the Falkland Islands and Kerguelen Islands (Spencer, 2002).

European production of *M. edulis* from aquaculture and natural fisheries totalled 196,665 tons in 2011 (FAO, 2014) accounting for 83.5% of world production, as well as representing 23.5% of all bivalve species produced in Europe. Based upon statistics provided by the Food and Agriculture Organization of the United Nations the vast majority of production (75.6%) is derived from aquaculture, with natural fisheries providing a small but significant contribution, although this appears to have declined in recent years (FAO, 2014). However, it is reportedly almost impossible to locate a truly natural fishery that has not been subject to some level of human intervention at some stage (Gosling, 2003). The fisheries that are closest to being described as natural are those where seed are collected from natural sources and relayed on controlled beds for on-growing, with no further human input, such as those seen in the Wadden Sea area (Gosling, 2003). In Europe, mussel production is traditionally reliant upon collecting wild seed (around 20 mm shell length), either by dredging areas of natural settlement or using artificial substrates to act as collectors, and then on-growing to a marketable size (4-7 cm) in 1-3 years. On-growing is usually undertaken either using bottom culture or suspended culture on bouchots, rafts and long line systems (Spencer 2002; Maguire *et al*. 2007).

Figure 1.2: The blue mussel *Mytilus edulis.*

A key problem facing the commercial production of both *P. maximus* and *M. edulis* is the dependence on natural sources of seed animals. Natural variability in spat fall and limited windows of availability, as well as stringent conservation legislation in some areas, means there is a risk of limited production yields if spat falls are low and there is a resulting shortage of seed (Millican, 1997; de Vooys 1999; Kamermans and Smaal 2002; Spencer, 2002; Maguire *et al*. 2007). Furthermore, it is known that increasing demand for seed mussels is not being met by natural sources (Maguire *et al*. 2007). The obvious solution to meet demand and ensure a reliable supply of seed is to establish hatchery production as a source of high quality seed in sufficient quantities (Millican, 1997; Utting and Spencer 1991; Spencer 2002; Maguire *et al*. 2007). This would also offer producers the benefits of broodstock domestication, artificial selection for better growth and polyploidy manipulation (Utting and Spencer 1991; Brake *et al*., 2001; Spencer 2002; Beaumont and Hoare 2003).

The culture of both *P. maximus* and *M. edulis* is well documented (Loosanoff and Davis 1963; Gruffydd and Beaumont, 1970; Gruffydd and Beaumont, 1972; Beaumont and Budd, 1983; Utting and Spencer 1991; Millican, 1997; Bergh and Strand, 2001; Spencer, 2002; Helm and Bourne 2004; Torkildsen and Magnesen, 2004; Galley *et al*., 2010). Though still in its infancy, hatchery production of *P. maximus* seed has now been established in several European counties including Orkney in Scotland (Spencer, 2002), Norway (Bergh and Strand, 2001; Torkildsen and Magnesen, 2004), Ireland and France (Robert and Nicolas, 2000; REPROSEED, 2014). The Norwegian scallop aquaculture industry is now entirely reliant upon spat sourced from hatcheries (Torkildsen and Magnesen, 2004). In contrast seed production of *M. edulis* is currently restricted, as hatchery production has so far

been considered economically unfeasible, since production is based upon a low unit value (Brake *et al*., 2001). Hence, only a single hatchery in Europe, in the Netherlands, produces limited quantities for experimental purposes (P. Kamermans personal communication). However, in order to safeguard the industry the potential for hatchery production needs to be developed. Key challenges to the effective culture of both species however remain to be overcome, including synchronicity of larval development, survival and control of mobile seed.

Settlement and metamorphosis of larval bivalves

Most bivalves, like many marine invertebrates, have complex life histories that progress from a pelagic larval phase to a benthic juvenile and adult phase (Gosling, 2003). Many bivalves produce large numbers of pelagic larvae that are morphologically distinct from their adult forms and which spend their early life as part of the plankton, be that days, weeks or months (Widdows, 1991; Gosling, 2003; Knight *et al*., 2006). During this phase larvae undertake dispersal, the scale of which influences population dynamics and potentially links populations over large temporal and spatial scales (Knight *et al.,* 2006). Generally larvae remain locally within parcels of water close to adult habitats and are subjected to bottom currents, or they are dispersed over great distances by current advection influenced by wind-driven transport, with tidal conditions also playing a role upon distribution (Knight *et al*., 2006). Pelagic larvae continue to, feed, grow and develop until they reach a stage where they become competent to settle to the substratum and metamorphose into juveniles (Widdows, 1991; Gosling, 2003).

Settlement and metamorphosis are important events in controlling the population dynamics of benthic marine invertebrates, both of which are influenced by the interaction of abiotic and biotic factors over wide temporal and spatial scales (Hadfield, 1986; Pawlik, 1990; Pawlik, 1992; Rodríguez *et al*., 1993). Extensive early reviews on the control of settlement and metamorphosis in bivalves and other marine invertebrates are provided by Pawlik (1990), Pawlik (1992), and Rodríguez *et al*. (1993).

However, difficulties in the culture of bivalves have often been associated with this transitional period, as they move from one life stage to the next. In particular *M. edulis* is known to display variable growth rates, and delay settlement and metamorphosis for 5 to 6 weeks if conditions, based on temperature, salinity, pH and substrate availability, are unsuitable (Loosanoff and Davis 1963; Bayne 1965; Eyster and Pechenik, 1987). *P. maximus* exhibits protracted development over several

weeks, leading to a lack of development synchronicity and variable levels of survival in cultured animals (Nicolas *et al*., 1998; Robert and Nicolas, 2000). The ability to control settlement and metamorphosis in commercially important bivalves represents a powerful tool for the aquaculture sector (Baloun and Morse, 1984; Mesías-Gansbillar *et al*., 2008).

Larval *Pecten maximus* and *Mytilus edulis*

In the case of *P. maximus* and *M. edulis* both possess a similar larval model, releasing gametes into the water column where fertilisation occurs (Spencer, 2002; Gosling, 2003). The main difference between the two species is that *P. maximus* is a functional hermaphrodite, with the gonad divided into the white dorsal testis and orange ventral ovary (Gruffydd and Beaumont, 1972; Gosling, 2003), whilst *M. edulis* is dioecious meaning that the sexes are separate (Spencer, 2002; Gosling, 2003). In the laboratory *P. maximus* will often, but not always, discharge spermatozoa before they release eggs (Gruffydd and Beaumont, 1972). In both cases fertilised embryos develop through an initial flagellated trochophore stage, before developing into a straight-hinged veliger or D-larvae with prodissoconch I shell, which is secreted by the shell gland and mantle epithelium of the larvae (Figure 1.3) (Gruffydd and Beaumont, 1972; Widdows, 1991; Lutz and Kennish, 1992; Gosling, 2003)

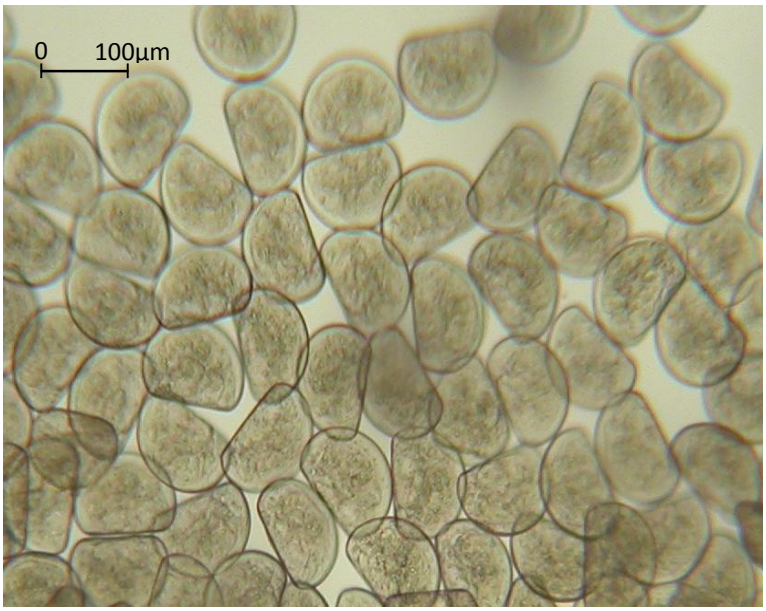

Figure 1.3: *Mytilus edulis* veliger larvae at 72 hours post-fertilisation.

At this stage veligers employ a ciliated velum for locomotion and feeding upon phytoplankton present within the water column. They also begin laying down the prodissoconch II shell (Bayne 1965: Gruffydd and Beaumont, 1972; Lutz and Kennish, 1992; Gosling 2003). Towards the end of the

larval cycle veligers develop a pair of eye-spots in the centre of each shell valve and a ciliated pedal organ, which 2 to 3 days later can be used for crawling. At this point the larvae are described as pediveligers, due to the coexistence of a functional foot and a functional velum, enabling them to alternate between swimming and crawling along the substratum as they search for a suitable site for settlement and metamorphosis (Bayne 1965: Gruffydd and Beaumont, 1972; Widdows, 1991; Lutz and Kennish, 1992; Gosling 2003).

The larval cycle lasts for several weeks, with larvae reaching a size of approximately 250-300µm, although the length of larval phase as well as larval survival is heavily dependent upon prevailing environmental conditions (Bayne, 1965; Gruffydd and Beaumont, 1972; Lutz and Kennish, 1992; Gosling, 2003). Under laboratory conditions it takes approximately 33 to 38 days for *P. maximus* at 16˚C, whilst it takes 16-20 days for *M. edulis* to reach the pediveliger stage at 16°C (Bayne, 1965; Gruffydd and Beaumont, 1972).

Settlement

The term settlement has been used to describe the overall transition from the pelagic to the benthic way of life, including decent from the plankton, substrate exploration, attachment and metamorphosis (Pawlik, 1990; Rodríguez *et al*., 1993). However, in the literature it is also commonly used to define the reversible, exploratory and a behavioural response of mature larvae as they descend and undertake active selection and differentiate it from the process of metamorphosis (Coon *et al*., 1985; Pawlik, 1990; Lutz and Kennish, 1992; Gosling, 2003). The settlement process of invertebrate larvae is seen as a passive delivery process to the surface with an active behavioural component at the surface, with possible active components in the water column (Boxshall, 2000; Knights *et al*., 2006).

In *M. edulis* and mussels, settlement can be characterised by non-permanent primary and permanent secondary settlement stages. Prior to selecting a permanent site for settlement, typically established mussel beds, stones, rocks, moorings and other hard surfaces, early settling larvae may undertake non-permanent settlement, whereby they repeatedly attach and de-attach themselves to several substrates, commonly filamentous algae, hydroids or other filamentous objects in the water column (Bayne, 1964a; Spencer, 2002). Juvenile *P. maximus* are generally found in shallow and sheltered bays attached to surfaces free of silt, including algae, invertebrates and materials such as coconut fibre, polypropylene rope, monofilament netting and pebbles, among

others (Minchin, 1992). Attachment is only temporary with most remaining attached up to 4-13mm in shell length, with few attached greater than 15mm, after which they recess into sediments and are subject to dispersal by water turbulence and swimming (Beninger and Le Pennec, 1991; Brand, 1991; Minchin, 1992).

Metamorphosis

Settlement culminates in attachment to the substratum and is followed by metamorphosis, a process which encompasses the morphological changes which occur within the larvae, including the reorientation of internal structures, increasing complexity of the organ systems and secretion of the adult shell, known as the dissoconch, as they develop into juvenile adults (Bayne, 1965; Coon *et al*., 1985; Widdows, 1991; Gosling 2003). A significant and distinctive changed during the onset of metamorphosis is the degeneration of the velum and the development of ciliated gill buds which become the feeding organ of the animal (Gruffydd and Beaumont, 1972; Gosling 2003; García-Lavandeira *et al*., 2005) (Figure 1.4).

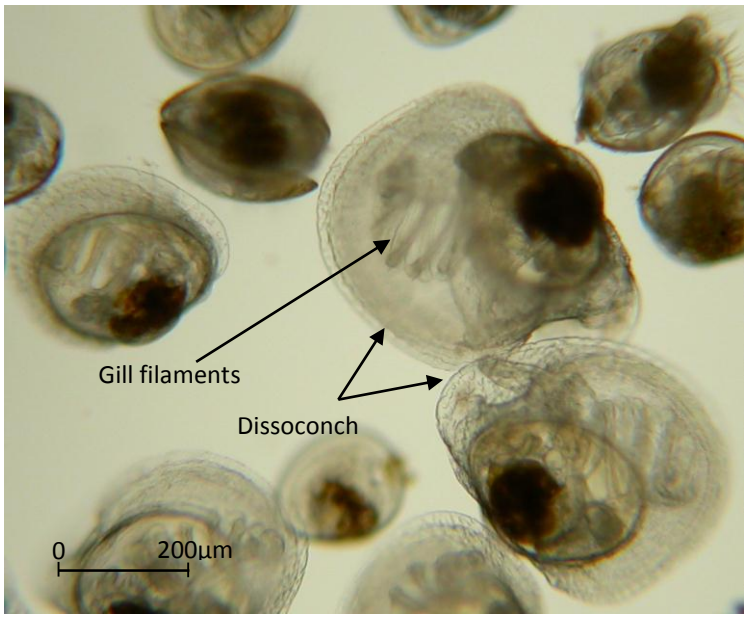

Figure 1.4: Post-larval *Pecten maximus* possessing elongated functional gill filaments and secondary dissoconch shell.

This change is thought to be controlled by a combination of neuronal and neuroendocrine activities and marks the end of the pelagic life phase and the beginning of the benthic life phase (Bayne, 1965; Widdows, 1991; Gosling 2003; García-Lavandeira *et al*., 2005). However, it does not necessarily mark the end of dispersal.

Control of settlement and metamorphosis

In general it is perceived that marine invertebrate larvae, including bivalves, are likely to move passively in hydrodynamic processes operating at large spatial scales (tens of metres to tens of kilometres) (Boxshall, 2000; Knights *et al*., 2006). Although it is recognised that some active regulation of current transport occurs over these scales as a consequence of vertical-tidal migration, as seen in the mussel *M. edulis* (Knights *et al*., 2006). At smaller scales (centimetres to metres) however, many invertebrate larvae possess the ability to undertake some form of active selection between favourable and unfavourable benthic environments, thereby maximising their chance of post-settlement survival (Eyster and Pechenik, 1987; Mullineaux and Butman, 1991; Grassle *et al*., 1992; Lutz and Kennish, 1992; Pawlik, 1992; Olivier *et al*., 1996; Daume *et al*., 1999; Ritson-Williams *et al*., 2009). This ability is a response to complex signals attributed to both physical and biological sources (Jackson, 1986; Wethey, 1986; Maldonado and Young, 1996; Eyster and Pechenik, 1987; Mullineaux and Butman, 1991; Pearce and Bourget, 1996; Moss, 1999; Boxshall, 2000), although a combination of factors may be required to determine acceptability of settlement sites (Pawlik, 1992).

The primary stimulus for induction of marine invertebrate settlement and metamorphosis has been determined to be chemical in nature, originating from biological sources (Morse and Morse, 1984). These cues are either surface-bound or water-bound, and have been associated with conspecifics (Highsmith, 1982; Zimmer-Faust and Tamburri, 1994; Pearce and Scheibling, 1990a; Zhao and Qian, 2002; Cob et al., 2010), biofilms (Satuito et al., 1995; Daume *et al*., 1999; Zhao and Qian, 2002; Zhao *et al*., 2003), bacteria (Bonar *et al*., 1990; Satuito *et al*., 1995), habitat and food (Morse and Morse, 1984; Pearce and Scheibling, 1990b; Swanson *et al*., 2004; Ritson-Williams *et al*., 2009).

A number of naturally derived chemicals have been isolated, including histamine from the red algae *Delisea pulchra* (Swanson *et al*., 2004), ammonia produced by bacteria, such as the marine gram-negative species *Alteromonas colwelliana* (Coon *et al*., 1988; Bonar *et al*., 1990) and jacaranone from the red algae *Delesseria sanguinea* (Yvin *et al*., 1985). The specific identity of many however is largely unknown or only partially characterised, though they often appear to be low molecular weight compounds (Highsmith, 1982; Hadfield, 1984; Morse and Morse, 1984; Fritt *et al*., 1990; Pearce and Scheibling, 1990a).

The identification of simple chemical inducers has the potential to provide routine, inexpensive and effective culture techniques for the induction and synchronisation of larval settlement and metamorphosis (Davis, 1990). This has led to a wide range of pharmacological agents being tested, including neurotransmitters, amines, amino acids, alcohols, carboxylic acids, sugars and amino sugars, ions and other compounds in a wide range bivalve and other marine invertebrate species. Effects on settlement and metamorphosis have ranged from the induction of normal settlement and metamorphosis, to abnormal or partial development, mortality or no effect at all (Morse *et al*., 1979; Hadfield, 1984; Coon *et al*., 1985; Pennington and Hadfield, 1989; Pawlik, 1990; Pires and Hadfield, 1991; Boettcher and Targett, 1998; Carpizo-Ituarte and Hadfield, 1998; Nicolas *et al*., 1998; Dobretsov and Qian, 2003; García-Lavandeira *et al*., 2005; Zhao *et al*., 2003; Yang *et al*., 2008; Yu *et al*., 2010).

Effective agents have been suggested to act as functional analogues of natural inducers, as precursors, or as active components within a signalling pathway (Yool *et al*., 1986; Pawlik, 1990). Some evidence indicates that the sites of action of effective chemicals can be biochemically distinct from one another, whilst there can be a cumulative effect on settlement and metamorphosis through the combination of chemical agents (Baxter and Morse, 1987). Settlement and metamorphosis in response to inducers including γ-aminobutyric acid (GABA), potassium chloride (KCl) and 3-isobutyl-1-methylxanthine, by the gastropod *Haliotis rufescens* can be amplified by combining with cholera toxin, GTP analog 5-guanylyl imidophosphate, the secondary messenger diacylglycerol, and certain diamino acids that stimulate separate regulatory and inductive pathways (Baxter and Morse, 1987).

However, the effectiveness of an inducer is influenced by the competence of the larvae to undergo settlement and metamorphosis (Chevolot *et al*., 1991). If larvae are not competent then the chemical is unlikely to stimulate a response. The level of larval competence in studies therefore has the potential to play a significant role in assessing the response of larvae to chemical agents. For *P. maximus* competence has previously been correlated, to larval size and development of a double ringed shell, with Nicolas *et al*. (1998) deeming cultures of larvae competent once 30-50% of larvae possessed a double ring and were retained by a 150µm mesh. For *M. edulis* competence has previously been correlated with the development of pigmented eye-spots (Eyster and Pechenik, 1987). Importantly though Coon *et al*. (1990a) demonstrated the temporal relationship between the onset of behavioural and morphogenetic competence in the larvae of the oyster *Crassostrea gigas* using L-3,4-dihydroxyphenylalanine (L-DOPA) and epinephrine as inducers. They found that larvae

display a settlement response to L-DOPA, before showing a metamorphic response to epinephrine, which in turn preceded a metamorphic response to L-DOPA. The temporal separation of the various facets of competence suggests that settlement and metamorphosis is a multi-step process with competence developing through different mechanisms (Coon *et al*., 1990a). Inclusion of an internal chemical control treatment, such as 10^{-4}M in assays with *C. gigas* oysters, has been recommended in order to allow comparison between the relative competence and inducability of batches of larvae, thereby enabling different batches of larvae to be compared (Bonar *et al*., 1990). However, this is only possible once a suitable treatment for use as an internal control has been identified.

Bivalve chemical assays

The methods adopted to test the effectiveness of different chemicals in bivalves has varied between studies, both in terms of scale and time. Many studies have tested small numbers of larvae ranging from 10 to 100 suspended in volumes of 1 to 10ml (Coon *et al*., 1990b; Fritt *et al*., 1990; Doroudi and Southgate, 2002; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005; Yu *et al*., 2008; Teh *et al*., 2012; Yang *et al*., 2013).Others have tested induction on larger scales in greater volumes with more larvae (Nicolas *et al*., 1996; Martinez *et al*., 1999; García-Lavandeira *et al*., 2005; Mesías-Gansbiller *et al*., 2008), including Coon *et al*. (1986) who induced 10^5 larvae at 20 larvae ml^{-1} in 4.5 litre cultures. However, it is recognised that there is inherent variability in the sensitivity and threshold of responses by larvae to chemical inducers within and between larval batches and with larval age, therefore absolute comparisons of inducability are only valid within individual sets of experiments, and data arising from small groups requires cautious interpretation (Bonar *et al*., 1990). In the literature exposure to chemicals ranges from 1-96 hours, whilst total study periods range from 24 hours to 1 week (Coon *et al*., 1985; Chevolot *et al*., 1991; Nicolas *et al*., 1996; Nicolas *et al*., 1998; Doroudi and Southgate, 2002; Yu *et al*., 2008). Larvae are either assessed immediately after the exposure period (Bonar *et al*., 1990; Doroudi and Southgate, 2002; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005; Yu *et al*., 2008) or after a further culture period (Coon *et al*., 1986; Fritt *et al*., 1990; Chevolot *et al*., 1991; Nicolas *et al*., 1996; Nicolas *et al*., 1998; Yang *et al*., 2008). It is apparent that the small larval numbers combined with short assay periods used in many studies may be insufficient to determine the full effect of chemical treatments.

The effectiveness of chemicals on bivalves has been evaluated in a range of ways. This includes their ability to induce settlement behaviours, such as sitting on a benthic surface with velum absorbed (Yu *et al*., 2008), attachment, either by byssus threads (Zhao *et al*., 2003) or by the inability to dislodge

with a jet of water (Martinez *et al*., 1999; García-Lavandeira *et al*., 2005; Mesías-Gansbiller *et al*., 2008). Assessment of metamorphosis varies with species. In *C. gigas* the cementation of larvae has been used to indicate metamorphosis, as this is irreversible (Coon *et al*., 1985; Coon *et al*., 1986), whilst in other species García-Lavandeira *et al*. (2005) used only the loss of the larval velum and use of the foot for pedal walking. The presence of post-larval shell growth (Chevolot *et al*., 1991; Yang *et al*., 2008) and differentiation of gill bars (Bonar *et al*., 1990; Fritt *et al*., 1990) have also been used as an indication of metamorphosis.

Effective chemical agents

A number of chemical agents have repeatedly proven successful at inducing settlement, metamorphosis or both in bivalves and other invertebrate larvae. These include the catecholamine neurotransmitter epinephrine and its precursor L-DOPA, the amino acid derivative GABA, the choline derivative acetylcholine, and potassium and ammonium ions. The following sections review the use of these chemicals in bivalves, with particular reference to *P. maximus* and *M. edulis.*

γ-aminobutyric acid

The amino acid derivative γ-aminobutyric acid, also referred to as GABA, has been implicated as a settlement and metamorphosis cue in many species of marine invertebrates, including gastropod molluscs (Morse *et al*. 1979;Moss and Tong, 1992; Bryan and Qian, 1998; Moss, 1999; Yu *et al*., 2010), echinoderms (Pearce and Scheibling, 1990b) and bryozoans (Yu *et al*., 2007)). It was initially identified by Morse *et al*. (1979) through the relationship between juveniles of the red abalone *H. rufescens,* and the crustose coralline algae *Lithothamnium* sp. and *Lithophyllum* sp.. In bivalves, GABA has been effective at inducing settlement, metamorphosis or both in a number of species (Table 1.1).

Table 1.1: Influence of GABA on the settlement and metamorphosis of bivalve larvae. FSW = filtered seawater.

Species	Concentration range tested	Optimum concentration	% Settlement (FSW control)	% Metamorphosis (FSW control)	Reference
Pinctada margaritifera	10^{-2}-10^{-5}M	10^{-4}M	~5-15%* (0%)	-	Doroudi and Southgate, 2002
Pinctada maxima	10^{-2}-10^{-5}M	10^{-3}M	~5-55%* (~5%)	-	Zhoa *et al*., 2003
Pinctada fucata martensii	10^{-2}-10^{-6}M	10^{-4}M	~<5-30%* (<5%)	-	Yu *et al*., 2008
Mytilus edulis	10^{-5}-10^{-6}M	x	-	0% (0%)	Cooper, 1982
	10^{-4}-10^{-6}M	x	0 (0%)	0 (0%)	Eyster and Pechenik, 1987
	10^{-2}-10^{-7}M	x	x(<9%)	-	Dobretsov and Qian, 2003
Mytilus galloprovincialis	10^{-4}-10^{-6}M	10^{-4}M	~5-28%* (~5%)	~65-68%* (~25%)	García-Lavandeira *et al*., 2005
	10^{-3}-10^{-6}M	10^{-3}M	~15-95%* (~20%)	-	Sánchez-Lazo and Martínez-Pita, 2012
Mytilus coruscus	10^{-4}-10^{-6}M	10^{-4}M	-	~2-6% (0%)	Yang *et al*., 2013
Venerupis pullastra	10^{-4}-10^{-6}M	10^{-4}M	~17-39%* (~10%)	~65-76%* (~55%)	García-Lavandeira *et al*., 2005
Ruditapes philippinarum	10^{-4}-10^{-6}M	10^{-4}M	~5-15%* (~2%)	~55-70%* (~20%)	García-Lavandeira *et al*., 2005
Ostrea edulis	10^{-4}-10^{-6}M	10^{-4}-10^{-5}M	~15-17%* (~5%)	~25-55%* (~15%)	García-Lavandeira *et al*., 2005
	10^{-4}-10^{-6}M	10^{-4}M	~45-65%* (~16%)	-	Mesías-Gansbiller *et al*., 2013
Crassostrea gigas	10^{-5}-10^{-6}M	X	0% (0%)	-	Beiras and Widdows, 1995
Chlamys varia	10^{-4}-10^{-6}M	10^{-6}M	~38-50%* (30%)	-	Mesías-Gansbiller *et al*., 2008

*Indicates a significance response at optimum concentrations above control.

x Indicates no significant response.

- not assessed.

The mode of action of GABA is unclear, however it is known to act as an inhibitory neurotransmitter in both vertebrates and invertebrates, including molluscs, increasing the permeability of membranes to chloride at postsynaptic sites (Baloun and Morse, 1984; Erdö and Wolff, 1990; Darlison *et al*., 1994; Watanabe *et al*., 2002; Zhao *et al*., 2003; Li and Xu, 2008). In some cases it has been found to inhibit the movement of the velar cilia in swimming invertebrate larvae, causing them to fall out of the water column and settle on the substratum (Rumrill and Cameron, 1983; Barlow, 1990). Other isoforms of GABA including γ-hydroxybutyric acid, δ-aminovaleric acid and ε-aminocaprioc acid have been found to possess GABA-like inducing properties (Morse *et al*., 1979; Laimek *et al*., 2008). However, structurally similar compounds can be less effective due to a reduction in molecular fit within a receptor specific for the GABA configuration (Morse *et al*., 1979). It has been identified that there is an absolute requirement for the carboxyl group of GABA and for specific substitution at the γ position, with any increase or decrease in chain length of GABA analog decreasing effectiveness (Morse *et al*., 1979).

In bivalves effectiveness is species-specific and influenced by concentration and length of exposure (Doroudi and Southgate, 2002; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005; Yu *et al*., 2008). For GABA, effective concentrations range from 10^{-3} to 10^{-6}M generally over exposure periods of 24 to 48 hours, although shorter periods of 1-2 hours have also proven effective (Doroudi and Southgate, 2002; García-Lavandeira *et al*., 2005; Yu *et al*., 2008; Mesías-Gansbiller *et al*., 2013). Above and below optimum concentrations induction is reduced. In terms of exposure, García-Lavandeira *et al*. (2005) and Mesías-Gansbiller *et al*. (2013) advocated an exposure of 48 hours for species including *Mytilus galloprovincialis, Venerupis pullastra, Ruditapes philippinarum* and *Ostrea edulis* in order to maximise settlement and metamorphosis, as does Mesías-Gansbiller *et al*., (2008) in *Chlamys varia*. Mesías-Gansbiller *et al*. (2013) demonstrated that whilst GABA was effective within 24 hours at 10^{-5}M in *O. edulis* resulting in 14.4% settlement compared to 6.9% in the control, maximum settlement was achieved after 48 hours at a concentration of 10^{-4}M with 64.8% settlement compared to 16.1% in the control. Settlement at alternative concentrations was effective, but lower, at approximately 47% at 10^{-5}M and 45% at 10^{-5}M. In contrast, *Pinctada margaritifera* benefits from short exposure periods of 1-2 hours to induce significant settlement (Doroudi and Southgate, 2002). Zhoa *et al*., (2003) reported that the required exposure period for *Pinctada maxima* varied from 24 to 72 hours to achieve settlement. In some cases similar induction levels can be achieved at lower concentrations by increasing the length of exposure (García-Lavandeira *et al.,* 2005). GABA has also been shown to have an extended latent affect, requiring several days following treatment for a response to be observed in some species (Yang *et al*., 2013).

However, there is also notable variation in reported induction levels between different studies, with levels of settlement reported for *M. galloprovincialis* and *O. edulis* varying greatly (García-Lavandeira *et al*., 2005; Sánchez-Lazo and Martínez-Pita, 2012; Mesías-Gansbiller *et al*., 2013).

GABA has also been found to have an impact upon the subsequent growth of post-larvae. In *O. edulis* larvae exposed to GABA at a concentration of 10^{-6}M for 4 hours leading to settlement of 70.7% compared to 38.8% in the untreated controls after 4 days subsequently displayed a significantly greater mean spat size of 700µm compared to 425µm in the controls by 15 days post-treatment (Mesías-Gansbiller *et al*., 2013). This has been attributed to the faster settlement and metamorphosis of larvae promoting synchronisation and spat growth.

Importantly it has been found that GABA can be toxic at high concentrations towards bivalves, particularly 10^{-2} and 10^{-3} M, and over long exposure periods, reducing settlement and causing high rates of mortality (Doroudi and Southgate, 2002; Yu *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012). *Pinctada fucata martensii* suffers up to 100% mortality at 10^{-2}M after 48hrs (Yu *et al*., 2008), whilst *P. margaritifera* suffers approximately 90% mortality within 24 hours at 10^{-2}M (Doroudi and Southgate, 2002). As for *M. galloprovincialis*, the induction of significant settlement must be offset be equally high rates of mortality, with approximately 80% mortality at the optimum settlement concentration of 10^{-3} and 10^{-4} M over 48hrs(Sánchez-Lazo and Martínez-Pita, 2012), a situation hatcheries would be unwilling to tolerate. Notably the level of mortality was not reported in the study by García-Lavandeira *et al.* (2005) for *M. galloprovincialis* which used similar GABA concentrations.

GABA has however, so far proven ineffective in the oyster *C. gigas* (Beiras and Widdows, 1995). Based upon work with GABA in *O. edulis* and comparisons to studies using GABA in other oyster species Mesías-Gansbiller *et al*. (2013) suggested that the mechanisms involved in cue reception are diverse since the response differs between related species, thereby supporting the idea that nervous system evolution diverges across taxa. GABA has also repeatedly been unsuccessful at inducing a response in *M. edulis* at concentrations of 10^{-2}M to 10^{-7}M, over exposure and monitoring periods of 24 to 48 hours (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). This is a stark contrast to the effect in the related species *M. galloprovincialis* (García-Lavandeira *et al*., 2005; Sánchez-Lazo and Martínez-Pita, 2012). Future investigations in *M. edulis* may benefit from shorter exposure periods, longer exposure at lower concentrations, or longer studies. Notably this chemical has yet to be tested with the scallop *P. maximus*. In other marine invertebrates, GABA has proven inconsistent in

the nudibranch *Phestilla sibogae* (Hadfield, 1984), and ineffective in the polychaete *Phragmatopoma lapidosa californica* (Pawlik, 1990) and the gastropod *Crepidula fornicata* (Pechenik and Heyman, 1987),

L-3,4-dihydroxyphenylalanine

L-3,4-dihydroxyphenylalanine, commonly known as L-DOPA, is the precursor to the catecholamine group of neurotransmitters. It rapidly induces settlement searching behaviour in bivalves (Coon *et al*., 1985; Yu *et al*., 2008), and has proven effective at inducing settlement or metamorphosis in a number bivalve species (Table 1.2). It has also proven effective in other marine invertebrates including gastropods (Boettcher and Targett, 1998; Yu *et al*., 2010), polychaetes (Pawlik, 1990), and bryozoans (Yu *et al*., 2007). L-DOPA can alter normal larval behaviour, as some *C. gigas* exposed to it will metamorphose without first attaching (Coon *et al*., 1985; Beiras and Widdows, 1995).

In bivalves concentrations that are too low or too high, or exposures that are too short or too long, can limit or inhibit a response, or increase larval mortality (Coon *et al*., 1985; Kingzett *et al*., 1990; Chevolot *et al*., 1991; Nicholas et al., 1998; Dobretsov and Qian, 2003; Teh *et al*., 2012; Mesías-Gansbiller *et al*., 2013). *P. maximus* requires exposure for 24 to 48 hours (Chevolot *et al*., 1991; Nicholas et al., 1998), whilst just 1 hour is optimum in *C. gigas* (Coon *et al*., 1985) and *Crassostrea iredalei* (Teh *et al*., 2012). In several studies higher concentrations (10^{-4}M) applied over short exposure periods (1 hour) have been highly effective, although there can be a delay in response (Coon *et al*., 1985; Beiras and Widdows, 1995; Pawlik, 1990; Teh *et al*., 2012). Higher concentrations over short periods can avoid the toxic consequences of long exposure (Beiras and Widdows, 1995). L-DOPA is known to auto-oxidise in solution, reducing its influence as an inducing agent over time, but also forming heterogeneous polymeric melanin pigments (Pawlik, 1990). At concentrations ≥$2x10^{-5}$M after 24 hours these pigments can form black precipitates that have been associated with incidence of increased larval mortality (Pawlik, 1990). L-DOPA has been highly toxic to bivalve larvae at high concentrations or after prolonged exposure (Coon et al., 1985; Teh et al., 2012), though this is species dependent. L-DOPA is toxic to *P. maximus* between 2.54 and 5.07 x 10^{-5}M (5 and 10mg litre^{-1}) after 24 hours exposure (Chevolot *et al*., 1991; Nicolas *et al*., 1998). In *M. edulis* L-DOPA is highly toxic at 10^{-2}M and 10^{-3}M after 48 hours exposure, whilst 10^{-4}M and below has no detrimental influence (Dobretsov and Qian, 2003).

Table 1.2: Influence of L-DOPA on the settlement and metamorphosis in bivalve larvae. FSW = Filtered seawater.

Species	Concentration	Optimum concentration	% Settlement (FSW control)	% Metamorphosis (FSW control)	Reference
Crassostrea gigas	10^{-4}-10^{-6}M	10^{-5}M	-	~0-60%*(~5%)	Coon et al., 1985
	10^{-4}-10^{-5}M	$3x10^{-5}$ - 10^{-4}M	-	~27-50%* (0%)	Beiras and Widdows, 1995
	$5.07x10^{-5}$ - 10^{-7}M	$2.54x10^{-6}$	-	~1-25%* (~15%)	Nicholas et al., 1998
Crassostrea iredalei	10^{-3}-10^{-7}M	10^{-6}M	0-~55%* (~35%)	-	Teh et al., 2012
Patinopecten yessoensis	10^{-4}-10^{-6}M	10^{-4}M	-	1.5-16.0%* (~0%)	Kingzett et al., 1990
Pinctada maxima	10^{-2}-10^{-5}M	x	<5% (<0%)	-	Zhao et al., 2003
Pinctada fucata martensii	10^{-2}-10^{-5}M	x	~0-10% (<5%)	-	Yu et al., 2008
Mytilus edulis	10^{-5}-10^{-6}M	10^{-5}M	-	12.2-36.9%* (0%)	Cooper, 1982
	10^{-2}-10^{-7}M	10^{-5}M	<5-~60%* (<10%)	-	Dobretsov and Qian, 2003
Pecten maximus	$5.07x10^{-5}$ - 10^{-7}M	$5.07x10^{-6}$M	-	~1-6%* (~1%)	Chevolot et al., 1991
	$5.07x10^{-5}$ - 10^{-7}M	$2.54x10^{-5}$	-	~3-12%* (2%)	Nicholas et al., 1998
Ostrea edulis	10^{-4}-10^{-6}M	10^{-4}M	~27-43%* (~16%)	-	Mesías-Gansbiller *et al*., 2013

*Indicates a significance response at optimum concentrations above control.

x Indicates no significant response.

- not assessed.

L-DOPA has been demonstrated to induce significant levels of settlement and metamorphosis in *M. edulis* (Cooper, 1982; Dobretsov and Qian, 2003) (Table 1.2). In *P. maximus* its influence is yet to be tested on settlement, however its influence on metamorphosis is relatively weak, with rates of just 6 to 12% achieved by 1 week (Chevolot *et al*., 1991; Nicolas *et al*., 1998) (Table 1.2). This may be a consequence of the slow rate of metamorphosis in *P. maximus*, with 10 to 30% of larvae typical metamorphosing in week 1, reaching 40 to 70% by the second and third weeks, in flowing seawater (Nicolas *et al*., 1998; Robert and Nicolas, 2000). Future studies may improve responses by applying alternative exposure and concentration combinations, as well as longer study periods.

The mode of action of L-DOPA has been linked to its conversion into dopamine (Bonar *et al*., 1990) and its oxidation into hydrogen peroxide (Pires and Hadfield, 1991; Boettcher and Targett, 1998). It has also been linked to the possession of quinones or quinone like units, or units that can be transformed into quinones by oxidation, leading to the suggestion that natural inducers are quinones or similar molecules (Chevolot *et al*., 1991). The analog D-DOPA, an isomer of L-DOPA, has also been tested and whilst ineffective at inducing metamorphosis in *C. gigas* (Coon *et al*., 1985) its influence was identical to L-DOPA in the polychaete *P. lapidosa californica* (Pawlik, 1990). Other analogs of L-DOPA have also influenced invertebrate metamorphosis, with varying results from partial to full metamorphosis or toxicity (Jensen and Morse, 1990; Pawlik, 1990).

Epinephrine

Epinephrine, a catecholamine hormonal neurotransmitter, and product of the methylation of the precursor norepinephrine, acts at chemical synapses and is involved in multiple biochemical actions (Shepherd, 1994; Devlin, 2006). It has proven to be a highly effective means of inducing settlement and metamorphosis in a number of bivalve species, particularly *C. gigas* (Table 1.3). It has also proven effective in the scallop *P. maximus,* although it has yet to be tested in the mussel *M. edulis*.

Table 1.3: Influence of epinephrine on settlement and metamorphosis in bivalve larvae. FSW = Filtered seawater.

Species	Concentration	Optimum concentration	% Settlement (FSW control)	% Metamorphosis (FSW control)	Reference
Crassostrea gigas	10^{-3}-10^{-6}M	10^{-4}M	-	<5->90%* (<5%)	Coon et al., 1985; Coon et al., 1986
	10^{-3}-10^{-6}M	10^{-4}M	-	0->90%* (0%)	Beiras and Widdows, 1995
	5.46x10^{-5} - 10^{-7}M	2.73x10^{-5}M	~10-35% (23%)	70-90%* (30%)	Nicholas et al., 1998
Crassostrea virginica	10^{-3}-10^{-6}M	10^{-4}M	~0-5% (0%)	~0-60%*	Coon et al., 1986
Crassostrea iredalei	10^{-3}-10^{-7}M	10^{-6}M	0-~60%* (~35%)	-	Teh et al., 2012
Pinctada margaritifera	10^{-2}-10^{-5}M	x	~0-<5% (0%)	-	Doroudi and Southgate, 2002
Mytilus galloprovincialis	10^{-4}-10^{-6}M	10^{-5}M	~5-12%* (~5%)	~55-65%* (25%)	García-Lavandeira et al., 2005
	10^{-4}-10^{-6}M	10^{-4}M	-	~15-85%* (0%)	Yang et al., 2008
	10^{-3}-10^{-6}M	10^{-4}M - 10^{-5}M	~15-95%* (~15%)	-	Sánchez-Lazo and Martínez-Pita, 2012
Mytilus coruscus	10^{-4}-10^{-6}M	10^{-4}M	-	~5-20%* (0%)	Yang et al., 2013
Venerupis pullastra	10^{-4}-10^{-6}M	10^{-5}M	~13-20%* (<10%)	~60-75%* (~35%)	García-Lavandeira et al., 2005
Ruditapes philippinarum	10^{-4}-10^{-6}M	10^{-5}M	~3-5% (~3%)	~37-78%* (~20%)	García-Lavandeira et al., 2005
Ostrea edulis	10^{-4}-10^{-6}M	10^{-5}M	~12-17%* (~5%)	~30-55%* (~11%)	García-Lavandeira et al., 2005
	10^{-4}-10^{-6}M	10^{-6}M	~25-43%*(~16%)	-	Mesías-Gansbiller *et al*., 2013
Pecten maximus	5.46x10^{-5} - 10^{-7}M	5.46x10^{-6}	-	~1-13%* (~1%)	Chevolot et al., 1991
	5.46x10^{-5} - 10^{-7}M	2.73 - 5.46x10^{-5}M	-	1-18%* (1%)	Nicholas et al., 1996
	5.46x10^{-5} - 10^{-7}M	5.46x10^{-5} - 10^{-6}M	1-32%* (1%)	1-32%* (1%)	Nicholas et al., 1998
Chlamys varia	10^{-4}-10^{-6}M	10^{-5}M	>30-55%* (~30%)	-	Mesías-Gansbiller et al., 2008
Argopecten purpuratus	10^{-4}-10^{-6}M	10^{-5}M	~15-45%* (<10%)	~5-40%* (<10%)	Martinez et al., 1999
Patinopecten yessoensis	10^{-4}-10^{-6}M	10^{-4}M	-	9.6-15.9%* (~0%)	Kingzett et al., 1990

*Indicates a significance response at optimum concentrations above control. x Indicates no significant response. - not assessed.

Epinephrine does not readily dissolve in seawater, and therefore it is typically dissolved in dilute HCl, usually 0.005N HCl, which is then diluted into larval cultures to give a solution concentration of 0.0005N HCl or less (Coon *et al*., 1986; García-Lavandeira *et al*., 2005). No buffering of either the stock solution or larval cultures to compensate for the inclusion of HCl is documented in the literature. Furthermore no reported impact has been discussed or attributed to the inclusion of HCl in induction assays in previous studies with species including *C. gigas* (Coon *et al*., 1986), *C. iredalei* (Teh *et al*., 2012), *C. varia* (Mesías-Gansbiller *et al*., 2008), *O. edulis, V. Pullastra, R. philippinarum* (García-Lavandeira *et al*., 2005), *M. galloprovincialis* (García-Lavandeira *et al*., 2005; Yang *et al*., 2008) and *M. coruscus* (Yang et al., 2013). Alternatively ethanol has been used to dissolve compounds with low water solubility and used in assays with *P. maximus* without effect on larvae (Chevolot *et al*., 1991). Additionally catecholamines such as epinephrine are known to be subject to oxidation and polymerisation in solution, and this is known to reduce their ability to act as inducing agents in marine invertebrate larvae (Pawlik, 1990). This means that the action of the chemical is possibly only effective for a short period of time, irrespective of whether the rate of settlement or metamorphosis continues to increase.

The response to epinephrine is species-specific. In many bivalve species epinephrine appears to be more influential on metamorphosis than on settlement (García-Lavandeira *et al*., 2005). In *Crassostrea virginica* and *C. gigas* application of epinephrine has consistently resulted in the development of "cultchless" spat, with most larvae rapidly metamorphosing without first attaching to a surface (Coon *et al*., 1985; Coon *et al*., 1986; Nicolas *et al*., 1998). Work by Nicholas *et al*. (1998) suggests the development of cultchless spat is influenced by concentration, with lower concentrations ($<2.73 \times 10^{-6}$M) having a higher incidence of attachment, although still proportionally lower than in untreated controls. The action of epinephrine may be mediated by receptors similar to vertebrate-type $alpha_1$-adrenergic receptors, and these receptors may influence a pathway that by-passes the induction of settlement (Bonar *et al*., 1990). Coon *et al*., (1985) suggested that L-DOPA or L-DOPA-mimetic molecules may initiate settlement, whilst epinephrine or similar compounds instigated metamorphosis in *C. gigas*. However, in other species, including *Argopecten purpuratus* (Martinez *et al*., 1999), *P. maximus* (Nicolas *et al*., 1996; Nicolas *et al*., 1998) and *C. varia* (Mesías-Gansbiller *et al*., 2008), *O. edulis* (Mesías-Gansbiller *et al*., 2013), as well as *C. iredalei* (Teh *et al*., 2012) epinephrine induces rates of settlement higher than in untreated controls (Table 1.3).

The response of larvae to epinephrine is dependent on concentration and exposure, however larval responses can vary within species between different studies (Chevolot *et al*., 1991; Nicolas *et al*.,

1996; Nicolas *et al*., 1998; García-Lavandeira *et al*., 2005; Yang *et al*., 2008; Mesías-Gansbiller *et al*., 2013). This may be a consequence of the variability in larval sensitivity apparent in groups of larvae (Bonar et al., 1990). In bivalves effective concentrations range from 10^{-4}M to 10^{-6}M, over exposure periods of 10 minutes to 72 hours (Coon *et al*., 1985; Coon *et al*., 1986; García-Lavandeira *et al*., 2005; Mesías-Gansbiller *et al*., 2008; Yang *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012). Concentrations too high or too low, or exposure periods too short or too long fail to induce a response, inhibit induction, suppressed larval activity and growth or increase larval mortality (Coon *et al*., 1985; Coon *et al*., 1986; Nicolas *et al*., 1998; Martinez *et al*., 1999; García-Lavandeira *et al*., 2005; Yang *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012). In several species, including *C. gigas*, *C. virginica*, *C. iredalei* and *Patinopecten yessoensis,* a significant response can be induced following short exposure for 1 to 2 hours, thereby maximising induction whilst minimising larval mortality (Coon *et al*., 1985; Zimmer-Faust and Tamburri 1994; Kingzett *et al*., 1990; Teh *et al*., 2012). Effective exposures can be even shorter, as Coon *et al*. (1985) achieved >50% metamorphosis after just 10 minutes exposure in *C. gigas* at 10^{-4}M, whilst Beiras and Widdows (1995) achieved an induction rate of >80% after exposure for just 15 minutes at 10^{-4}M within 48 hours. However maximum induction may be obtained only after longer exposure (Coon *et al*., 1986). The rate of response is also species-specific. In some, a measurable response can be rapid, such as in *C. gigas* (Coon *et al*., 1985) whilst in others a response can take time and require an additional culture period, as in *P. maximus* (Nicolas *et al*., 1998). In *A. purpuratus* settlement is rapid, with high settlement in the first 48 hours, however larvae required longer after treatment to metamorphose (Martinez *et al*., 1999).

Epinephrine can also be toxic to bivalve larvae, and the influence on mortality is dependent upon concentration and species susceptibility. In *P. maximus* concentrations of ≥2.73×10^{-5}M are highly toxic to pre-metamorphic larvae a week after exposure for 24 hours, however concentrations up to 5.46×10^{-5}M have no apparent effect on post-larvae (Nicolas *et al*., 1998). Survival in *A. purpuratus* is significantly improved by concentrations below 10^{-4}M (Martinez *et al*., 1999), as is survival in *P. margaritifera* after exposure for 24 hours (Doroudi and Southgate, 2002). *M. galloprovincialis* can tolerate epinephrine at concentrations of 10^{-4}M for 72 hours with mortality <5% (Yang et al., 2008). In *C. gigas* levels as high as 10^{-3}M are not significantly toxic, although above 10^{-4}M larval development and activity is suppressed (Coon *et al*., 1985). Post-treatment, no negative effect was reported in *C. gigas* or *C. virginica* by Coon *et al*. (1986) over a 12 month study period after induction with epinephrine. Further work is required to clarify if this is the case in other species.

Potassium chloride

Seawater naturally contains an ambient level of potassium, reportedly 9mM in the form of potassium chloride (Kang *et al*., 2004). However, elevated potassium ion (K^+) levels, typically applied as a chloride (KCl) have been shown to induce settlement, metamorphosis or both in a wide range of invertebrate species, including many gastropods (Baloun and Morse, 1984; Yool *et al*., 1986; Baxter and Morse, 1987; Pechenik and Heyman, 1987; Davis, 1990; Bryan and Qian, 1998; Gallardo and Sánchez, 2001; Kang *et al*., 2004; Cob *et al*., 2010; Yu *et al*., 2010), as well as polychaetes (Yool *et al*., 1986; Carpizo-Ituarte and Hadfield, 1998) over concentrations of 5 to 50mM. In bivalves, KCl has been effective at inducing settlement, metamorphosis or both in a number of species (Table 1.4). Continuing work suggests a potentially shared sensitivity to K^+ as an inductive cue in invertebrates, leading to suggestions that excess K^+ may provide a simple and economical method for inducing settlement and metamorphosis (Baloun and Morse, 1984; Yool *et al*., 1986).

K^+ has been identified as a direct agent in inducing morphological changes in invertebrate larvae (Pechenik and Heyman, 1987). It is thought that excess KCl acts by increasing the external K^+ concentration depolarising external membrane potentials. The consequence of which is the exciting of cells involved in the perception of an inductive stimuli, or by-passing the normal receptor-stimulus interaction to activate the morphogenetic pathways controlling settlement and metamorphosis, or alternatively affecting target tissues directly (Baloun and Morse, 1984; Yool *et al*., 1986; Carpizo-Ituarte and Hadfield, 1998). A second messenger such as cAMP or inositol trisphosphate (IP_3), triggered by the initial chemical, may also be involved, regulating ion channel activity or phosphorylation of proteins and catabolic enzymes (Martinez *et al*., 1999). The influence of K^+ is reliant upon functioning K^+ channels in invertebrate neurons, with blockage of Ca^+ activated K^+ transport inhibiting metamorphosis. It is also apparent that K^+ influence is sensitive to alterations in external Ca^+ concentration, with induction inhibited by reduced levels (Baloun and Morse, 1984; Yool *et al*., 1986; Carpizo-Ituarte and Hadfield, 1998).

Table 1.4: Influence of excess potassium ions (K^+) on the settlement and metamorphosis of bivalve larvae. FSW = filtered seawater.

Species	Concentration	Optimum concentration	% Settlement (FSW control)	% Metamorphosis (FSW control)	Reference
Argopecten purpuratus	5-30mM	10mM	~5-45%* (~6%)	~10-45%* (~15%)	Martinez et al., 1999
Pinctada maxima	10-70mM	10 to 30mM	10-45%* (~5%)	-	Zhao et al., 2003
Pinctada fucata martensii	10-70mM	20mM	~<5-40%* (~<5%)	-	Yu et al., 2008
Mytilus edulis	5-20mM	x	0 (0%)	0 (0%)	Eyster and Pechenik, 1987
	10-40mM	x	x(<9%)	-	Dobretsov and Qian, 2003
Mytilus galloprovincialis	1-50mM	30mM	-	0-39%* (0%)	Yang et al., 2008
	10-40mM	20mM	~15-100% (~18%)	-	Sánchez-Lazo and Martínez-Pita, 2012
Mytilus coruscus	10-50mM	50mM	-	~<5-20%* (0%)	Yang et al., 2013

*Indicates a significance response at optimum concentrations above control.

x Indicates no significant response.

- not assessed.

In bivalve the response is species-specific, and influenced by concentration and length of exposure (Yang *et al*., 2008; Yu *et al*., 2008). Optimum concentrations in bivalves range from 10mM to 30mM, over exposures of 24 to 72 hours (Martinez *et al*., 1999; Zhao *et al*., 2003; Yang *et al*., 2008; Yu *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012; Yang *et al*., 2013). Outside specific concentrations and lengths of exposure the response is limited, not stimulated or inhibited (Zhao *et al*., 2003; Yang *et al*., 2008; Yu *et al*., 2008; Yang *et al*., 2013). Previous work has shown that the influence of K^+ can be significantly amplified by incorporating the use of the diamino acids 1-oleoyl-2-acetylglycerol and L-α,β-diamionpropionic acid that act by stimulating separate regulatory and inductive pathways, especially at low concentrations of excess K^+ (Baxter and Morse, 1987).

This ion can be toxic to invertebrate larvae at high concentrations and over long exposure periods. In several bivalves species concentrations of 30mM or greater have proven toxic to larvae (Martinez *et al*., 1999; Zhao *et al*., 2003; Yu *et al*., 2008). There does appear to be some variation in the toxic influence between studies. In *M. galloprovincialis* Sánchez-Lazo and Martínez-Pita (2012) reported mortality increasing with concentration from zero at 10mM to approximately 80% at 40mM after exposure for 48 hours, however no adverse effects on survival was reported up to 50mM after exposure for up to 72 hours in the same species by Yang *et al*. (2008).

In the *Mytilus* genus excess K^+ ions have been an effective metamorphic inducer in *M. galloprovincialis* (Yang *et al*., 2008) and in *Mytilus coruscus* (Yang *et al*., 2013) after exposure for 24 hours, although it has not been found to induce a settlement or metamorphic response in *M. edulis* at levels from 5 to 40mM following exposure periods of 24 to 48 hours (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). Induction of settlement behaviour and settlement can be rapid, within the chemical exposure period, however a metamorphic response can take longer to be observed (Martinez *et al*., 1999; Yu *et al*., 2008). In *M. galloprovincialis* (Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013), metamorphosis was recorded following a lag period of 2 to 3 days following exposure to the chemical for 24 hours. Importantly it has been found that continuous exposure for 48 to 96 hours can inhibit metamorphosis (Yang *et al*., 2008). Future investigation in *M. edulis* may benefit from shorter exposure, longer exposure at lower concentrations and a longer study period. Notably this chemical has yet to be tested with the scallop *P. maximus*, but has proven effective in the scallop *A. purpuratus* (Martinez *et al*., 1999) with metamorphosis following a lag period of 4 days after exposure to the chemical for 48 hours.

Ammonium chloride

NH_4Cl has proven to be an inductive cue for settlement and metamorphosis in several bivalve species (Table 1.5). The level of influence in bivalves appears to be limited compared to agents such as GABA and epinephrine, however the assessment of chemical effectiveness has been comparably limited and further investigation using NH_4Cl is advocated. Notably it has also proven to be effective at inducing settlement in other invertebrate species, including the bryozoan *Bugula neritina* (Yu *et al*., 2007) and gastropod *Haliotis diversicolor supertexta* (Yu *et al*., 2010).

The mode of action of NH_4Cl remains unclear and the effectiveness of this chemical may be related to several factors, as well as species specificity. It is unclear whether it is ammonium (NH_4^+) or ammonia (NH_3), or possibly both, that acts to induce a response (Berking 1988; Coon *et al*., 1990b). When using NH_4Cl the proportion of NH_4^+ and NH_3 that makes up the total contribution is dependent upon the pH of the solution, and even at a pH of 8 a proportion of NH_4Cl will be converted into NH_3 (Coon *et al*., 1990b). Early studies identified that ammonia (NH_3) arising from bacterial supernatants, rather than ammonium (NH_4^+), was the active molecule responsible for substratum exploration by larvae of the oyster *C. gigas,* and therefore recruitment (Coon *et al*., 1988; Bonar *et al*., 1990; Coon *et al*., 1990b). It was suggested that NH_3 acted as a chemokinetic cue to bring larvae within contact of substrates where other inductive cues stimulated final settlement and metamorphosis (Bonar *et al*., 1990; Coon *et al*., 1990b; Zimmer-Faust and Tamburri, 1994; Yu *et al*., 2008). Solutions of NH_4Cl containing >100µM of NH_3 have been found to elicit a behavioural response, whilst an optimum concentration of 400µM has resulted in a response of up to 100% within 5 minutes in *C. gigas* (Coon *et al*., 1988; Bonar *et al*., 1990). However, NH_4^+, a weak acid, has a role as a signalling agent within the nervous systems of vertebrates and invertebrates, crossing many cell membranes via ion channels or on membrane transporters (Marcaggi and Coles, 2001), acting as a substitute for or competing with potassium ions (Kuffler *et al*., 1984; Marcaggi and Coles, 2001). In a process driven by environmental pH, it is known to increase the intracellular pH by penetrating the cell membrane and disassociating to the weak base ammonia (NH_3) $+H^+$ ions, which cycles across membrane until an equilibrium is achieved (Coon *et al*., 1990b; Marcaggi and Coles, 2001). Weak bases have been identified to induce larval settlement behaviour and therefore ultimately recruitment (Coon *et al*., 1990b). Berking (1988) found that the uptake of NH_4^+ was required to trigger metamorphosis rather than NH_3 in the hydroid *Hydractinia.* Ultimately if induction is influenced by intracellular alkalisation through the application of NH_4^+ or NH_3, low culture pH may inhibit this process. At a low pH, insufficient NH_3 may be formed from NH_4^+ for

induction (Yu *et al*., 2008), since Berking (1988) found that at a pH of 7.7 a higher concentration of NH_4Cl was required than at a pH of 8.2.

As with all chemicals, concentration and length of exposure are critically important. In *C. gigas* the percentage of larvae exhibiting settlement behaviour increased with increasing concentration, whilst the time for maximum percentage of larvae to respond decreased (Coon *et al*., 1990b). Concentrations effective at inducing settlement or metamorphosis in bivalves range from 10^{-2} to 10^{-3}M (1 to 10 mM) over exposure periods of 30 minutes to 24 hours (Kingzett *et al*., 1990; Yang *et al*., 2008; Yu *et al*., 2008; Yang *et al*., 2013). Length of exposure period is species-specific, with 24 hours highly effective in *M. galloprovincialis* (Yang *et al*., 2008), whilst in *M. coruscus* 3 hours was optimum, with 24 hours exposure reducing metamorphosis and increasing larval mortality (Yang *et al*., 2013). In all cases concentrations too high or too low, and exposure for too little or too long, can limit or inhibit a response, or increase larval mortality (Kingzett *et al*., 1990; Yang *et al*., 2008; Yu *et al*., 2008; Yang *et al*., 2013). However a metamorphic response can take time to transpire. In *M. galloprovincialis* (Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013) metamorphosis was recorded following a lag period of 48 hours following exposure to the chemical for 24 hours. Observed results can also be highly variable (Kingzett *et al*., 1990). This chemical is yet to be tested on the larvae of either *M. edulis* or *P. maximus.*

Table 1.5: Influence of excess ammonia ions (NH_4^+) on the settlement and metamorphosis of bivalve larvae. FSW = Filtered seawater.

Species	Concentration range	Optimum concentration	% Settlement (FSW control)	% Metamorphosis (FSW control)	Reference
Crassostrea virginica	0.00031 – 1mM	x	x	-	Zimmer-Faust and Tamburri, 1994
Crassostrea gigas	0.001-0.01mM	x	-	X	Beiras and Widdows, 1995
Pinctada maxima	0.1 -50mM	x	~<5-15% (~5%)	-	Zhao et al., 2003
Pinctada fucata martensii	1-50mM	10mM	0-10%* (0%)	-	Yu et al., 2008
Patinopecten yessoensis	1-10mM	5mM	-	6.4-16.3%* (above controls)	Kingzett et al., 1990
Mytilus galloprovincialis	1-50mM	10mM	-	~63-78%*(0%)	Yang et al., 2008
Mytilus coruscus	1-50mM	1mM	-	~4-8%* (0%)	Yang et al. 2013

*Indicates a significance response at optimum concentrations above control.

x Indicates no significance response

- not assessed.

The influence of this chemical is species-specific, and has had little or no influence in a number of bivalve species including *C. virginica* (Zimmer-Faust and Tamburri, 1994), *C. gigas* (Beiras and Widdows, 1995), *P. maxima* (Zhao *et al*., 2003), and *P. fucata martensii* (Yu *et al*., 2008) as well as several gastropod species (Pechenik and Heyman, 1987; Bryan *et al*., 1997). In the case of *C. virginica* and *C. gigas* however this may be a consequence of the concentrations being too low to induce a response (Zimmer-Faust and Tamburri, 1994; Beiras and Widdows, 1995).

Importantly the toxicity of NH_4Cl to bivalves has been demonstrated to increase with concentration and length of exposure (Zhao *et al*., 2003; Yu *et al*., 2008). Both NH_4^+ and NH_3 play an important role in aquatic toxicity (Boardman *et al*., 2004), with NH_3 recognised as being highly toxic towards aquatic freshwater and marine vertebrates and invertebrates, due to its ability to enter cells more readily than NH_4^+ (Epifanio and Srna, 1975; Chen *et al*., 1990; Marcaggi and Coles, 2001; Zhao *et al*., 2003; Boardman *et al*., 2004; Bermudes and Ritar, 2008; Yu *et al*., 2008). Concentrations as low as 0.1mM have led to significant toxic effects after exposure for 96 hours in marine invertebrates (Yu *et al*., 2010), whilst in bivalves concentrations as low as 1mM over extended periods can be toxic (Yu *et al*., 2008). In some cases the toxicity of the chemical outweighs any benefit to induction, as found in *P. fucata martensii* where an optimum effective concentration of 10mM NH_4Cl over 24 hours resulted in the mortality of approximately 75% of larvae (Yu *et al*., 2008). However, toxicity is species-specific, as *M. galloprovincialis* can withstand concentrations of 10^{-2}M for 24 hours without incurring larval mortality, and suffers <5% mortality in those exposed for 48 hours (Yang *et al*., 2008).

Acetylcholine

The influence of choline derivatives was first recorded by Bonar (1976), with succinylcholine chloride stimulating metamorphosis in the nudibranch *P. sibogae*. Since then other choline derivatives have demonstrated inductive abilities in a number of marine invertebrate species, including gastropods (Hadfield, 1978; Levantine and Bonar, 1986), bryozoan (Yu *et al*., 2007), as well as bivalves (Beiras and Widdows, 1995; Dobretsov and Qian, 2003; Zhao *et al*., 2003; Yu *et al*., 2008). Notably, larvae can respond differently to choline derivatives than to natural inducers, with higher concentrations of choline compounds required, whilst the response can take longer to occur (Hadfield, 1978; Pawlik, 1990). In the bryozoan *B. neritina* acetylcholine will induce larval settlement but will inhibit normal attachment (Yu *et al*., 2007).

One choline derivative that has repeatedly been assessed in a number of bivalve species is acetylcholine (Table 1.6). Acetylcholine is known to act as a neurotransmitter in both vertebrates and invertebrates, bridging the neuron-neuron and neuron-muscle synapses by simultaneously increasing the permeability of postsynaptic membranes to potassium and sodium, leading to a depolarising potential (Kuffler *et al*., 1984).

Effective concentrations range from 10^{-2}M to 10^{-7}M over exposure periods of 24 to 96 hours, although this varies with species (Dobretsov and Qian, 2003; Zhao *et al*., 2003; Yu *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012). A response to choline compounds, such as settlement searching behaviour, can be relatively quick occurring within 6 hours, however actual attachment or metamorphosis may be slow, taking 2 to 3 days to be observed (Yu *et al*., 2008; Yang *et al*., 2013). Acetylcholine has proven to be particularly effective as a settlement cue (Dobretsov and Qian, 2003; Zhao *et al*., 2003; Yu *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012). As a metamorphic inducer its influence appears more limited, although further investigation with other bivalve species is required. It has been relatively effective in *C. gigas* with larval metamorphosis increased to 27% after treatment at 10^{-4}M for 48 hours, with 100% of post-larvae cementing themselves to the culture vessels, unlike larvae exposed to epinephrine and L-DOPA (Beiras and Widdows, 1995). However, the larval response to acetylcholine can be inconsistent, as Coon *et al*. (1985) identified limited and variable inductive activity in their study with *C. gigas*. In cases where acetylcholine has been effective, concentrations too low or too high, and exposure for too little or too long, has limited or inhibited a response, or increased larval mortality (Beiras and Widdows, 1995; Dobretsov and Qian, 2003; Yu *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012). Acetylcholine has however failed to induce settlement or metamorphosis in a number of other marine invertebrate groups including gastropods (Morse *et al*., 1979; Hadfield, 1978; Yu *et al*., 2010) and polychaetes (Pawlik, 1990).

Table 1.6: Influence of acetylcholine on settlement and metamorphosis in bivalve larvae. FSW = Filtered seawater.

Species	Concentration	Optimum concentration	% Settlement (FSW control)	% Metamorphosis (FSW control)	Reference
Crassostrea gigas	10^{-4}-10^{-6}M	x	-	Inconsistent	Coon et al., 1985
	10^{-3}-10^{-6}M	10^{-4}M	-	0 - 27%* (0%)	Beiras and Widdows (1995)
Mytilus edulis	10^{-2}-10^{-7}M	10^{-6}M	<10 - ~35%* (<10%)	-	Dobretsov and Qian, 2003
Mytilus galloprovincialis	10^{-2}-10^{-5}M	10^{-2}M	~40-95% (~25%)	-	Sánchez-Lazo and Martínez-Pita, 2012
Mytilus coruscus	10^{-4} to 10^{-6}M	10^{-5}M	-	~1-8%*(0%)	Yang et al., 2013
Pinctada maxima	10^{-2}-10^{-5}M	10^{-3}M	~<5-45%* (~5%)	-	Zhao et al., 2003
Pinctada fucata martensii	10^{-2}-10^{-5}M	10^{-4}M	~0-45%*(<5%)	-	Yu et al., 2008

*Indicates a significance response at optimum concentrations above control.

x Indicates no significant response.

- not assessed.

In bivalves toxicity is species-specific and concentration and exposure dependent. *M. coruscus* can withstand concentrations of 10^{-4} to 10^{-6}M for 24 hours with larval mortality <5% after 96h hours (Yang *et al*., 2013), whilst *P. maxima* is able to tolerate acetylcholine without significant mortality at concentrations up to 10^{-3}M for 72 hours (Zhao *et al*., 2003). Acetylcholine is highly toxic at high concentrations (10^{-2}M and 10^{-3}M) or over continuous exposure for 96 hours (Dobretsov and Qian, 2003; Yu *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012). Notably in *P. fucata martensii* maximum settlement induction is achieved at 10^{-3}M over 96 hours, however the consequence is significant larval mortality, reaching approximately 25%. The compromise is to use a concentration of 10^{-4}M and achieve a lower although significant induction rate that does not impact upon survival (Yu e*t al*., 2008). This has also been the case with *M. galloprovincialis,* with adoption of a concentration of 10^{-3}M over 10^{-2}M recommended (Sánchez-Lazo and Martínez-Pita, 2012). Alternatively as found by Yang *et al*. (2013) with NH_4Cl in *M. coruscus* the highest rate of metamorphic induction may be achieved by using a higher concentration of acetylcholine but over a short exposure period and allowing larvae time to develop (Yang *et al*., 2013).

Whilst acetylcholine is effective, Hadfield (1978) demonstrated that it is the choline moiety that is the critical active element for induction and that susceptibility to different choline derivatives varies. In *P. sibogae* choline chloride induces high rates of metamorphosis (60-85%), almost equal to that of the derivative succinylcholine chloride (70-90%), whilst methacholine (acetyl-β-methylcholine) induces variable low rates of metamorphosis (10-40%), and acetylcholine fails to induce a response (Hadfield, 1978). However, susceptibility to choline and its derivatives is species-specific, as Levantine and Bonar (1986) found that in the gastropod *Ilyanassa obseleta* succinylcholine, acetylcholine and choline induced minor metamorphic activity whilst acetyl-β-methylcholine induced 80-100% metamorphosis. Therefore assessment of alternative choline compounds is equally as important, and may prove beneficial.

Additional chemical agents

There is a long list of chemical agents that have been used in assays in an attempt to influence settlement and metamorphosis in invertebrate larvae, some of which are shown in Table 1.7. This includes amino sugars, amino acids, other neurologically active amines, ions, alcohols and other compounds (Morse *et al*., 1979; Coon *et al*., 1985; Hadfield, 1984; Pennington and Hadfield, 1989; Pawlik, 1990; Boettcher and Targett, 1998; Yang *et al*., 2008; Yu *et al*., 2010; Yang *et al*., 2013).

Table 1.7: List of additional chemicals tested as inducers of settlement and metamorphosis in marine invertebrate larvae (Morse *et al*., 1979; Coon *et al*., 1985; Hadfield, 1984; Pennington and Hadfield, 1989; Pawlik, 1990; Boettcher and Targett, 1998; Yang *et al*., 2008; Yu *et al*., 2010; Yang *et al*., 2013).

Chemicals		
Amines	**Carboxylic acids**	**Other compounds**
Ethanolamine	*n*-Butyric acid	Synephrine
Triethanolamine	*n*-Pentanoic acid	Hydrogen peroxide
β-Hydroxyphenethylamine	Succinic acid	Sodium orthovanadate
n-Butylamine	**Ions**	Picrotoxin
Tyramine	Magnesium	*db*-cAMP
Octopamine	Sodium	Phenylephrine
Amino acids	Rubidium	Clonidine
Alanine	Caesium	Isoproterenol
Arginine	Lithium	Dobutamine
Asparagine	Calcium	Methoxyphenamine
L-Aspartic acid	**Sugars and amino sugars**	Metanephrine
L-Glutamic acid	Glucose	Vanillymandelic acid
D- Glutamic acid	Galactose	Adrenochrome
L-Glutamine	Galactosamine	Ethanol
Glycine	**Other neurotransmitters and effectors**	Methanol
Histidine	α-Aminobutyric acid	Ethylene glycol
Homoserine	β- Aminobutyric acid	n-propanol
Hydroxylysine	Serotonin (5-HT)	Accetonitrile
Isoleucine	Histamine	Dimethyl sulfoxide
Leucine	Indole-3-butyric acid	Acetone
Lysine	Indole-3-acetic acid	Hexane
Methionine	Indole-3-propionic acid	Cholera toxin
Phenylalanine	Indole-3-acrylic acid	
Serine	L-Thyroxine	
Threonine	Succinylcholine chloride	
Tryptophan	Methacholine chloride	
Tyrosine	Carbamylcholine	
Valine	3-Iodo-tyrosine	
α-Aminoisobutyric acid	γ-Guanidinobutyric acid	
4-Hydroxyproline	Catechol	
	γ-Hydroxybutyric acid	
	δ-Amino-*n*-valeric acid	
	ε- Amino-*n*-caproic acid	

Some of these have proven to be effective induction agents and should therefore be considered for assessment with bivalves including *P. maximus* and *M. edulis* in future studies. In particular in bivalves the compounds methoxyphenamine, clonidine, serotonin, as well as organic solvents including ethanol, methanol ethylene glycol and acetonitrile have proven effective metamorphic

inducers in the mussels *M. galloprovincialis* (Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013). Whilst serotonin has also been effective in the oyster *P. maxima,* inducing settlement up to 90%, without any toxic effect (Zhao *et al*., 2003). Yu *et al*. (2008) has also reported a limited response to magnesium ions (Mg^{2+}) in *P. fucata martensii*. In other marine invertebrates the chemicals γ-Hydroxybutyric acid, δ-Amino-*n*-valeric acid and ε-Amino-*n*-caproic acid were effective metamorphic inducers in the gastropod *H. rufescens* (Morse *et al*., 1979). Whilst alcohols including ethanolamine and triethanolamine, as well as acetonitrile, acetone, dichloromethane and toluene, have reportedly shown inductive metamorphic properties in the nudibranch mollusc *P. sibogae* (Hadfield, 1984; Pennington and Hadfield, 1989). The chemical bromomethane has also been tested and proven effective with the abalone *H. discus hannai* (Kang *et al*., 2004). The chemical histamine has also been found to be effective in the sea urchin *Holopneustes purpurascens* (Swanson *et al*., 2004). A wide range of amino acids have also been tested, with isoleucine and valine being found to induce significant normal metamorphosis in the queen conch *Strombus gigas* (Boettcher and Targett, 1998). Hydrogen peroxide and sodium orthovanadate have both proven effective at inducing *S. gigas* to metamorphose and may act by modulating secondary messenger pathways (Boettcher *et al*., 1997; Boettcher and Targett, 1998).

Interaction of factors

The influence of chemical cues cannot be considered in isolation. Studies have demonstrated that bivalves settle or metamorphose more readily in the presence of seawater turbulence and suitable substrates (Eyster and Pechenik, 1987; Nicolas *et al*., 1996; Nicolas *et al*., 1998). Notably Eyster and Pechenik (1987) demonstrated a dramatic cumulative improvement in attachment by *M. edulis* larvae by combining the provision of filamentous substrates and water agitation, with substrate being particularly critical. The provision of adequate nutrition, both in terms of quantity and quality is also critical to larval development, with sufficient energy reserves needed to undertake and survive metamorphosis (Nicolas and Robert, 2001; Tremblay *et al*., 2007). Combining multiple factors, including chemical and physical elements offers greater potential, with a cumulative effect on settlement and metamorphosis. Nicolas *et al*. (1998) found that combining seawater turbulence with the chemical agent L-DOPA increased metamorphosis in *P.* maximus indicating a cumulative effect of the variables. Whilst Doroudi and Southgate (2002) also demonstrated improved settlement in *P. margaritifera* by combining GABA and plastic filaments over either alone. There is significant potential in the investigation of combinations of stimuli, in an effort to further improve induction.

Conclusion

The use of exogenously applied chemicals as a tool for controlling the settlement and metamorphosis of many larval bivalve species is clearly effective in many cases. However systematic investigation of each potential agent is required, as the responses to the different chemicals are as varied as the methods used to assess their influence. It is also apparent that whilst some chemicals have proven to be effective and others have not, they have often been tested within a limited range of concentration, length of exposure or study period. It may therefore be beneficial to broaden the methodological approach used to test potential chemicals, including those that have already been tested. Additionally it is important to consider the potential implications of induction on subsequent development and survival. Ultimately however there is a long list of chemicals yet to be tested and this research is likely to be ongoing for some time.

Bivalve seed mobility and dispersal

The classical life cycle model for benthic marine invertebrates is characterised by a planktonic larval phase, which is seen as the primary dispersal period, followed by settlement and metamorphosis into the benthic juvenile-to-adult phase (Baker and Mann, 1997). Whilst this model may hold roughly true for some groups of marine invertebrates, including oysters, barnacles, hermatypic corals and ascidians, for many others it is not as simple (Baker and Mann, 1997). Recommended background information on post-larvae stages is provided by Günther (1992) in a short review of dispersal in invertebrates in general, whilst Baker and Mann (1997) provide an extensive review on the post-larval bivalve phase, including dispersal.

Oysters are unique amongst bivalves in appearing to conform to the classical model. The planktonic larval phase represents the primary means of dispersal, and is followed by irreversible settlement and metamorphosis as juveniles permanently cement to a substrate (Gruffydd *et al*., 1975; Gosling, 2003). However, evidence suggests the life cycle of most bivalves is far more complex and involves additional highly motile life stages between the pediveliger stage and the sedentary juvenile stage that facilitate secondary dispersal following initial settlement (Sigurdsson *et al*., 1976; Sörlin, 1988; Beukema and de Vlas, 1989; Roper *et al*., 1995; Baker and Mann, 1997; Lundquist *et al*., 2004). Secondary dispersal represents an important tool for selecting favourable habitats, with dispersal potentially more important than mortality for population dynamics of juvenile bivalves over small

and meso spatial-time scales (Norkko *et al*., 2001). The causes of secondary dispersal behaviour in bivalves remain unclear, though primary causes are thought to be in response to unfavourable conditions or a shift in habitat requirement (Sörlin, 1988; Beukema and de Vlas, 1989; Baker and Mann, 1997; Lundquist *et al*., 2004).

From an aquaculture perspective it is a priority to maximise seed retention, since the inefficient use of valuable and finite resources makes production more costly and unpredictable, and can lead to large production and economic losses (Carton *et al*., 2007). Therefore understanding how mobility and dispersal are influenced, and how they can be affected by husbandry activities, will increase production control and efficiency. Ultimately seed that fail to attach or subsequently detach can be lost from production systems and this represents a financial loss.

Post-settlement dispersal

Post-settlement dispersal of marine invertebrates is considered to be an important process in determining spatial and temporal distribution patterns of benthic species (Lundquist *et al*., 2004). Secondary dispersal is seen in both juveniles and adults across a wide range of marine invertebrate taxa, including polychaetes (Tamaki, 1987; Günther, 1992; Olivier *et al*., 1996; Shull, 1997; Stocks, 2002), crustaceans (Hedvall *et al*., 1998; Moksnes *et al*., 1998; Blackmon and Eggleston, 2001; Moksnes, 2002), gastropods (Levinton, 1979; Levinton *et al*., 1995) and especially bivalves (Sigurdsson *et al*., 1976; Lane *et al*., 1985; Beukema and de Vlas, 1989; Roper *et al*., 1995; Lundquist *et al*., 2004; Carton *et al*., 2007). Influential factors include hydrodynamic pressures (Tamaki, 1987; Levinton *et al*., 1995; Blackmon and Eggleston, 2001), population density (Levinton, 1979), adult conspecifics (Olivier *et al*., 1996), habitat preference (Olivier *et al*., 1996; Hedvall *et al*., 1998; Moksnes, 2002), food (Stocks, 2002), disturbance (Günther, 1992; Commito *et al*., 1995; Dunn *et al*., 1999), temperature (Sörlin, 1988) and predation (Hedvall *et al*., 1998; Moksnes *et al*., 1998; Moksnes, 2002). However, it also appears that it is a response to changing habitat requirements resulting in migrations from initial sites to secondary sites, which somehow benefit the animal, possibly with higher growth rates and improved survival (Bayne, 1964a; Beukema and de Vlas, 1989). Dispersal has in the past been defined either as a passive or an active process. Several studies have suggested that a number of species, including copepods (Palmer, 1984), bivalves (Palmer and Gusf, 1985; Rankin *et al*., 1994), and polychaetes (Tamaki, 1987), are dispersed passively by water currents. In bivalve clams a correlation has been established between post-settlement transport and sediment transport, which is affected by flow speed and sediment characteristics (Emmerson

and Grant, 1991; Rankin *et al*., 1994; Commito *et al*., 1995; Roegner *et al*., 1995; Hunt, 2004; Hunt 2005; Jennings and Hunt, 2009). Bivalve species such as *Mya arenaria*, *Mercenaria mercenaria* and *Gemma gemma* utilise bedload transport as an effective means of dispersal (Commito *et al*., 1995; Hunt *et al*., 2007; Jennings and Hunt, 2009). However, it is now apparent that active processes commonly play a role, with a behavioural response regulating transport either by promoting or decreasing its occurrence (Palmer, 1984; Lundquist *et al*., 2004). Burrowing into sediment (Tamaki, 1987; Roegner *et al*., 1995; Jennings and Hunt, 2009) and byssus attachment (Allen *et al*., 1976; Dolmer and Svane, 1994; Carton *et al*., 2007; Babarro *et al*., 2008) reduce the chance for dispersal by preventing entrainment in water currents. Behaviours which promote transport include emergence from sediment to promote re-suspension and water column transport (Sörlin, 1988; Roper *et al*., 1995; Lundquist *et al*., 2004) and production of drifting byssus threads (Sigurdsson *et al*., 1976; Lane *et al*., 1985; Beukema and de Vlas, 1989; Beaumont and Barnes, 1992; Olivier *et al*., 1996).

Post-larval motility in bivalves

Many bivalves display highly motile post-larval phases between the planktonic stage and the sedentary juvenile stage, characterised by pedal walking, temporary byssus attachment and secretion of drifting byssus (Sigurdsson *et al*., 1976; Lane *et al*., 1985; Carton *et al*., 2007). Baker and Mann (1997) categorised this time into two functionally and behaviourally distinct life stages, the benthic plantigrade and the planktonic post-larvae, between which individuals may alternate.

The plantigrade stage describes post-larvae locomotion called pedal crawling, using the muscular extension and retraction of the foot and byssal attachment to crawl between and across benthic surfaces (Allen *et al*., 1976; Baker and Mann, 1997; Gosling, 2003). It was initially described in the burrowing clam *M. mercenaria* in which post-metamorphic individuals too small to burrow, crawled between sand grains and across surfaces. Behaviourally distinct, there is however no sharp morphological transition between it and the juvenile phase (Baker and Mann, 1997). Plantigrade locomotion is known to occur in the post-larvae of *M. edulis* (Lane and Nott, 1975; Broad, 1983), *P. maximus* (Gruffydd and Beaumont, 1972), *Cerastoderma edule*, *C. glaucum* (Yankson, 1986), *Perna canaliculus* (Carton *et al*., 2007), *Macomona liliana* (Roper *et al*., 1995), *Austrovenus stutchburyi* (Lundquist *et al*., 2004), *M. arenaria* and *Panope generosa* (Baker and Mann, 1997). In the case of the burrowing clams *M. arenaria* and *P. generosa,* both are very active as plantigrades using their foot and byssus to enable movement to a size of 10-12mm, after which mobility is lost (Baker and Mann, 1997). Similarly plantigrade *C. glaucum* alternate between crawling and byssal climbing of

vegetation to achieve dispersal, whilst the closely related *C. edule* employs crawling alternated with byssal drifting (Yankon, 1986).

Pelagic dispersal in many bivalves is characterised by two distinct periods of pelagic existence, the first being the initial pelagic larval stages before first settlement, and the second phase of much larger post-larvae (Beukema and de Vlas, 1989). Whilst secondary dispersal is considered optional it has been found that a number of bivalve species re-enter the plankton on a population-wide scale (Bayne, 1964a; Beukema and de Vlas, 1989; Baker and Mann, 1997). In the case of *Macoma balthica* populations of post-metamorphic animals in the Wadden Sea are known to re-enter the plankton and drift for distances of up to 15km to new recruitment areas (Beukema and de Vlas, 1989). A similar population migration was reported by Bayne (1964a) in *M. edulis,* which initially settles upon filamentous objects before migrating to adult mussel beds. This means that initial settlement of pediveligers cannot be used to predict adult distribution, as changing requirements result in a niche migration (Baker and Mann, 1997).

A widespread mechanism for drifting in post-metamorphic bivalves is the secretion of long threads that function by increasing the viscous drag exerted upon the young animals, enabling them to be carried on relatively weak currents (Sigurdsson *et al*., 1976; Lane *et al*., 1982; Lane *et al*., 1985; Armonies, 1992; Beaumont and Barnes, 1992; Baker and Mann, 1997; Gosling, 2003). The mechanism is commonly known as byssal drifting. These threads are typically many times longer than the length of the secreting animal (Sigurdsson *et al*., 1976; Lane *et al*., 1985; Beukema and de Vlas, 1989). As seen in *M. edulis* these long monofilament threads appear to be a form of byssus secreted from separate specialised glands within the foot that are both significantly longer and structurally distinct from the byssus used to undertake substrate attachment (Lane *et al*., 1982; Lane *et al*., 1985). Beukema and de Vlas (1989) reported that *M. balthica* uses hyline mucous threads for byssal drifting up to 100 times the length of the secreting animal. The ability to drift using byssus has been seen in animals several millimetres in size, although seed up to 10mm in shell length may possess this ability, as seen in *M. balthica* (Beukema and de Vlas, 1989; Armonies, 1992). Byssal drifting allows post-larvae to take advantage of sporadic up currents, potentially maintaining dispersal within the water column for several months (Lane *et al*., 1985; Beukema and de Vlas, 1989). Deployment of these threads is rapid, when tested in long static water filled vertical tubes, allowing post-larvae to quickly modify their descent. Lane *et al*. (1985) reported a swift initial deceleration in vertical decent, followed by a gradual decline towards a terminal velocity, which in the case of *M. edulis* appears to be in the region of 1mm s^{-1}. *P. maximus,* tested under similar

conditions, deploy byssus although deployment can be inconsistent and is generally restricted to individuals below 500µm in size, though other forms of activity appear to slow initial decent (Beaumont and Barnes, 1992). In similar laboratory trials with small *M. balthica* in vertical tubes drifting could be prolonged by gentle turbulence (Beukema and de Vlas, 1989). Post-larvae are also known to detach from drifting threads at will, potentially to aid positional control or due to disturbance (Sigurdsson *et al*., 1976; Lane *et al*., 1985; Barnes and Beaumont, 1992). These drifting threads may be polysaccharide in nature (Sigurdsson *et al*., 1976; Lane *et al*., 1982; Beukema and de Vlas, 1989). Species that produce these threads can be reared in the laboratory without displaying this behaviour, indicating it is not an obligatory life stage (Loosanoff and Davis, 1963).

Sörlin (1988) describes another form of drifting in *M. balthica.* Animals up to 14mm in shell length appeared to increase buoyancy by extending their foot to allow brief floatation. This mechanism represents a second dispersal mechanism for this species allowing the relocation of older individuals. However this is over shorter distances enabling smaller modification in settled habitat, whilst thread drifting appears to allow dispersal on a larger scale, but maybe restricted to smaller animals (Sörlin, 1988; Beukema and de Vlas, 1989). Planktonic drifting is particularly important for some species of viviparious bivalve, such as the clam *Transenella tantilla* and species of *Musculus* and *Lasaea* (Martel and Chia, 1991), in which passive drifting within the plankton using byssus threads is their only means of pelagic dispersal. However difficulties exist in visually identifying the deployment of drifting byssus and mucous threads as they are incredibly thin; notably drifting byssus threads are only 1-3µm in diameter (Lane *et al*., 1985; Baker and Mann, 1997). Threads can be stained with Alcian blue (Sigurdsson *et al*., 1976) or viewed with a transmission electron microscope (Lane *et al*., 1985), whilst Beaumont and Barnes (1992) demonstrated their presence through the connection between drifting individuals and the water surface on mounted needles.

Some bivalves lose the ability for movement as adults whilst others retain high motility throughout their life (Baker and Mann, 1997; Gosling, 2003). In the case of *M. edulis* and *P. maximus*, both species possess highly mobile post-larvae, but also retain motility throughout their lives. In *P. maximus* the modes of locomotion between benthic plantigrade and adult stages change. Early post-larvae plantigrades alternate between periods of attachment and periods of pedal crawling (Gruffydd and Beaumont, 1972). Juveniles less than 500µm in shell length are also able to employ drifting byssus to disperse (Beaumont and Barnes, 1992). As *P. maximus* grow, their foot organ degenerates to a point where it plays no further role in either locomotion or attachment, and consequently there are few individuals greater than 15mm found attached (Beninger and Le Pennec,

1991; Brand, 1991; Minchin, 1992). However it retains locomotion due to their ability to swim, with juveniles as small as 3mm actively swimming or jumping by ejecting water from the mantle cavity to propel them through the water column, an ability retained throughout their benthic existence (Brand, 1991; Minchin, 1992). In other species the adult mode of locomotion does not appear to differ from that of the plantigrade, as is the case with *M. edulis.* In fact *M. edulis* remains motile by employing several methods (Baker and Mann, 1997). As in many species, young post-larval *M. edulis,* up to at least 2.5mm in size, are able to sever their byssus attachment and secrete long monofilament drifting byssus (Sigurdsson *et al*., 1976). However, whilst the ability to utilise byssus drifting is lost, *M. edulis* retains the abilities to secrete and sever attachment byssus, and use the muscular action of their foot organ to crawl across surfaces (Dare and Davies, 1975; Petraitis, 1987; Baker and Mann, 1997).

Factors influencing seed behaviour

In bivalves a number of environmental factors have been highlighted to stimulate behaviour resulting in an increase in post-larval dispersal. In the wild, transport of post-larval juveniles appears to have a seasonal or tidal variable. Field observations in the North Sea using plankton nets to collect drifting benthos found that in the bivalves *M. balthica, C. edule* and *Ensis directus*, as well as the gastropod *Hydrobia ulvae* dispersal occurs on a population-wide scale and has been associated with seasonal, diurnal and lunar cycles, with factors such as turbulence and reproductive events playing a role (Beukema and de Vlas, 1989; Armonies, 1992), as well as temperature (Sörlin, 1988). *M. balthica,* a cold-sensitive species, is known to show seasonal dispersal, undertaking winter migrations from tidal flats to warmer sub-tidal areas (Günther, 1992; Armonies, 1992). Laboratory studies by Sörlin (1988) found fluctuations in temperature influenced drifting and that winter migrations were stimulated by low temperatures. In the laboratory natural rhythmic patterns have been maintained for 4 days in collected animals, with surfacing from sediments by *M. balthica* initially correlated to dark periods, after which non-rhythmic behaviour is seen (Sörlin, 1988).

A number of bivalves have been observed to elicit increased pedal crawling in response to environmental factors, particularly unfavourable habitats. In laboratory scale experiments Roper *et al*. (1995) found that juveniles of the burrowing bivalve *M. liliana* (mean length 1.3mm) would surface and crawl away from sites where sediment was contaminated with zinc (Zn) and copper (Cu). The response was concentration sensitive between 40 to 80 mg Zn kg^{-1} of dry sediment and 10 to 25 mg Cu kg^{-1} dry sediment. In water flume experiments Lundquist *et al*. (2004) demonstrated that

juveniles (<8mm in length) of both *M. liliana* and the venerid burrowing bivalve *A. stutchburyi* undertook active dispersal behaviour, influenced by substrate type and flow velocity. Experiments were long (48 hours) and used recently collected bivalves (<24 hours) in order to ensure that natural endogenous tidal and diurnal behaviours would be maintained and encompassed within the study. They showed that at a slow flow rate of 4.8 cm second^{-1} most individuals (>95%) remained within their substrate cores with many burying themselves, whilst only a few would surface and crawl away from substrate cores of natural sediment and glass beads.. At higher flow speeds of 11.0 and 16.6 cm second^{-1} dispersal consistently increased with velocity, however mode and frequency differed markedly between species. For *A. stutchburyi* crawling at low flow speeds changed to bedload transport at higher speeds, whilst for *M. liliana* crawling and bedload transport gave way to drifting and greater bedload transport, although this was dependent upon seed size with drifting restricted to small *M. liliana* (<4mm). This demonstrated that size-related differences in transport exist, with small animals more likely to disperse. It was also clear than bivalves would actively migrate from unsuitable substrates, with higher dispersal from glass beads for *A. stutchburyi* at all flow, and for *M. liliana* at the lowest velocities compared to natural sediment. The work by Lundquist *et al*. (2004) importantly demonstrated the inter-connectivity of different factors on influencing dispersal behaviours.

Density is known to play a significant role on bivalve recruitment and dispersal patterns (Commito *et al*., 1995; Turner *et al*., 1997; Hunt and Mullineaux, 2002). *M. edulis* live in dense assemblages and show little tendency to move within established groups (Okamura, 1986; Gosling, 2003). However, populations of benthic suspension feeders, including bivalves will naturally undertake self-thinning as a consequence of space availability (Fréchette and Lefaivre, 1990), and the high seed losses of *M. edulis* in the first year, particularly in the first winter, after settlement has been correlated with density (McGrorty *et al*., 1990). Evidence for high dispersal rates in juvenile bivalves has been provided by Norkko *et al*. (2001). They reported a 50% turnover for post-larvae <1mm within 17.4 hours and of 1-4mm juveniles in 31.5 hours from sandflat habitats with an area of 0.25m^2, with active dispersal playing a significant role. Increased dispersal with increasing density has been observed in other species including the scallop *Argopecten irredians concentricus* (Powers and Petersen, 2000), the clam *M. arenaria* (Hunt and Mullineaux, 2002), as well as the gastropod *Hydrobia ventrosa* (Levinton, 1979). Notably Powers and Petersen (2000) demonstrated that at water velocities, up to 0.28m second^{-1}, dispersal of the scallop *A. irredians concentricus* increased with density of scallop, with 0% at 12 scallop m^2, 60% at 25m^2 and 71% at 62m^2.

Bivalve seed attachment using byssus threads and the loss of attachment have been postulated to be a consequence of exposure to potential stressors, which may lead to the triggering of secondary dispersal (Carton *et al*., 2007). Byssus attachment is known to be influenced by factors including animal size, temperature, salinity, hydrodynamic conditions and food availability in seed, juveniles and in adults (Allen *et al*., 1976; Dolmer and Svane, 1994; Paul, 1980a,b; Price, 1982; Christophersen and Strand, 2003; Babarro *et al*., 2008; Lachance *et al*., 2008), with attachment strength in *M. edulis* showing seasonal variation (Price, 1982; Moeser and Carrington, 2006).

Food availability is believed to influence bivalve seed attachment and retention. Sörlin (1988) speculated that a lack of available food sources for settled animals in laboratory tests was a potential cause of increased drifting dispersal. The metabolic requirement for byssus formation has been estimated to be up to 8% of monthly energy expenditure in mussels and must compete with the energetic demands of other biological activities (Hawkins and Bayne, 1985; Carrington, 2002). In natural adult mussel populations attachment strength shows seasonal variation, and has been linked to reproductive cycles, with tenacity low during periods of high reproductive development (Carrington, 2002; Moeser and Carrington, 2006). It is suggested that byssogenesis can only occur when energetic resources are available and there is a trade-off between tenacity and other biological functions such as reproductive effort (Carrington, 2002). Whilst the energy reserves of spat stage animals will not be compromised by reproduction, the conflicting demands for limited resources, such as for growth, may impact on byssus production. Carton *et al*. (2007) established a link between nutritionally compromised *P. canaliculus* seed and the reduced ability to remain attached. It was suggested that artificial feeding of seed for a short period (2-4 days) prior to seeding out could substantially improve retention of nutritionally compromised seed. Starved mussels are known to continue to partition resources to the production of fewer threads, whilst below maintenance levels energy is transferred solely to byssus production, as opposed to soft tissues (Clarke, 1999). As seen in juvenile *M. galloprovincialis,* starvation has been reported to reduce byssus secretion and attachment force due to depletion of glycogen stores (Babarro *et al*., 2008). This is possibly a consequence of a constant carbon ration between soft tissue and byssus, even under stress (Clarke, 1999; Babarro *et al*., 2008).

Desiccation also has a significant influence on seed retention. Carton *et al*. (2007) found that short periods of desiccation (5 hours) resulted in a significant decrease in retention of *P. canaliculus* seed. Whilst Webb and Heasman (2006) demonstrated that desiccation events for as little as 2 hours could significantly reduce activity in seed of the same species, with emersion for 4 hours potentially

causing lasting damage. This indicates that extreme care is required to keep animals moist during husbandry activities. The impact of emersion may also be related to food availability, as the ability to withstand desiccation has been directly related to the availability of food reserves, as well as the ability to respire anaerobically and the tolerance of waste products (Akberali and Trueman, 1985).

Salinity and temperature also play a critical role in byssus formation and attachment (Allen *et al*., 1976; Paul, 1980a,b; Christophersen and Strand, 2003). Several studies have shown that salinities below 30‰ reduces attachment. Paul (1980a) and Christophersen and Strand, (2003) found that attachment of *Chlamys opercularis* and *P. maximus* respectively fell with reducing salinity. A reduction in salinity to 25‰ could reduce attachment by 80% or more compared to 30‰, with no attachment at 20‰. A similar reduction in thread production has been seen in *M. edulis* seed and juveniles with decreasing salinity below full strength seawater (33.9‰) (Allen *et al*., 1976). The rate of thread production increases with increasing temperature for seed, juveniles and adults up to an optimum after which it declines (Allen *et al*., 1976; Paul, 1980b). It has been shown in *C. opercularis* that at 9°C and 24°C byssus attachment is slow and variable (~80% attachment within 140 minutes), however towards 18°C time to attach and variability in attachment diminishes. At 18°C 80% attached within ~25 minutes (Paul, 1980b). Christophersen and Strand (2003) found that attachment of *P. maximus* decreased over time at 15˚C compared to 18˚C, at a salinity of 30‰. It is possible that animals in the wild must rely on threads laid during the warmer summer months to remain attached during the winter, the time of greatest mechanical stress (Allen *et al*., 1976). High and stable attachment rates have been associated with conditions where high growth and survival occur (Paul, 1980a,b; Christophersen and Strand, 2003). Byssus formation is clearly sensitive to environmental conditions.

Illumination also plays a prominent role in the recruitment and dispersal of marine invertebrates, influencing both larval and juvenile locomotion and attachment (Bayne, 1964b; Thorson, 1964; Maldonado and Young, 1996). In the bivalve *M. edulis,* competent larvae become photonegative with crawling pediveligers attaching to algal substrates more readily in the dark, with attachment inversely proportional to light intensity (Bayne, 1964b). The impact of illumination is therefore an important factor for consideration.

Hydrodynamic conditions also have a strong influence on bivalve dispersal and play a major role in recruitment and population structure (Martel and Chia, 1991; Commito *et al*., 1995; Hunt *et al*., 2007; Jennings and Hunt, 2009). Lundquist *et al*. (2004) found that *M. liliana* would undertake

increasing migration with increasing flow speeds, and displayed drifting at speeds of 11.0 and 16.6 cm second^{-1} although this was also influenced by substrate type. Without a water current, dispersal can be prevented, as was the case for *M. balthica* drifting using pedal buoyancy (Sörlin, 1988). Whilst current alone may not induce a response, it allows migration in response to other factors. A bivalve behavioural response to unsuitable habitats can be influenced and modified by changing water velocities, with incidence of pedal crawling or byssus drifting increasing in conjunction with water velocity (Roper *et al*., 1995; Lundquist *et al*., 2004). Hydrodynamic pressures also have a significant role in stimulating byssus attachment. In larvae, Eyster and Pechenik (1987) demonstrated that water agitation contributed to improve early attachment over static conditions. It has been repeatedly demonstrated that attachment by *M. edulis,* in terms of both byssus production and strength, increases in response to increasing water agitation, turbulence or flow velocity, with attachment significantly reduced in static water environments (Glaus, 1968; Price, 1982; Dolmer and Svane, 1994; Lachance *et al*., 2008). In natural assemblages the seasonal cycle of mussel tenacity generally mirrors the seasonal cycle of wave intensity, with decreased byssus strength associated with a reduction in wave action (Price, 1982; Carrington, 2002).

A number of factors are implicated in stimulating retention, detachment and active migration, and it appears likely that a number of these act in concert to effect a response. However, further investigation is required in order to assess and identify the multitude of influential factors, particularly in commercially important species such as *M. edulis* and *P. maximus*.

Seed behaviour as a sign of quality

Conversely a behavioural response by bivalve seed to environmental stimuli has been correlated to seed quality and fitness (Maguire *et al*., 1999a,b). Establishment of links between seed quality and seed behaviour through simple non-destructive tests has been seen as offering the potential for predictive indicators of future performance (Paul, 1980a; Carton *et al*., 2007), thereby providing aquaculture with further useful tools. Webb and Heasman, (2006) showed that the uptake of fast green dye by the mussel *P. canaliculus* was inversely proportional to spat health and fitness. Staining proportions were low for unstressed controls, but significantly rose in spat exposed to stressors including emersion, elevated temperature and ethanol, with lethally stressed spat showing the highest level of staining. Righting and recessing behaviour of *P. maximus* is directly proportional to length of desiccation event and the density of individual seed (Maguire *et al*., 1999a,b). Recessing speed could be used to detect decreases in quality due to increasing short-term acute stress

associated with desiccation, and increasing long-term chronic stress due to stocking density, with time taken to recess increasing with level of stress (Maguire *et al*., 1999a). Carton *et al*. (2007) demonstrated that attachment rate could be used as a measure of future retention performance, since *P. canaliculus* seed that rapidly attached within 20 minutes in a flume tank at 6-7 litre min^{-1} had significantly higher retention rates on ropes than seed which failed to attach in the flume, 63.3±0.5% retention compared to 47.8±0.6%. There is huge potential to apply these simple and practical selection techniques for seed with improved quality, and therefore similar links should be explored in other species.

Thesis aims

The first sections of the thesis set out to investigate effects of chemical agents on the metamorphosis, mortality and growth of two commercially important bivalves, *P. maximus* and *M. edulis*. The impact of chemicals including potassium chloride (KCl), ammonium chloride (NH_4Cl), acetylcholine chloride, γ-aminobutyric acid (GABA), 3,4-dihydroxyphenylalanine (L-DOPA) and epinephrine were investigated. This included assessing a range of concentrations and in the case of *M. edulis* exposure periods. The experiments encompassing this work are detailed in Chapter 2 (*P. maximus*) and Chapter 3 (*M. edulis*). The second half of this thesis addressed the problem of attachment and detachment of mobile bivalve seed in hatcheries. Specifically this investigated the influence of variables including substrate type, substrate conditioning, attachment period, agitation, illumination, food availability, animal activity, animal density and water velocity. The experiments encompassing this work are detailed in Chapter 4 (*M. edulis*) and Chapter 5 (*P. maximus*).

The specific aims and objectives of the four experimental chapters, plus the general discussion are detailed below.

Chapter 2 - Influence of exogenous chemicals on larval development and survival of the king scallop *Pecten maximus* (L.)

Chapter aim: Investigate the influence of potential chemical agents on the development of the king scallop *P. maximus*.

- Objective 1: Assess the effectiveness of a range of concentrations of KCl, NH_4Cl, acetylcholine chloride, GABA, L-DOPA and epinephrine applied over a 48 hour period on their ability to induce metamorphosis in competent larvae.
- Objective 2: Assess the relative toxicities of KCl, NH_4Cl, acetylcholine chloride, GABA, L-DOPA and epinephrine on *P. maximus*.

Chapter 3 - Influence of exogenous chemicals on larval metamorphosis, survival and growth of the blue mussel *Mytilus edulis* (L.)

Chapter aim: Investigate the influence of concentration and exposure period of potential chemical agents on the development of the blue mussel *M. edulis*.

- Objective 1: Assess the effectiveness of a range of concentrations of KCl, NH_4Cl, L-DOPA and epinephrine applied over 24, 48 and 72 hours on their ability to induce metamorphosis in competent larvae.
- Objective 2: Assess the relative toxicities of KCl, NH_4Cl, L-DOPA and epinephrine on *M. edulis*.
- Objective 3: Assess the impact of KCl, NH_4Cl, L-DOPA and epinephrine on the growth of *M. edulis* post-treatment.

Chapter 4 - Investigation of mobile seed behaviour to increase the security of settlement and ease of management of the blue mussel *Mytilus edulis* (L.).

Chapter aim: Investigate the influence of husbandry and environmental pressures on attachment, retention and mortality in seed of the blue mussel *M. edulis* within an experimental benthic system.

- Objective 1: Examine the impact of substrates type, attachment periods, food availability and seed density on attachment, retention and mortality.
- Objective 2: Assess pedal crawling behaviour as a means of estimating future performance of seed based upon subsequent attachment, retention and mortality.

Chapter 5 - Impact of environmental conditions on the attachment and detachment of juvenile *Pecten maximus* (L.).

Chapter aim: Investigate the influence of husbandry and environmental pressures on attachment and retention of *P. maximus* juveniles.

- Objective 1: Assess the impact of variables likely to be encountered during seed handling on seed attachment, including substrate type and pre-conditioning, attachment period, illumination, water agitation and feeding level.
- Objective 2: Assess the impact of water flow velocity on the level of seed detachment and retention, and the level of inter-connection with secondary environmental conditions including substrate type, substrate pre-conditioning and feeding level.

Chapter 6 – General Discussion

Chapter aim: To discussion the work conducted within this study its context within bivalve aquaculture, and to identify future direction for research in this field.

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Chapter 2

Influence of exogenous chemicals on larval development and survival of the king scallop *Pecten maximus* (L.)

Abstract

The commercially important king scallop, *Pecten maximus* (L.), is an emerging aquaculture species in Europe and the focus of ongoing research to optimise culture practices. At present, difficulties in the culture of this species are commonly associated with the transition from the larval to juvenile stages, with protracted metamorphosis, low development synchronicity and variable survival. The use of exogenously applied chemical agents, as demonstrated in other bivalve species, has been viewed as a means of resolving these issues. The present study evaluated the effects of the chemicals KCl, NH_4Cl, acetylcholine chloride, GABA, L-DOPA and epinephrine on the induction of larval metamorphosis and larval toxicity. A range of concentrations for each chemical, applied over a 48 hour period, were assessed. Larval metamorphic response was low, and concentration-dependent, whilst all chemicals proved toxic within the range of concentrations tested. Among the tested chemicals, KCl at 20mM and L-DOPA at 10^{-6}M induced significantly ($P<0.05$) higher rates of larval development, improving development by 208% and 128% respectively compared to the controls after 1 week. However, whilst the KCl treatment was toxic, reducing survival by 33% compared to the control ($P<0.05$), the L-DOPA treatment was found to significantly increase survival by 49% compared to the control ($P<0.05$). Furthermore, the influence of these two chemicals on larval development varied, with KCl only promoting dissoconch growth and L-DOPA only promoting gill development, suggesting that the pathways influenced by these two chemicals maybe distinct. In contrast, NH_4Cl, acetylcholine chloride, GABA and epinephrine all proved ineffective in inducing metamorphosis in the range of concentrations tested. Work remains to fully realise the ability to completely synchronise larval metamorphosis in *P. maximus* suitable for hatchery application, however the results of the present study provide further evidence for the potential use of chemicals.

Keywords

Pecten maximus, Larvae, Chemical, Induction, Metamorphosis, Survival

Abbreviations

ACH	Acetylcholine chloride
ANOVA	Analysis of variance
GABA	γ-aminobutyric acid
HCl	Hydrochloric acid
L-DOPA	L-3,4-dihydroxyphenylalanine
KCl	Potassium chloride
M	Molar
NH_4Cl	Ammonium chloride

Introduction

The king scallop *Pecten maximus* is a commercially important species in Europe. Annual production reached 63,776 tons by 2011, more than doubling over the previous 10 years (FAO, 2014). However, nearly all production is from wild fisheries in Europe, which currently provide over 99% of the supply (Millican, 1997; Spencer, 2002; FAO, 2013). Cultivation of *P. maximus* has generally depended on the collection of naturally settled juvenile spat using collector materials, such as mesh bags filled with monofilament netting, suspended just prior to expected spatfall in areas of natural settlement (Spencer, 2002). These are then on-grown to a marketable size using suspended pearl and lantern nets, ear-hanging, or bottom culture (Millican, 1997; Spencer, 2002). Nevertheless, the risk of unpredictable spat fall, and seasonal constraints on availability have led to recognition of a need for hatcheries to support the expansion of cultivation, to sustain the industry during times of short supply, and offer a potential means of combating dwindling wild stocks (Millican, 1997; Spencer, 2002). Culture of *P. maximus* is well documented (Gruffydd and Beaumont, 1970; Gruffydd and Beaumont, 1972; Beaumont and Budd, 1983; Millican, 1997; Bergh and Strand, 2001; Spencer, 2002; Torkildsen and Magnesen, 2004), and although considered in its infancy, hatchery production for this species has been carried out in several European counties including Scotland (Spencer, 2002), Norway (Bergh and Strand, 2001; Torkildsen and Magnesen, 2004), Ireland and France (Robert and Nicolas, 2000; REPROSEED, 2014). The Norwegian scallop aquaculture industry is now entirely reliant upon spat sourced from hatcheries (Torkildsen and Magnesen, 2004).

Difficulties in the culture of bivalves have often been associated with settlement and metamorphosis, the period during which mature larvae undertake exploratory behaviour as they search for a suitable substratum, before undergoing permanent morphological changes allowing them to adapt to their new benthic habitat (Bayne, 1965; Pawlik, 1990; Lutz and Kennish, 1992; Gosling, 2003). In the hatchery environment, *P. maximus* exhibits a protracted development over several weeks, leading to a lack of synchronous development and variable levels of survival within larval batches (Nicolas *et al*., 1998; Robert and Nicholas, 2000). In the natural environment, control of settlement and metamorphosis of marine invertebrates is controlled by a range of interrelated biotic and abiotic factors operating over different temporal and spatial scales (Jackson, 1986; Pawlik, 1990; Pawlik, 1992, and Rodríguez *et al*., 1993). Chemical cues and triggers have been identified as major influential factors on settlement and metamorphosis in marine invertebrates (Pawlik, 1992), and have been associated as originating from conspecifics (Pearce and Scheibling, 1990a; Zhao and Qian, 2002), biofilms (Satuito *et al*., 1995; Zhao and Qian, 2002; Zhao *et al*., 2003), bacteria (Bonar *et*

al., 1990; Fitt *et al*., 1990; Satuito *et al*., 1995), specific habitats (Pearce and Scheibling, 1990b; Swanson *et al*., 2004; Cob *et al*., 2010) and food sources (Steneck, 1982; Ritson-Williams *et al*., 2009). Whilst a number compounds have been isolated, including histamine from the red algae *Delisea pulchra* (Swanson *et al*., 2004), ammonia from bacteria (Coon *et al*., 1988; Bonar *et al*., 1990) and jacaranone from the red algae *Delesseria sanguinea* (Yvin *et al*., 1985), the specific identity of many of these naturally-derived cues remains largely unknown. From an aquaculture perspective it has been recognised that the identification of chemical agents with inductive properties could provide an effective means of controlling settlement and metamorphosis in commercially important species (Cooper, 1982; Baloun and Morse, 1984; Pawlik, 1990; Mesías-Gansbillar *et al*., 2008).

A wide range of chemical agents have been tested with varying effects, ranging from increased induction of normal settlement and metamorphosis, to abnormal or partial development, death or no effect at all (Morse *et al*., 1979; Hadfield, 1984; Pawlik, 1990; Pires and Hadfield, 1991; Carpizo-Ituarte and Hadfield, 1998; Garca-Lavandeíra *et al*., 2005; Zhao *et al*., 2003; Yang *et al*., 2008). These chemicals are thought to act either as functional analogues of natural inducers, as precursors, or as active components within a signalling pathway (Yool *et al*., 1986; Pawlik, 1990; Garcia-Lavandeira *et al*., 2005). A number of compounds have proven effective at stimulating settlement, metamorphosis, or both, in bivalves, including the neurotransmitter γ-aminobutyric acid (GABA) (García-Lavandeira *et al*., 2005; Mesías-Gansbillar *et al*., 2008; Yu *et al*., 2008), the catecholamine epinephrine (Coon *et al*., 1985; García-Lavandeira *et al*., 2005; Mesías-Gansbillar *et al*., 2008), and its precursor L-DOPA (Coon *et al*., 1985; Kingzett *et al*., 1990; Nicholas *et al*., 1998; Dobretsov and Qian, 2003), as well as excess potassium (Martinez *et al*., 1999; Zhao *et al*., 2003; Yu *et al*., 2008), ammonium (Coon *et al*., 1990; Kingzett *et al*., 1990; Yang *et al*., 2008), and choline derivatives (Dobretsov and Qian, 2003, Zhao *et al*., 2003; Yu *et al*., 2008). However, effectiveness of these compounds is influenced by concentration, length of exposure and by variations in larval sensitivity (Doroudi and Southgate, 2002; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005; Yu *et al*., 2008). Therefore extensive testing of chemical agents with candidate bivalve species is essential.

In the case of *P. maximus* previous studies have shown that competent larvae have been induced to settle and metamorphose in the presence of the algae extract jacaranone (Chevolot *et al*., 1991; Nicholas *et al*., 1998), epinephrine (Chevolot *et al*., 1991; Nicolas *et al*., 1996; Nicolas *et al*., 1998) and L-DOPA (Chevolot *et al*., 1991; Nicolas *et al*., 1998). However, so far this species seems less sensitive to the few chemicals tested to date and induction levels remain relatively low. Further investigation is essential to assess the potential of chemical agents and identify an effective

application method. Therefore, the aim of this study was to assess the effectiveness of several chemical agents that had shown inductive abilities in molluscs on their ability to induce the morphological transition in competent *P. maximus* larvae. Importantly however, this study also aimed to assess the relative toxicities of these agents towards the king scallop *P. maximus* as a measure of their suitability. This includes the examination of novel chemicals yet to be applied to *P. maximus*, as well as clarifying the influence of some previously tested agents.

Materials and Methods

Larval culture

Veliger larval *P. maximus* (202 ±19µm in shell length)(Figure 2.1) were obtained from the Scalpro AS hatchery (Rong, Norway) and transported by airfreight to the marine laboratory at the Centre for Marine Sciences, Bangor University (Anglesey, Wales). On arrival, imported larvae were assessed, before stocking at a density of 5 larvae ml^{-1} in 65-litre static polyethylene tanks, filled to a volume of up to 45-litres with 1µm filtered, UV-light irradiated seawater (FSW), at a salinity of 33‰. Culture temperature was maintained at16±1˚C. Three times a week the larvae were sieved onto a 45µm mesh screen and inspected, and the containers cleaned before the larvae were restocked Larvae were fed with a mixed microalgae diet equivalent to 30 cells μl^{-1} day^{-1}, consisting of *Pavlova lutheri* (PLY75), *Isochrysis* sp. (clone T-ISO) (PLY506A) and *Chaetoceros calcitrans* (PLY537) at a ratio of 1:1:1. Veligers were reared until they reached competence to metamorphose, within 2 to 7 days of import.

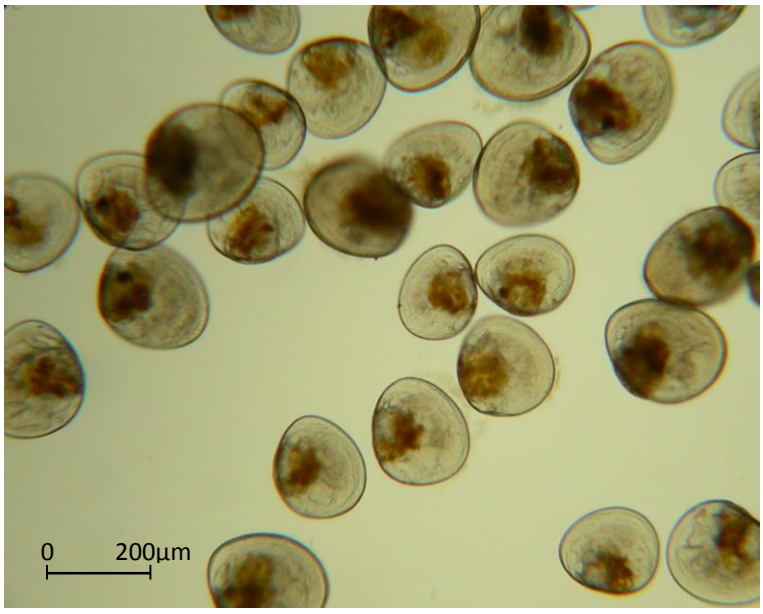

Figure 2.1: *Pecten maximus* veliger larvae imported from Scalpro AS, Norway.

Competence of pediveligers to undertake metamorphosis was assessed based upon the presence of eye-spots (Figure 2.2), with experiments initiated once the eye-spot ratio reached approximately 35-50%. Three batches of larvae were used for the assays carried out in this study. Notably no size grading of larval cultures was undertaken. Assessment of larval size (µm) and percentage survival at competence, immediately prior to use, was also determined in order to provide additional reference indicators for this species of bivalve. Size was based upon the shell length (µm) of approximately 30 larvae, with measurements made from digital analysis of photomicrographs using the image analysis software Image J. Survival was estimated by comparing stocked number of imported larvae and final numbers within cultures using sub-sample counts.

In larval batch 1, larvae measured 236.8±25.5µm at competence, with survival to this point estimated at 100%. In batch 2 larvae measured 233.3±20.3µm at competence, with survival to this point estimated at 100%. In batch 3 larvae measured 206.8±19.2µm at competence, with larval survival to this point estimated at 92.7%. Cultures with signs of larvae mortality >90% and ciliate infestation where not used in assays.

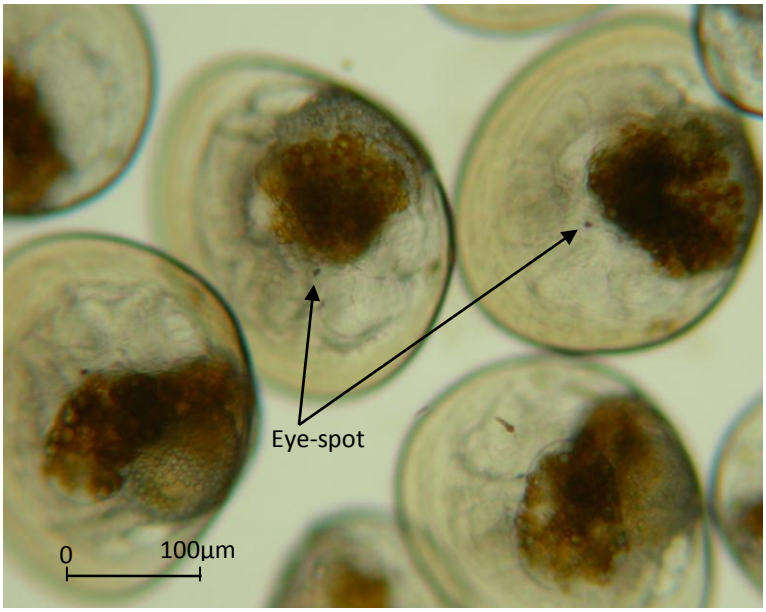

Figure 2.2: Veliger *Pecten maximus* larvae with the distinct eye-spot.

Chemical agents

All chemicals, potassium chloride (KCl), ammonium chloride (NH_4Cl), acetylcholine chloride (ACH), γ-aminobutyric acid (GABA), L-3,4-dihydroxyphenylalanine (L-DOPA) and epinephrine, were obtained from Sigma-Aldrich (Poole, UK). Concentrated stock solutions of KCl (1Molar), NH_4Cl (1M), ACH (10^{-1}M), GABA (10^{-2}M) and L-DOPA (10^{-2}M) were prepared by dissolving the chemicals in FSW, whilst

epinephrine (10^{-2}M) was dissolved in a solution of FSW containing 0.009 N Hydrochloric acid (HCl). All stock solutions were freshly prepared on the same days as the assay. Stock solutions were diluted into the FSW containing the larvae to achieve the experimental test concentrations. KCl was assayed at concentrations of 10, 20, 30 and 40mM; NH_4Cl at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}M; ACH at 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}M; and GABA, L-DOPA and epinephrine at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}M. For epinephrine, the concentration of HCl was adjusted to be equal in each treatment (0.0009N HCl), including the control. A second control treatment without HCl was also run to test the influence of HCl.

Chemical assays

Assays were carried out in 1-liter Pyrex-glass beakers containing 500ml of static FSW, stocked at a density of 5 larvae ml^{-1} and maintained at 16±1˚C (Figure 2.3). For all experiments, each chemical treatment was conducted in triplicate. All assays were conducted in the light, with the exception of epinephrine which was conducted in the dark during the initial treatment period to reduce photo-oxidation. Larvae were exposed to each test compound for 48 hours, after which they were thoroughly rinsed on a 45µm mesh screen to remove residual chemicals and restocked in clean beakers of FSW. Further water changes were conducted every 2-3 days. During the experiments, larvae were fed with a mixed microalgae diet as described for larval rearing. Larval batch 1 was utilised for assays of KCl, NH_4Cl and ACH; larval batch 2 for assays of GABA and L-DOPA; and larval batch 3 for the assay of epinephrine. Triplicated control treatments were included within each batch.

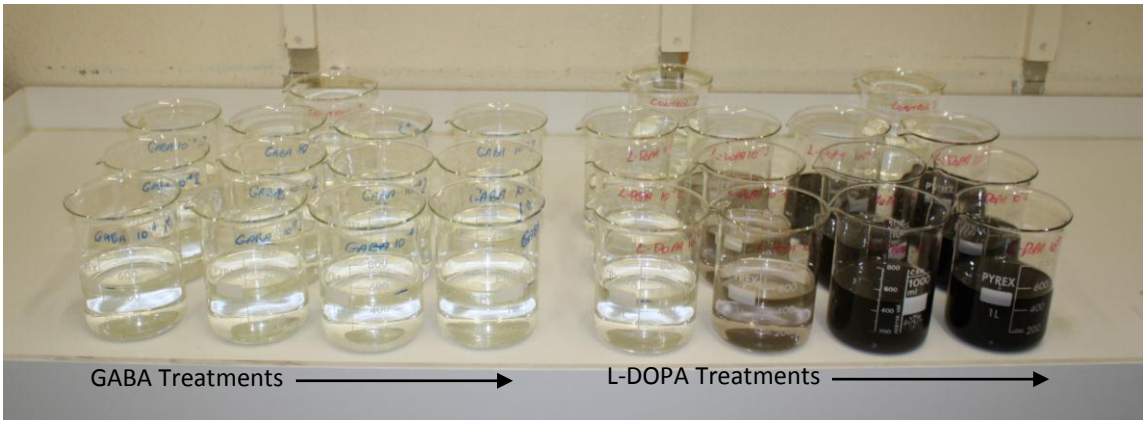

Figure 2.3: Exogenous chemical assay cultures of *Pecten maximus* larvae assessing the impact of γ-aminobutyric acid (GABA) on the left and L-3,4-dihydroxyphenylalanine (L-DOPA) on the right on metamorphosis and survival. The L-DOPA cultures show increasing discolouration due to oxidation of the chemical proportional to concentration.

Triplicate sub-samples of larvae were taken from each replicate beaker after the 48 hour induction period and at the conclusion of the experiment after a total experimental period of 7 days (5 days post-treatment). Immediately prior to sampling a small brush was used to dislodge any attached post-larvae and the culture vessels were agitated to suspend all animals within the water column. Utilising a Leica DME binocular microscope the number of metamorphosed post-larvae was assessed and the total remaining number of live individuals counted to determine survival. Percentage metamorphosis and percentage survival were both calculated relative to the initial stocking density. Larval metamorphosis was measured based upon larvae possessing elongated and functional gill filaments and on post-larvae shell (dissoconch) growth (Nicholas *et al*., 1998) (Figure 2.4).

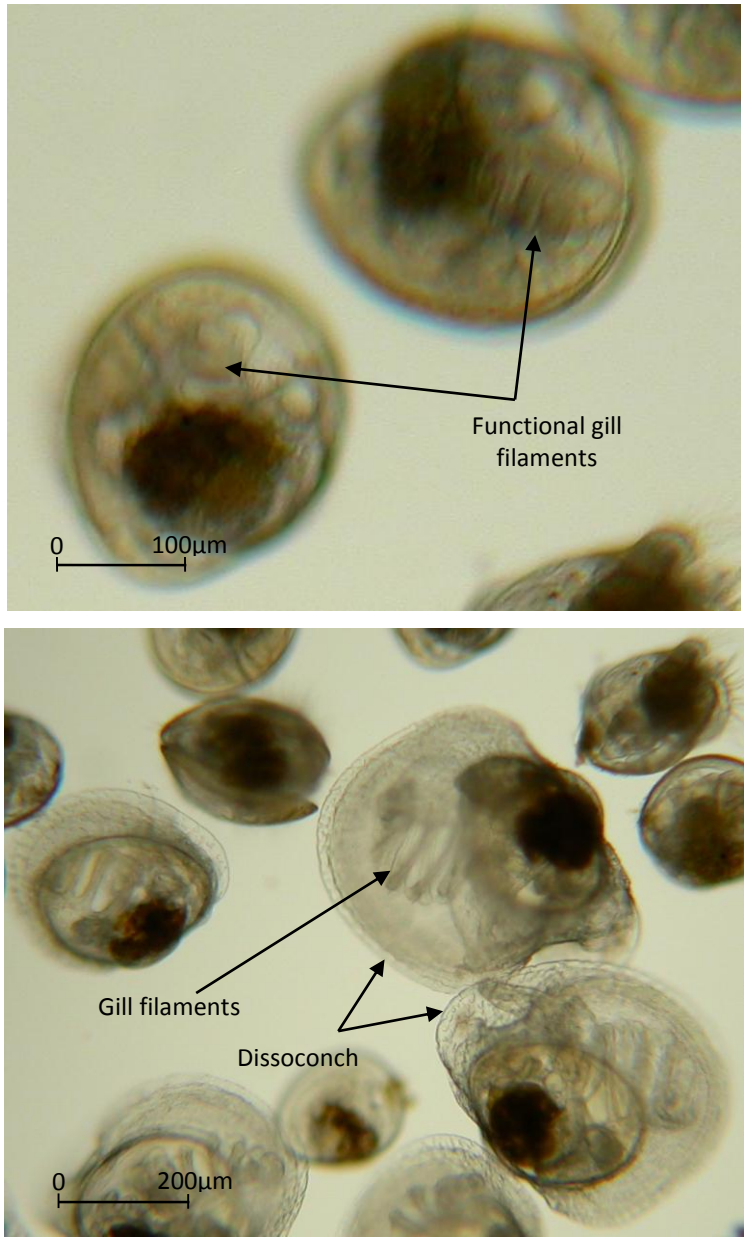

Figure 2.4: Post-larval *Pecten maximus* possessing elongated functional gill filaments and secondary dissoconch shell. Development of functional gill filaments preceded growth of the dissoconch.

Statistical analyses

All data sets are described as the percentage of larval metamorphosis, based upon both gill filament development and secondary shell growth, and larval survival. Before analysing, all percentage data sets were converted by arcsine square root transformation. The data presented in all figures is untransformed. Mean data sets were tested using the Anderson-Darling test to investigate departure from normality and Bartlett's test to assess heteroscedasticity before applying any test of comparison (Sokal and Rohlf, 1995). ANOVA tests were used to determine if there was any significant difference among treatments, followed by pairwise comparisons between treatments using Fisher's Least Significant Difference test (LSD). All results were considered to be significantly different when $P<0.05$. Analyses were undertaken using the statistical package Minitab®.

Results

Influence of potassium chloride

Figures 2.5 and 2.6 show the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively in response to testing a range of KCl concentrations over an exposure period of 48 hours. At the beginning of the assay 46.9 ±6.0% of larvae possessed eye-spots, with 2.5 ±2.3% showing some signs of early gill formation and none showing any secondary shell growth.

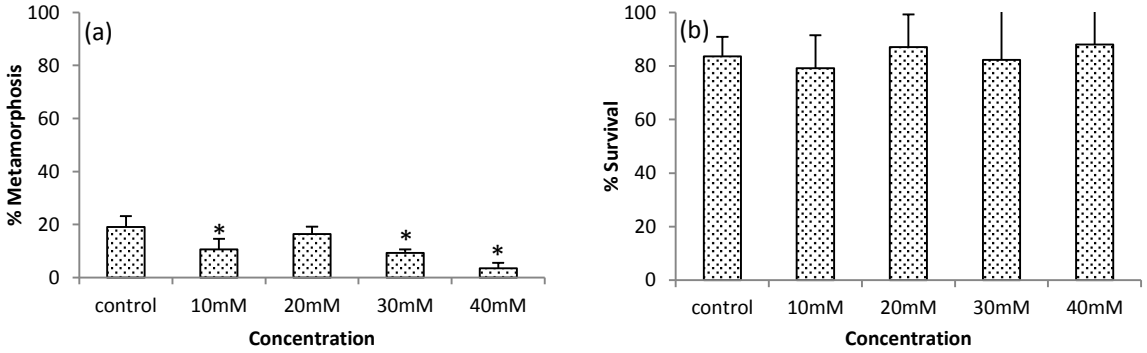

Figure 2.5: Percentage metamorphosis of larval gill filaments (a) and larval survival (b) following exposure to 10mM, 20mM, 30mM and 40mM of KCl for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

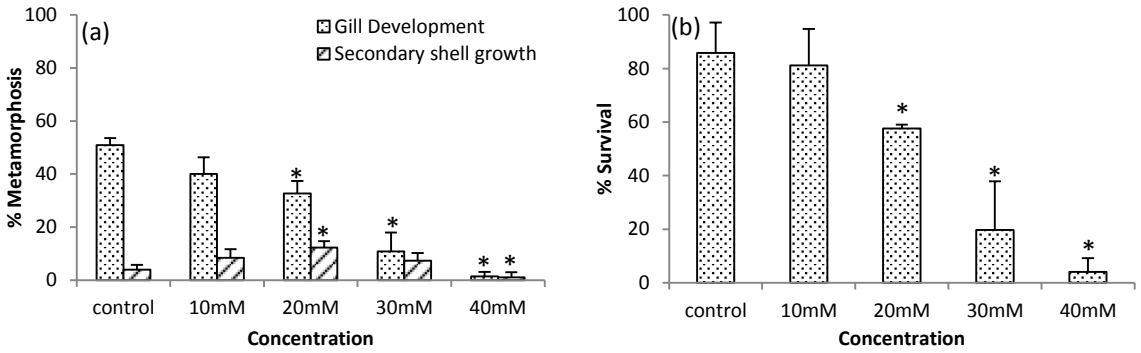

Figure 2.6: Percentage metamorphosis of larval gill filaments and secondary shell growth (a), and larval survival (b) after 7 days following exposure to 10mM, 20mM, 30mM and 40mM of KCl for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

After the initial 48 hour treatment period no KCl treatment induced larval metamorphosis greater than the control, although notably larvae in concentrations of 10mM, 30mM and 40mM all had significantly lower gill development (Figure 2.5a, Fisher's $P<0.05$). At this point there was no significant difference in survival compared to the control (ANOVA $F=0.37$, $P=0.872$) (Figure 2.2b). After 7 days, both gill development and survival were significantly impaired at concentrations of 20mM and above (Figure 2.6a, 2.6b) (Fisher's $P<0.05$). However, development of secondary shell occurred at a significantly higher frequency (208% higher) in larvae exposed to 20mM KCl compared to the control (Fisher's $P<0.05$), although survival at this level was approximately 33% lower than the control.

Influence of ammonium chloride

Figures 2.7 and 2.8 show the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively in response to testing a range of NH_4Cl concentrations over an exposure period of 48 hours. At the beginning of the assay the proportion of larvae possessing eye-spots and showing early gill formation was the same as for the KCl assay.

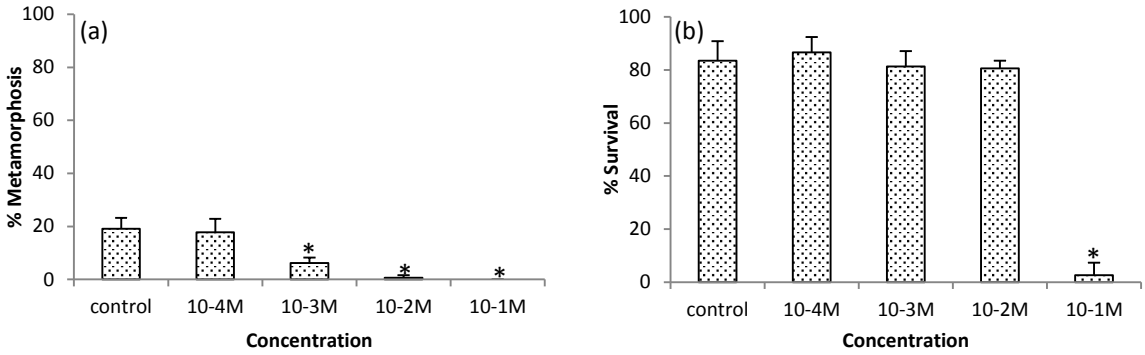

Figure 2.7: Percentage metamorphosis of larval gill filaments (a) and larval survival (b) following exposure to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}M of NH_4Cl for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

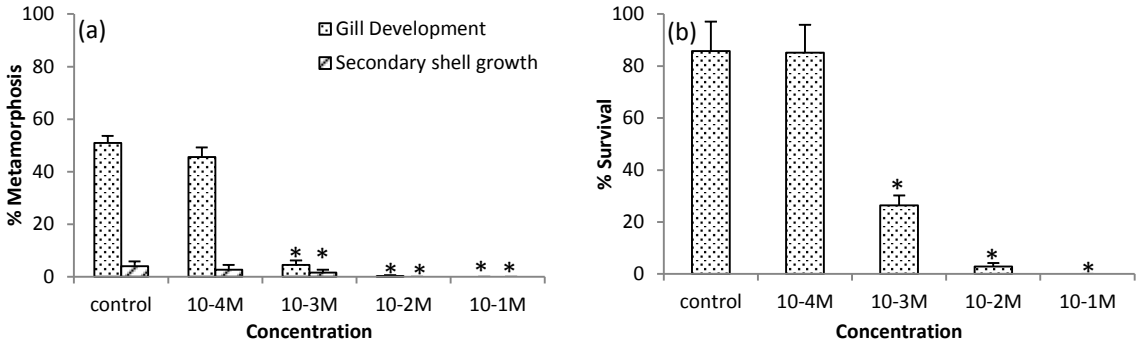

Figure 2.8: Percentage metamorphosis of larval gill filaments and secondary shell growth (a), and larval survival (b) after 7 days following exposure to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}M of NH_4Cl for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

NH_4Cl did not elicit a significant improvement in larval metamorphosis in terms of either gill development or secondary shell growth over the study period (Figure 2.7a and 2.8a). Concentrations of 10^{-3}M and above significantly decreased larval development at both sample points, compared to the control (Fisher's $P>0.05$). A concentration of 10^{-1}M NH_4Cl was highly toxic within 48 hours (Figure 2.7b, Fisher $P<0.05$), while after 7 days concentrations 10^{-3}M and above resulted in significantly lower survival compared to the control (Figure 2.8b, Fisher's $P<0.05$).

Influence of acetylcholine chloride

Figures 2.9 and 2.10 show the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively in response to testing a range of ACH concentrations over an exposure period of 48 hours. At the beginning of the assay the proportion of larvae possessing eye-spots and showing early gill formation was the same as for the KCl assay.

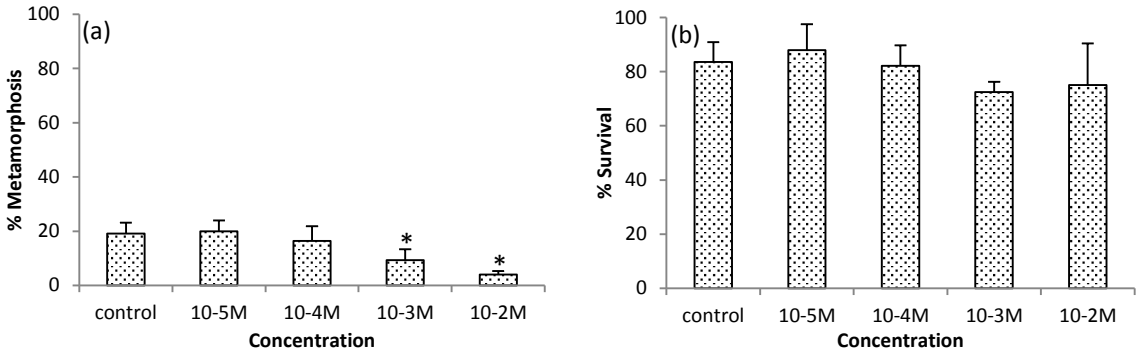

Figure 2.9: Percentage metamorphosis of larval gill filaments (a) and larval survival (b) following exposure to 10^{-2}, 10^{-3}, 10^{-4}M and 10^{-5}M of acetylcholine chloride for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control (P<0.05).

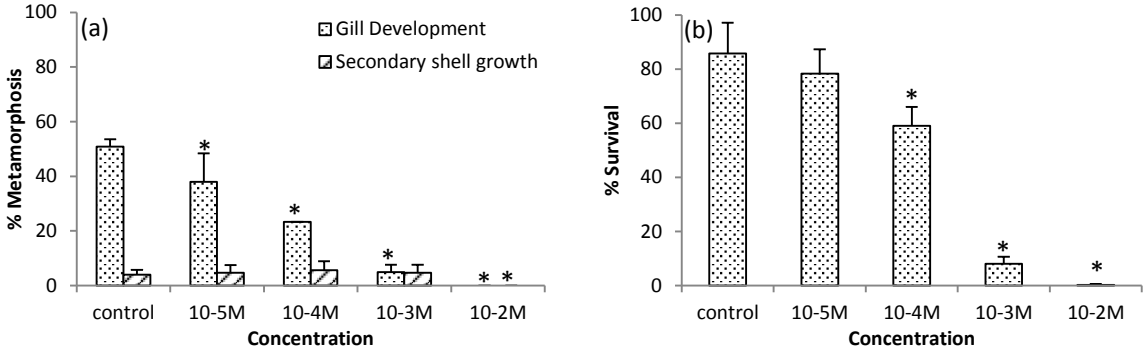

Figure 2.10: Percentage metamorphosis of larval gill filaments and secondary shell growth (a), and larval survival (b) after 7 days following exposure to 10^{-2}, 10^{-3}, 10^{-4}M and 10^{-5}M of acetylcholine chloride for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control (P<0.05).

ACH over the tested concentrations did not elicit a significant improvement in either gill development or secondary shell growth over the study period (Figure 2.9a and 2.10a). After 1 week gill development by larvae at all concentrations of ACH was significantly lower than seen in the control (Fisher's P<0.05), although secondary shell growth was only significantly lower at the highest concentration of 10^{-2}M (Figure 2.10a), with almost total mortality experienced at this concentration (Figure 2.10b). Exposure to acetylcholine significantly reduced larval survival at concentrations of 10^{-4}M and above but only after 7 days, (Fisher's P<0.05), with the level of toxicity increasing with concentration (Figure 2.10b).

Influence of γ-aminobutyric acid

Figures 2.11 and 2.12 show the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively in response to testing a range of GABA concentrations over an

exposure period of 48 hours. At the beginning of the assay 39.7 ±3.4% of larvae possessed eye-spots, with 2.8 ±1.8% showing some signs of early gill formation and none showing any secondary shell growth.

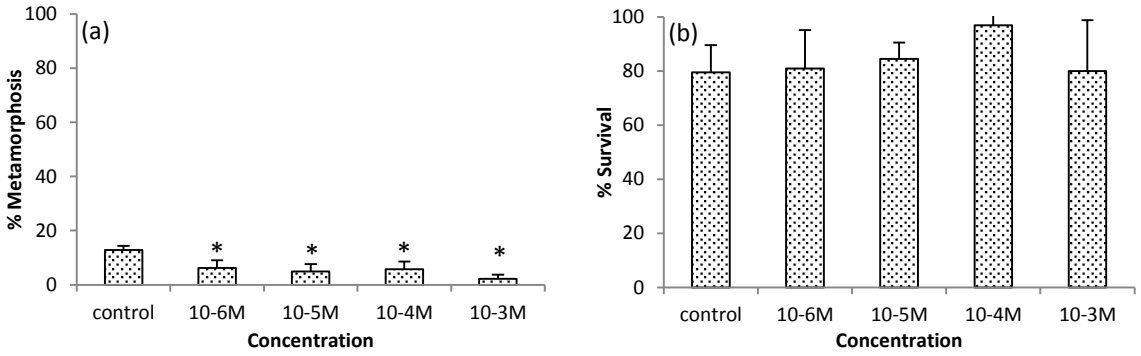

Figure 2.11: Percentage metamorphosis of larval gill filaments (a) and larval survival (b) following exposure to 10^{-3}, 10^{-4}, 10^{-5}M and 10^{-6}M of γ-aminobutyric acid for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control (P<0.05).

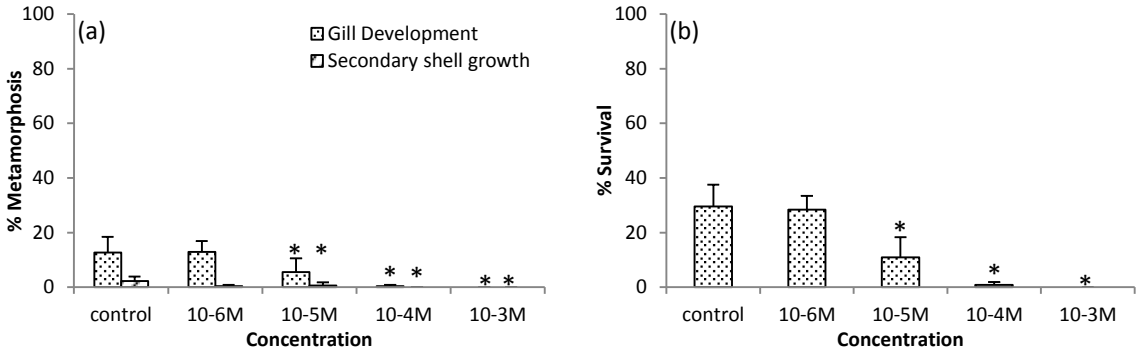

Figure 2.12: Percentage metamorphosis of larval gill filaments and secondary shell growth (a), and larval survival (b) after 7 days following exposure to 10^{-3}, 10^{-4}, 10^{-5}M and 10^{-6}M of γ-aminobutyric acid for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control (P<0.05).

Over the tested concentrations GABA did not induce a significant improvement in gill development or secondary shell growth during the study period, but inhibited larval development in most cases (Figure 2.11a and 2.12a). As with ACH, the toxicity of GABA was not apparent at 48 hours, but survival after 7 days was reduced in treatments exposed to concentrations of 10^{-5}M and above (Fisher's P<0.05), with the level of toxicity increasing with concentration (Figure 2.12b). Only a concentration of 10^{-6}M did not depress development or survival.

Influence of L-3,4-dihydroxyphenylalanine

Figures 2.13 and 2.14 show the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively in response to testing a range of L-DOPA concentrations over an exposure period of 48 hours. At the beginning of the assay the proportion of larvae possessing eye-spots and showing early gill formation was the same as for the GABA assay.

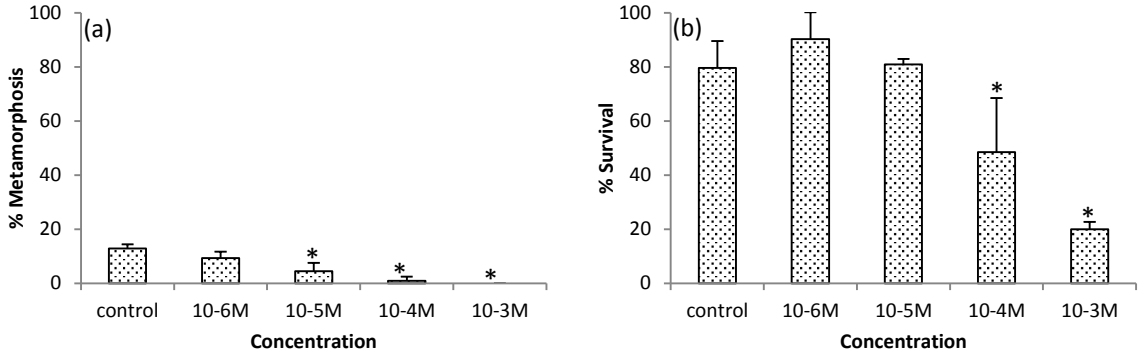

Figure 2.13: Percentage metamorphosis of larval gill filaments (a) and larval survival (b) following exposure to 10^{-3}, 10^{-4}, 10^{-5}M and 10^{-6}M of L-DOPA for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

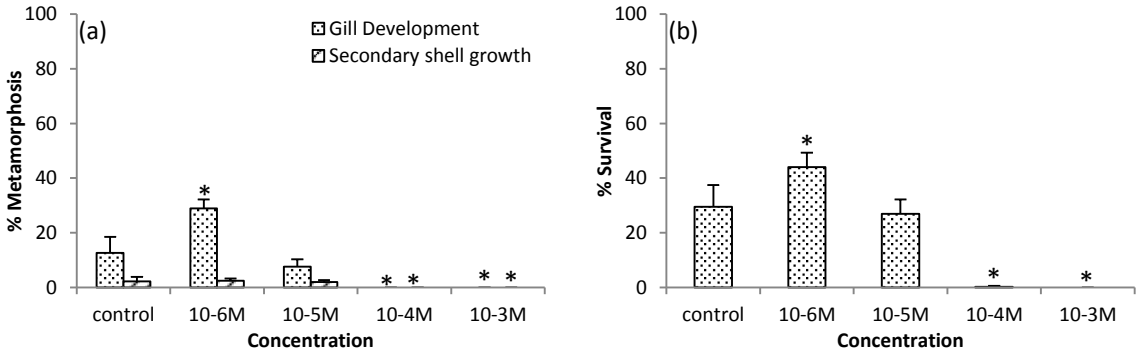

Figure 2.14: Percentage metamorphosis of larval gill filaments and secondary shell growth (a), and larval survival (b) after 7 days following exposure to 10^{-3}, 10^{-4}, 10^{-5}M and 10^{-6}M of L-DOPA for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

L-DOPA was found to significantly and positively influence larval metamorphosis and survival. At 48 hours no L-DOPA treatment had induced increased larval metamorphosis, with concentrations of 10^{-5}M and above showing significantly reduced gill formation (Figure 2.13a). However by 7 days significantly greater gill development was observed in larvae exposed to 10^{-6}M compared to all other treatments (Fisher's $P<0.05$) (Figure 2.14a). At this level, metamorphosis was over double that

observed in the control. No other treatment significantly increased gill development, whilst no treatment significantly encouraged the development of secondary shell growth above that seen in the control. The lack of metamorphosis of larvae at concentrations of 10^{-4}M and 10^{-3}M reflects the toxicity of the chemical at these levels (Figures 2.13b and 2.14b), with survival significantly lower than in the control at these levels after 48 hours (Fisher's $P<0.05$). After 7 days, few or no larvae survived at these L-DOPA concentrations. In this larval batch survival by 1 week was relatively low, with a 62% drop in survival between 48 hours and 1 week in the control. However, survival was significantly improved by L-DOPA at 10^{-6}M, with a 49% improvement over the control (Fisher's $P<0.05$) (Figure 2.14b).

Influence of epinephrine

Figures 2.15 and 2.16 show the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively in response to testing a range of epinephrine concentrations over an exposure period of 48 hours. At the beginning of the assay 35.6 ±10.2% of larvae possessed eye-spots, with 8.9 ±9.6% showing some signs of early gill formation and none showing any secondary shell growth.

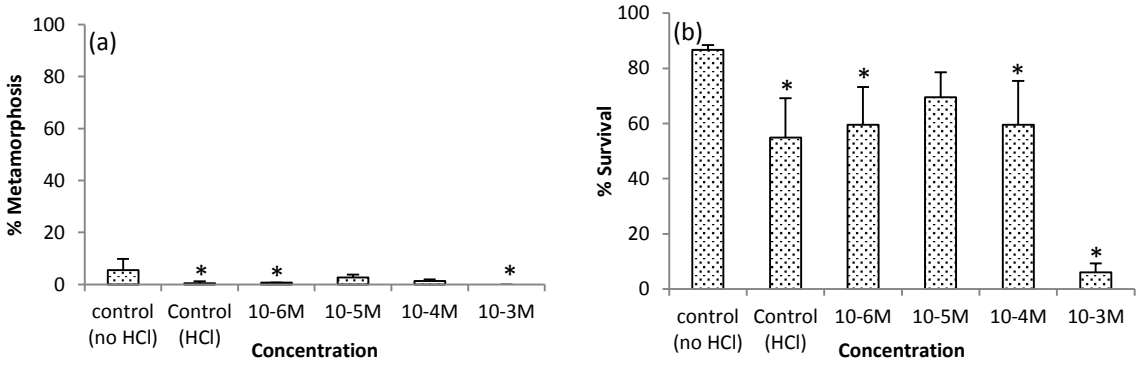

Figure 2.15: Percentage metamorphosis of larval gill filaments (a) and larval survival (b) following exposure to 10^{-3}, 10^{-4}, 10^{-5}M and 10^{-6}M of epinephrine for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control (no HCl) ($P<0.05$).

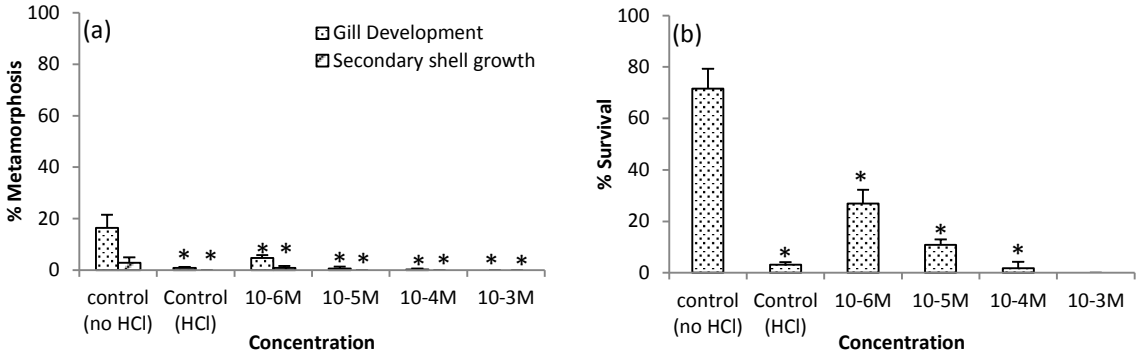

Figure 2.16: Percentage metamorphosis of larval gill filaments and secondary shell growth (a), and larval survival (b) after 7 days following exposure to 10^{-3}, 10^{-4}, 10^{-5}M and 10^{-6}M of epinephrine for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control (no HCl) ($P<0.05$).

Over the tested concentrations, epinephrine did not induce a significant improvement in larval metamorphosis or survival over the study period. However, the inclusion of HCl detrimentally influenced the results of this experiment. Comparison with the control containing no HCl showed that by 48 hours larval metamorphosis and survival was significantly lower in most treatments containing HCl, including the control with HCl, and after 7 days larval metamorphosis and survival were significantly lower in all treatments (Fisher's $P<0.05$). In fact metamorphosis was almost zero in treatments containing HCl, whilst survival was extremely low by the conclusion of the experiment. Notably, concentrations of epinephrine of 10^{-6}M significantly improved survival compared to the control containing HCl (Fisher's $P<0.05$).

Discussion

The ability to synchronise bivalve larval progression through the transitional phases of settlement and metamorphosis using exogenously applied chemical agents would benefit the commercial culture of many species. However, the effectiveness of different chemicals has proven to be highly species-specific, typically requiring extensive testing for every species of interest (Kingzett *et al*., 1990; Chevolot *et al*., 1991; Nicholas *et al*., 1998; Dobretsov and Qian, 2003; Zhao *et al*., 2003; Yu *et al*., 2008). *P. maximus* is known to exhibit protracted metamorphosis over a 2 to 3 week period, during which 35 to 70% of larvae will metamorphose, with metamorphosis in the first week often not exceeding 5% (Nicholas *et al*., 1996; Nicholas *et al*., 1998; Robert and Nicholas, 2000). Several investigations have previously demonstrated that a number of chemical agents have inductive settlement and metamorphic properties in *P. maximus*. However induction levels and

synchronisation remain relatively low, with induced metamorphosis ranging from just 5 to 32% after 1 week compared to approximately 5% in control groups (Chevolot *et al*., 1991; Nicholas *et al*., 1996; Nicholas *et al*., 1998). Examination of alternative chemicals, as well as re-assessing the influence of some previously tested agents, is therefore essential for this species, and the focus of this study.

We found no positive influence of any chemical treatment within the first 48 hours, with these only exhibited after 7 days, reflecting the slow larval development in this species (Robert and Nicholas, 2000). Larval development in control treatments ranged from 5.6 to 19.1% with functional gills in the first 48 hours, reaching 12.7 to 50.9% with functional gills and 2.2 to 2.9% with dissoconch growth after 7 days. This is similar to experiences of *P. maximus* development reported in previous studies. Notably differences between the chemical assays in the present study and previous studies could be a consequence of differences in their respective competence, or larval batch variation. As with all bivalve larvae the effectiveness of chemical induction appears to be related to the competence of larvae to undergo metamorphosis (Coon *et al*., 1990; Chevolot *et al*., 1991); if larvae are immature then the chemical is unlikely to stimulate a response. There is a correlation between competence and morphological features, such as size and the development of eye-spots; however variations to this are apparent, highlighting the individual variability within groups of larvae (Bonar et al., 1990). For *P. maximus* competence has previously been correlated to the presence of double shell rings at the margin of the shell, which correspond to the peripheral groove to which the dissoconch shell attaches, as well as grading larvae with mesh screens to >150µm in size (Chevolot *et al*., 1991; Nicolas *et al*., 1998). A larval size exceeding 212µm has also been associated with ready to settle *P. maximus* (Robert and Gérard, 1999). In this study the mean size of each batch of *P. maximus* larvae was over 200µm, in line with previous studies. Double shell rings are also not the only criteria used for assessing competence in bivalves and the present study assessed the level of competence based upon the development of pigmented eye-spots as an alternative indicator. Eye-spot development has been used to characterise the end of larval development in other bivalve species including *Crassostrea gigas* (Coon *et al*., 1990), *Pinctada margaritifera* (Doroudi and Southgate, 2002), *Pinctada maxima* (Zhao *et al*., 2003) and *Mytilus edulis* (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003).

The application of excess K^+, typically applied as a chloride, has been found to effectively induced settlement, metamorphosis or both in a range of bivalve species (Martinez *et al*., 1999; Zhao *et al*., 2003; Yang *et al*., 2008; Yu *et al*., 2008; Yang *et al*., 2013) as well as other molluscan species (Baloun and Morse, 1984; Yool *et al*., 1986; Pechenik and Heyman, 1987; Cob *et al*., 2010). It has also proven

effective in other marine invertebrate taxa (Yool *et al*., 1986). Its wide spread effect has led to the suggestion that there may be a shared sensitivity to K^+ as an inductive cue in marine invertebrates (Yool *et al*., 1986). The exact mode of action remains to be ascertained although it is thought to be a consequence of the depolarisation of external cell membrane caused by the influx of K^+ (Baloun and Morse, 1984). In bivalves K^+ has previously induced metamorphosis in *Argopecten purpuratus* (Martinez *et al*., 1999), *Mytilus galloprovincialis* (Yang *et al*., 2008) and *Mytilus coruscus* (Yang *et al*., 2013), though in each case development has been observed following a lag period of 2 to 4 days following a treatment window of 24 to 48 hours. In contrast continuous exposure, in the case of *M. galloprovincialis* for 48 to 96 hours, has been shown to inhibit metamorphosis (Yang *et al*., 2008). This indicates shorter exposure periods may prove more beneficial. Our study represents the first instance of excess K^+ tested with *P. maximus,* and results indicate that it can be added to the list of species positively affected by this ion. A three-fold increase in the rate of dissoconch growth was observed at a concentration of 20mM (12.3±2.4%) compared to the control (4.0±1.8%) after 7 days. However, no other concentration demonstrated a positive influence, and no treatment produced a positive result after just 48 hours. In *A. purpuratus* KCl was effective at a concentration of 10mM over an exposure of 48 hours, utilising a similar protocol (Martinez *et al*., 1999). However, whilst Martinez *et al*. (1999) induced approximately 45% of larvae to metamorphose, the level of synchronous larval development in the current study remains low. In other marine invertebrates sensitivity to K^+ as a metamorphic inducer has ranged from approximately 15% in the gastropod *Haliotis diversicolor (*Bryan and Qian, 1998) to 100% effective in the gastropods *Crepidula fornicata* (Pechenik and Heyman, 1987), and *Strombus canarium* (Cob *et al*., 2010), whilst it has notably failed to induce a response in the bivalve *M. edulis* (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). In the current study the positive influence was not replicated in the rate of gill development, which was instead inhibited at concentrations of 20mM and above. Assessment of metamorphosis in *P. maximus* has typically relied upon the presence of post-larval shell growth as an indication (Chevolot *et al*., 1991; Nicholas *et al*., 1998), however the differentiation of functional gill bars has also been used as an indication in pectinidae and other bivalves (Bonar *et al*., 1990; Fritt *et al*., 1990; Kingzett *et al*., 1990; Martinez *et al*., 1999). In addition to shell growth, gill formation represents a clear, distinctive change involving the degeneration of the velum and the development of ciliated gill buds which become the feeding organ of the animal (Gruffydd and Beaumont, 1972; Gosling 2003; García-Lavandeira *et al*., 2005). Importantly prolonged exposure to excess K^+ at concentrations of 50mM and above are known to be toxic to some bivalve larvae (Martinez *et al*., 1999; Zhao *et al*., 2003; Yu *et al*., 2008). In the current study any positive influence must be offset against a drop in survival of approximately 33% at an effective induction concentration (20mM), with concentrations of 20mM

and above proving toxic to some degree towards larvae. Whilst showing promise further testing is required to establish the full potential of KCl as an inductive agent for use in aquaculture.

Previous work has shown ammonium (NH_4^+), to be an effective inducer of settlement and metamorphosis in a number of marine invertebrates, including the bivalves *Patinopecten yessoensis* (Kingzett *et al*., 1990), *Pinctada fucata martensii* (Yu *et al*., 2008), *M. galloprovincialis* (Yang *et al*., 2008) and *M. coruscus* (Yang *et al*. 2013). In particular it was found to induce almost 80% metamorphosis in *M. galloprovincialis* compared to 0% in the control following exposure to 10^{-2}M for 24 hours (Yang *et al*., 2008). However, its influence appears species-specific, as no inductive influence was reported in the oyster *P. maxima* (Zhao *et al*., 2003) or the gastropod *C. fornicata* (Pechenik and Heyman, 1987). In the present study, the first known test with this compound on *P. maximus*, NH_4^+ showed no positive influence after 48 hours or by 1 week. Concentrations of 10^{-3}M and above were clearly detrimental towards both larval development and survival, although this was not necessarily evident until after 7 days. The mode of action of NH_4^+ remains unclear although is thought to be related to intracellular alkalisation (Coon *et al*., 1990). In a process driven by environmental pH, it is known to increase the intracellular pH by penetrating the cell membrane and disassociating to the weak base ammonia (NH_3) +H^+ ions, which cycles across the membrane until an equilibrium is reached (Coon *et al*., 1990; Marcaggi and Coles, 2001). It is unclear whether it is NH_4^+ or NH_3 which is the active element, however Berking (1988) found that the uptake of NH_4^+ was required to trigger metamorphosis rather than NH_3 in the hydroid *Hydractinia.* Ultimately if induction is influenced by intracellular alkalisation, low culture pH may inhibit this process with insufficient NH_3 formed from NH_4^+ for induction (Yu *et al*., 2008). Berking (1988) found that at a pH of 7.7 a higher concentration of NH_4Cl was required than at a pH of 8.2. Future studies must take this into account. Notably both NH_4^+ and NH_3 play an important role in aquatic toxicity (Boardman *et al*., 2004). The toxicity of NH_4Cl to bivalves has been demonstrated to increase with concentration and length of exposure (Zhao *et al*., 2003; Yu *et al*., 2008) and it is feasible that the concentrations tested in the present study may be too high for larvae to endure, becoming inhibitory as a consequence of disturbing normal biochemical functions. This is also likely linked to the length of exposure, since Kingzett *et al*. (1990) determined that just 30 minutes exposure could be effective, whilst Yang *et al*. (2008) showed that development was induced after exposure for 24 hours but impeded if exposed for longer. *P. maximus* may benefit from a reduced length of exposure, which may in turn reduce toxicity, thereby allowing potential induction.

Acetylcholine is known to act as a neurotransmitter in both vertebrates and invertebrates and has been shown to induce settlement and metamorphosis in marine bivalves (Beiras and Widdows, 1995; Dobretsov and Qian, 2003; Zhao *et al*., 2003; Yu *et al.*, 2008). This includes *C. gigas,* in which Beiras and Widdows (1995) found that larval metamorphosis increased to 27% after treatment at a concentration of 10^{-4}M, with 100% of post-larvae cementing themselves to the culture vessels, contrasting to no metamorphosis or attachment in untreated controls. However, larval response to this chemical is also known to be inconsistent (Coon *et al*., 1985), and has previously failed to induce a response in other molluscan groups including the nudibranch *Phestilla sibogae* (Hadfield, 1978), and the gastropods *Haliotis rufescens* (Morse *et al*., 1979) and *H. diversicolor supertexta* (Yu *et al*., 2010). In the present study ACH showed no inductive effects on larval metamorphosis for *P. maximus* after 48 hours or within 7 days, however the degree of negative influence was visibly greater after 7 days. Hadfield (1978) demonstrated in *P. sibogae* that it is the choline moiety that is the critical active element for induction and that susceptibility to different choline derivatives varies. At concentrations equivalent to 0.1% of test solution choline chloride induced high rates of metamorphosis (60-85%), almost equal the derivative succinylcholine chloride (70-90%), whilst methacholine (acetyl-β-methylcholine) induced variable low rates of metamorphosis (10-40%), and acetylcholine failed to induce a response. Similarly in the eastern mudsnail, *Ilyanassa obsoleta*, choline, acetylcholine and succinylcholine induced limited metamorphosis, while methacholine induced 80-100% (Levantine and Bonar, 1986). Further investigation of alternative choline derivatives may therefore prove beneficial.

GABA has proven effective at inducing larval settlement, metamorphosis or both in a number of bivalves, including the oysters *P. margaritifera* (Doroudi and Southgate, 2002), *P. maxima* (Zhoa *et al*., 2003), *P. fucata martensii* (Yu *et al*., 2008), and *Ostrea edulis,* the mussel *M. galloprovincialis*, the clams *Venerupis pullastra* and *Ruditapes philippinarum* (García-Lavandeira *et al*., 2005), and the scallop *Chlamys varia* (Mesías-Gansbiller *et al*., 2008). However, effectiveness is influenced by concentration, length of exposure and by variations in larval sensitivity (Doroudi and Southgate, 2002; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005; Yu *et al*., 2008). Effective concentrations range from 10^{-3} to 10^{-6}M over exposure for 24 to 48 hours, although short exposure periods of 1-2 have also proven effective (Doroudi and Southgate, 2002; García-Lavandeira *et al*., 2005; Yu *et al*., 2008). Induction is reduced outside optimum concentrations. GABA has however, proven ineffective at inducing a response in the bivalves *M. edulis* (Cooper, 1982; Eyster and Pechenik, 1987; Dobretsov and Qian, 2003) and *C. gigas* (Coon *et al*., 1985), and provided inconsistent results in the nudibranch *P. sibogae* (Hadfield, 1984). This study is the first instance of GABA being tested

with *P. maximus*, however at present it can be added to the list of those currently showing no inductive influence. Only a concentration of 10^{-6}M exhibited development and survival rates equal to the control after 1 week. All other treatments proved detrimental to development and toxic to larvae. Previous studies have demonstrated the toxicity of this chemical, particularly at high concentrations (10^{-2} and 10^{-3} M) and over long exposure periods, reducing settlement and causing high rates of mortality (Doroudi and Southgate, 2002; Yu *et al*., 2008). Our results show that GABA was toxic in *P. maximus* at concentrations ≥10^{-5}M, but this was only apparent after 7 days.

L-DOPA has been proven effective at inducing metamorphosis in a number of bivalves including *C. gigas* (Coon *et al*., 1985; Nicholas *et al*., 1998), *M. edulis* (Cooper, 1982), *P. yessoensis* (Kingzett *et al*., 1990), as well as *P. maximus* (Chevolot *et al*., 1991; Nicolas *et al*., 1998), although larval response is concentration and exposure dependent. Induction in previous studies has been low in some cases, and in both *C. gigas* and *P. maximus* L-DOPA has proven less effective than the catecholamine epinephrine (Coon *et al*., 1985; Kingzett *et al*., 1990; Chevolot *et al*., 1991; Nicolas *et al*., 1998). In *P. maximus,* previous studies have shown L-DOPA to induce higher rates of metamorphosis than in untreated larvae at concentrations of 0.51 and 2.54 x 10^{-5}M over exposures of 24 to 48 hours, with optimum dissoconch growth ranging from just 6 to 12% by 1 week (Chevolot *et al*., 1991; Nicolas *et al*., 1998). In contrast, the results of the present study showed no increase in dissoconch growth at any of the tested concentrations after 7 days. In fact dissoconch growth was lower at concentrations of 10^{-4}M and above compared to the control. The results did however show that gill development was more rapid and survival higher at a concentration of 10^{-6}M. Previously higher concentrations of 5.07 x 10^{-5}M have been found to reduce larval metamorphosis in *P. maximus* (Chevolot *et al*., 1991; Nicolas *et al*., 1998). The results of this study clearly demonstrate that L-DOPA at 10^{-4}M and above is highly toxic, reducing survival, which is in line with studies on other species of bivalve (Coon *et al*., 1985; Zhoa *et al*., 2003; Yu *et al*., 2008). Although the site of action of L-DOPA remains unclear, our results suggest the pathway influenced by L-DOPA maybe morphologically distinct to that of excess K^+ in *P. maximus.* Although both were effective at inducing a larval response, L-DOPA induced increased gill development compared to increased dissoconch growth induced by KCl. Further investigation is however required to confirm this theory.

Epinephrine has proven to be widely effective at inducing larval metamorphosis in bivalves, including the oysters *C. gigas* (Coon *et al*., 1985; Coon *et al*., 1986; Nicholas *et al*., 1998), *Crassostrea virginica* (Coon *et al*., 1986), *Crassostrea iredalei* (Teh *et al*., 2012) and *O. edulis* (García-Lavandeira *et al*., 2005), the mussel *M. galloprovincialis* (García-Lavandeira *et al*., 2005; Yang *et al*., 2008), and the

clams *V. pullastra* and *R. philippinarum* (García-Lavandeira *et al*., 2005; Sumin *et al*., 2006). It has also proven effective at inducing settlement in *M. galloprovincialis, V. pullastra, O. edulis* (García-Lavandeira *et al*., 2005), *C. varia* (Mesías-Gansbiller *et al*., 2008) and *A. purpuratus* (Martinez *et al*., 1999) although the level of response is typically below that seen for metamorphosis (Nicholas *et al*., 1998; García-Lavandeira *et al*., 2005). Metamorphic response is species-specific and is influenced by concentration and length of exposure, with effective concentrations ranging from 10^{-6}M to 10^{-4}M, over exposure periods from 1 to 48 hours. Low concentrations can fail to induce a response, whilst high concentrations can inhibit a significant response (Coon *et al*., 1986; Nicolas *et al*., 1998; Martinez *et al*., 1999; García-Lavandeira *et al*., 2005). In the past it has been postulated that the action of epinephrine is mediated by receptors similar to vertebrate-type $alpha_1$-adrenergic receptors, and that these receptors may influence a pathway that by-passes the induction of settlement (Coon *et al*., 1986; Bonar *et al*., 1990). In *C. gigas* and *C. virginica* application of epinephrine has consistently resulted in the development of "cultchless" spat, with larvae rapidly metamorphosing without first attaching to a surface (Coon *et al*., 1985; Coon *et al*., 1986; Bonar *et al*., 1990; Nicolas *et al*., 1998). However, in species of pectinidae, including *A. purpuratus* (Martinez *et al*., 1999), *P. maximus* (Nicolas *et al*., 1996; Nicolas *et al*., 1998) and *C. varia* (Mesías-Gansbiller *et al*., 2008) epinephrine has been shown to induce significant attachment, indicating a potentially alternative route of action in scallops. In *P. maximus* previous studies have shown that epinephrine is effective at inducing metamorphosis of larvae at concentrations between 0.55 to 5.46 x 10^{-5}M following exposure for 24 to 48 hour, with subsequent development determined 5 to 6 days post-treatment (Chevolot *et al*., 1991; Nicolas *et al*., 1996; Nicolas *et al*., 1998). However, it has also proven to be toxic at high concentrations, and less than 2.73 x 10^{-5}M over an exposure of 24 hours has been advocated to prevent excessive mortality (Nicolas *et al*., 1998). Epinephrine can produce a response following short exposure for 1 to 2 hours in some species, thereby maximising induction whilst minimising larval mortality (Coon *et al*., 1986; Nicolas *et al*., 1998; Teh *et al*., 2012), although in *P. maximus,* at 0.55 x 10^{-5}M, optimum induction was achieved at 24 hours (Nicolas *et al*., 1998). Maximum induction in *P. maximus* was recorded by Nicholas *et al*. (1998) at a rate of 32% at 5.46 x 10^{-5}M, after exposure for 24 hours, with all larvae being attached, compared to a control showing just 1% metamorphosis. However, effective concentration level and rate of metamorphosis is known to vary with larval batch, since Nicholas *et al*. (1998) found optimal concentrations could vary between 0.55, 1.09 and 5.46 x 10^{-5}M with metamorphic rates of 12% (control 2%), 18% (29% control) and 32% (1% control) respectively. In separate studies Nicolas *et al*. (1996) recorded induction rates of 18% at 2.73 to 5.46 x 10^{-5}M, whilst Chevolot *et al*. (1991) showed that optimum induction of 13% was achieved at 0.55 x 10^{-5}M. The lower induction in the earlier studies may be a consequence of a

longer exposure period (48 hours). The response to epinephrine has also been found to vary between studies in other species including *M. galloprovincialis* and *R. philippinarum* (García-Lavandeira *et al*., 2005; Sumin *et al*., 2006; Yang *et al*. 2008) indicating susceptibility fluctuates from larval group to larval group. It is recognised that there is inherent variability in the sensitivity and threshold of responses within and between larval batches to chemical inducers, and care must be taken with interpreting data arising from single or very small larval groups (Bonar *et al*., 1990). The results of the present study must be treated with caution, due to the clear influence of a secondary factor, HCl. As epinephrine does not readily dissolve in seawater, it is typically dissolved in dilute HCl, usually 0.005N HCl, which is then diluted into larval cultures to give a solution concentration of 0.0005N HCl or less (Coon *et al*., 1986; García-Lavandeira *et al*., 2005). In many previous studies with species including *C. gigas* (Coon *et al*., 1986), *C. iredalei* (Teh *et al*., 2012), *C. varia* (Mesías-Gansbiller *et al*., 2008), *O. edulis, V. Pullastra, R. philippinarum* (García-Lavandeira *et al*., 2005), *M. galloprovincialis* (García-Lavandeira *et al*., 2005; Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013) no reported impact has been discussed or attributed to the inclusion of HCl in induction assays. In this study epinephrine was dissolved in 0.009N HCl to generate a stock solution, below which it would not dissolve. This lead to assay treatments with a higher HCl concentration, equivalent to 0.0009N HCl, and this may account for the obtained results. Our results show that compared to a control treatment containing no HCl, in which 16% of larvae developed functional gill structures, 3% showed dissoconch growth and survival was 72%, the inclusion of HCl alone lead to almost 100% larval mortality, and no meaningful development. In treatments with epinephrine, larval development was significantly reduced, both in terms of dissoconch growth and gill formation, with high toxicity, increasing with concentration. The degree of negative influence was visibly greater by 1 week. It is clear that the presence of HCl in this study was detrimental, irrespective of epinephrine inclusion. However, it is notable that dissoconch growth, gill formation and survival of larvae was improved at a concentration of 10^{-6}M compared to the HCl containing control, indicating a potential beneficial influence of the chemical, if the overriding negative influence of the HCl could be addressed. Alternatively ethanol has been used to dissolve compounds with low water solubility and used in assays with *P. maximus* without effect on larvae (Chevolot *et al*., 1991). Although untested in this study it is possible that the HCl caused a critical drop in culture pH, and therefore this may be alleviated by buffering the culture solution with a suitable alkali, although this theory requires verification. At present, it is impossible to accurately compare the results of the present study with other studies using epinephrine.

The influence of chemical cues cannot be considered in isolation to other factors. Several studies have demonstrated that bivalves settle or metamorphose more readily in the presence of seawater turbulence (Eyster and Pechenik, 1987; Nicolas *et al*., 1996; Nicolas *et al*., 1998). In *P. maximus* cultures in still water conditions and without chemical induction, the metamorphosis rate after 1 week rarely exceeds 5%, however turbulence alone has increased metamorphosis in *P. maximus* by as much as 16% (Chevolot *et al*., 1991; Nicolas *et al*., 1996; Nicolas *et al*., 1998). Additionally the provision of adequate nutrition, both in terms of quantity and quality, is also critical to larval development in *P. maximus* (Nicolas and Robert, 2001; Tremblay *et al*., 2007). Tremblay *et al*. (2007) determined that in *P. maximus* the composition of microalgae diets influenced the rate of metamorphosis, and that the incorporation of *Rhodomonas salina* or the replacement of *Chaetoceros gracilis* by *Skeletonema costatum* in traditional hatchery diets (*Isochrysis aff. galbana, P. lutheri and C. gracilis*) could lead to an improved metamorphosis rate. However, it is the combining of multiple factors which is possibly of the greatest interest. Previous studies have demonstrated a cumulative effect on settlement and metamorphosis by several factors, in addition to the use of combinations of chemical agents (Baxter and Morse, 1987). Nicolas *et al*. (1998) found that combining seawater turbulence with the chemical agent L-DOPA increased metamorphosis over each variable individually, although the reverse was true for the chemical epinephrine. Therefore there is potential to investigate the combination of stimuli in an effort to further improve induction.

In conclusion the results of the present study increase our knowledge of induced metamorphosis in the scallop *P. maximus*. This study provides a clear assessment of the impact of six chemical agents on metamorphosis and survival. KCl can be added to the list of effective agents, alongside L-DOPA, although their larval responses differed. In addition the benefit of KCl must be off-set against a drop in survival, unseen for L-DOPA. All other tested chemicals proved unsuccessful, although the results for epinephrine appear to be critically affected by the inclusions of HCl. However, it is also clear that the full impact of each chemical is not necessarily evident immediately after the conclusion of chemical exposure, with this only becoming clear after a further culture period. What is clear is that the induction of synchronous larval development in this species remains unachieved, with sensitivity to tested chemical agents low. Recommendations for application within a hatchery environment cannot therefore be made at this point. Further investigation of these and other chemical agents, as well as their method of application, is recommended to determine whether a sufficiently effective and economically viable solution to the long development period in *P. maximus* can be found. However, it requires consideration whether all chemical agents or just specific agents can or should be used within the cultivation of animals intended for human consumption.

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Chapter 3

Influence of exogenous chemicals on larval metamorphosis, survival and growth of the blue mussel *Mytilus edulis* (L.)

Abstract

The commercially important blue mussel, *Mytilus edulis*, is the focus of on-going research aimed at reducing the reliance on natural sources of seed animals and improving the economic viability of future hatchery operations. This study examined the use of the exogenously applied chemical agents L-DOPA, epinephrine, KCl and NH_4Cl as a means of inducing larval metamorphosis, and assessed their influence on survival and growth. A range of concentrations for each chemical were tested over exposure periods of 24, 48 and 72 hours. However, larval response to these chemicals proved to be limited, as none of the assessed chemicals induced a significant lasting improvement to metamorphosis rate compared to the untreated control. High concentrations generally proved to be toxic and led to lower levels of development. Excess K^+ did however induce a slight improvement in larval growth at concentrations of $1.3x10^{-3}$M and $1.3x10^{-2}$M after exposure for 24 and 48 hours. The ability to synchronise metamorphosis in *M. edulis* using a chemical agent remains to be realised. However the results of the present study provide additional information on the role of chemicals in this species and the genus as a whole. Further research of alternative agents is recommended.

Keywords

Mytilus edulis, Larvae, Chemical, Induction, Metamorphosis, Survival, Growth

Abbreviations

ANOVA	Analysis of variance
HCl	Hydrochloric acid
KCl	Potassium chloride
L-DOPA	3,4-dihydroxyphenylalanine
NH_4Cl	Ammonium chloride

Introduction

The behavioural and morphological responses of a range of marine invertebrate species to exogenously applied chemical agents has provided insights into the complex biochemical mechanisms involved in settlement and metamorphosis (Baloun and Morse, 1984; Hadfield, 1984; Coon *et al*., 1985; Yool *et al*., 1986; Baxter and Morse, 1987; Pawlik, 1990). Whilst ecologically significant, this has also led to important advances in the culture of commercially important species, including addressing the endogenous timing of metamorphosis in many marine bivalves. Metamorphosis is a challenging phase during bivalve culture, as larvae undergo a progression of permanent morphological changes, including development of a foot organ, disintegration of the velum, formation of gill structures, and reorientation of the mantle cavity (Lutz and Kennish, 1992; Gosling, 2003). Metamorphosis is only initiated once larvae reach a sufficient developmental stage and have become "competent" to undertake the transition between the larval and adult life stages, and only then if stimulated by exogenous cues originating from the environment (Bayne 1965; Hadfield, 1978; Fitt *et al*., 1990). However, individual larvae vary in growth rate, even within identical conditions, and consequently some larvae reach metamorphosis sooner than others (Loosanoff and Davis 1963; Bayne 1965). Furthermore metamorphosis is often associated with protracted development periods, low larval synchronicity (Martinez *et al*., 1999; Robert and Nicholas, 2000; García-Lavandeira *et al*., 2005), delayed metamorphosis (Bayne 1965; Coon *et al*., 1990a) and variable survival (Martinez *et al*., 1999; Nicholas *et al*., 1998).

The use of chemical agents has proven to be a successful means of inducing metamorphosis, as well as settlement, in bivalves including the oysters *Crassostrea gigas* (Coon *et al*., 1985), *Crassostrea iredalei* (Teh *et al*., 2012), *Crassostrea virginica* (Coon *et al*., 1986) and *Ostrea edulis* (García-Lavandeira *et al*., 2005), the scallops *Argopecten purpuratus* (Martinez *et al*., 1999) and *Pecten maximus* (Nicolas *et al*., 1998), and the clams *Venerupis pullastra* and *Ruditapes philippinarum* (García-Lavandeira *et al*., 2005) Chemical agents are seen as an effective and routine way of increasing larval development and synchronicity (Coon *et al*., 1985; García-Lavandeira *et al*., 2005). However, effectiveness is influenced by concentration, length of exposure and by variations in species-sensitivity to different chemicals (Coon *et al*., 1986; Nicholas *et al*., 1998; Martinez *et al*., 1999; García-Lavandeira *et al*., 2005; Yu *et al*., 2008); therefore significant testing of potential inducers for each species is essential.

The blue mussel *Mytilus edulis* is a commercially important bivalve in Europe, with production reaching 196,665 tons in 2011 (FAO, 2014). However production remains dependent on natural sources of seed for on-growing. This means the industry risks limited production yields if spat falls are low, and places constraints on future expansion to meet growing demand (de Vooys 1999; Kamermans and Smaal 2002). The obvious solution is the development of hatchery sources of seed (Utting and Spencer 1991; Spencer 2002). This can allow producers to take advantage of associated benefits such as broodstock domestication, artificial selection for better growth and polyploidy manipulation (Utting and Spencer 1991; Brake *et al*., 2001; Spencer 2002; Beaumont and Hoare 2003). Culture protocols for *M. edulis* are well established (Loosanoff and Davis 1963; Utting and Spencer 1991; Helm and Bourne 2004; Galley *et al*., 2010). However, hatchery production of *M. edulis* has been considered to be economically unfeasible, since production is based upon a low unit value (Brake *et al*., 2001). Hence, only a single hatchery in Europe, in the Netherlands, produces limited quantities for experimental use (P. Kamermans personal communication). Globally Mytilidae mussels have been cultured in hatcheries, with *M. edulis* one of three mussel species to have been cultured in China (Zhang, 1984), whilst hatchery culture has been undertaken for M*ytilus galloprovincialis* in North America (Brake *et al*., 2001; Penn Cove Shellfish, 2014) and Australia (Jahangard *et al*., 2010). In these cases, hatchery production proved to be more economical viable than relying on unreliable, limited and distant wild-sources. In Europe increasing demand and reliance on unpredictable natural supplies, necessitates the continued effort to develop improved culture practices, which may in turn make the culture of *M. edulis* a more economically attractive prospect.

Currently there is a lack of information on the influence of exogenous chemicals on metamorphosis in *M. edulis*. This species is known to display variable growth rates, and delay settlement and metamorphosis if conditions, based on temperature, salinity, pH and substrate availability, are unsuitable (Loosanoff and Davis 1963; Bayne 1965; Eyster and Pechenik, 1987). Previous studies in *M. edulis* have assessed a handful of chemicals over concentrations ranging from 5 to 40mM for KCl and 10^{-2}M to 10^{-7}M for other chemicals over exposure periods of 24 to 48 hours (Cooper, 1982; Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). However, these studies were carried over short time periods, concluding after 48 to 72 hours, and were primarily focused on the settlement response of larvae (Cooper, 1982; Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). These showed that the catecholamine precursor 3,4-Dihydroxy-L-phenylalanine (L-DOPA), the xanthine derivative 3-isobutyl-1-methylxanthine (IBMX) and acetylcholine chloride all stimulate increased settlement at concentrations of 10^{-5}M, 10^{-6} to 10^{-4}M, and 10^{-7} to 10^{-5}M respectively. Whilst KCl and

γ-aminobutyric acid (GABA) failed to induce a settlement or metamorphic response within the study periods. Only Cooper (1982) has so far reported an increase in metamorphosis in *M. edulis* using the chemical L-DOPA at concentrations of 10^{-5}M and 10^{-6}M or extracts of the algae *Platythamnion villosum*. Notably however, the short time-scales employed in previous studies with *M. edulis* may not have been sufficiently long enough to characterise the full impact, either positive or negative, of using the different chemical agents. Responses to chemical agents, particularly metamorphosis, can take several days post-treatment to emerge (Yang *et al*., 2008; Yang *et al*., 2013). Furthermore several previous studies with *M. edulis* have relied upon the assessment of small numbers of larvae (≤20) (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003), as have assays in other Mytilidae species (Yang *et al*., 2008; Yang *et al*., 2013), however this may not be large enough to accurately determine the full effect of the chemical on the species due to the inherent variability within, as well as between, larval batches (Bonar et al., 1990). A number of chemical agents have however proven effective at inducing metamorphosis in related Mytilidae and may prove beneficial in *M. edulis*. These include epinephrine, methoxyphenamine, clonidine, serotonin, excess K^+, NH_4^+, organic solvents including methanol, ethanol and acetonitrile among others (García-Lavandeira *et al*., 2005; Yang *et al*., 2008; Yang *et al*., 2013). Further investigation is therefore essential to assess the potential of chemical agents as a means of optimising culture practices in *M. edulis*.

This study evaluated the effect of the previously untested chemicals, epinephrine and ammonium chloride (NH_4Cl), together with the influence of concentration and exposure period, on the metamorphosis, survival and growth of larval *M. edulis* over a study period of 1 week. The chemicals, L-DOPA and potassium chloride (KCl), were reassessed over an extended treatment range and study period compared to previous studies (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). The aim of the present study was to identify chemical agents with the potential for use in the culture of *M. edulis*, as well as increasing our understanding of the biochemical mechanisms controlling its development.

Materials and Methods

Broodstock, spawning and fertilisation

Broodstock adult *M. edulis* mussels were collected in the winter (October to December) of 2010 and 2011 from natural populations in the Menai Strait, North Wales, UK. To aid reproductive maturation these were held sub-tidally, suspended in mesh sacks from a floating raft until March the following year. Mussels were then transferred to the laboratory, where they were cleaned of all encrusting epifauna and held in unfiltered flow-through seawater tanks at 6±1˚C until required for spawning (Galley *et al.* 2010). Mussels were supplemented with a mixed microalgae diet *ad libitum*. Prior to spawning, mussels were exposed to air for up to 21 hours at 5±1˚C. Spawning was induced by thermal shock treatment (Utting and Spencer 1991); mussels were immersed in 1µm filtered, UV-light irradiated seawater (FSW) at a salinity of 33‰, in individual 90ml plastic cups. Cups were transferred between temperatures of approximately 9±1˚C and 24±1˚C at 30-60 minute intervals. Fertilisation was accomplished as described by Beaumont *et al.* (2004) at a ratio of between 200-300 sperm egg^{-1}. Once polar bodies were detected (Figure 3.1), the activated egg suspensions were distributed into flat-bottomed trays and held undisturbed at 16±1˚C for 48 to 72 hours through the trochophore stage to the early veliger stage (Figure 3.2).

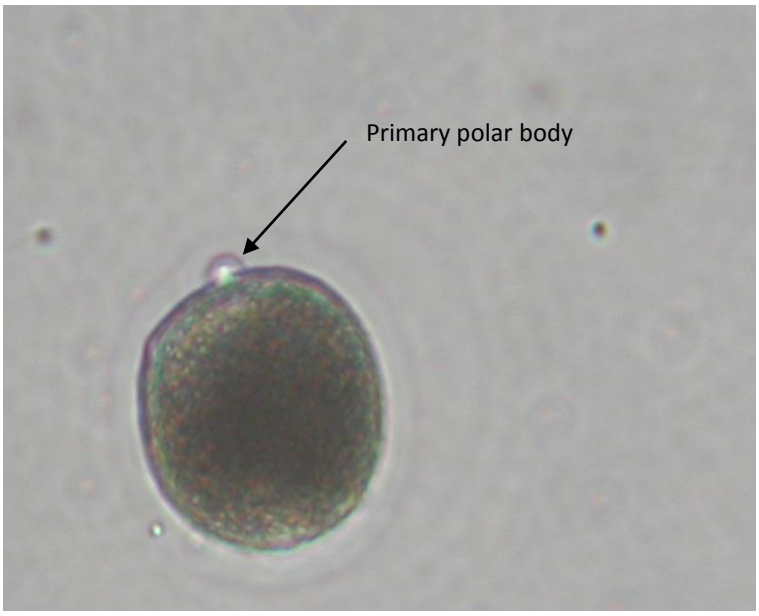

Figure 3.1: Fertilised *Mytilus edulis* egg with primary polar body.

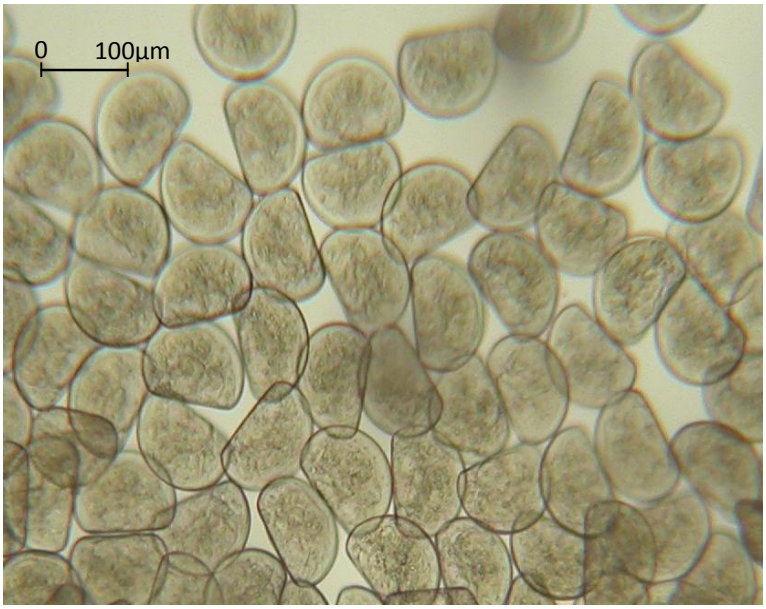

Figure 3.2: *Mytilus edulis* veliger larvae at 72 hours post-fertilisation.

Larval culture

Veliger larvae, with initial mean shell length ranging from 110.7±4.6µm to 115.3±5.2µm between larval batches, were stocked at a density of 10 larvae ml^{-1} in 65-litre polyethylene tanks filled to a volume of between 12 and 50-litres with FSW. Cultures were maintained at a temperature of 16±1˚C. Three times a week the larvae were sieved onto a 45 µm mesh screen and inspected, the containers cleaned, the FSW replaced and the larvae fed. Larvae were fed a mixed microalgae diet equivalent to a cell concentration of $3.75x10^4$ cells ml^{-1} day^{-1} of *Pavlova lutheri* (PLY75), *Isochrysis* sp. (clone T-ISO) (PLY506A) and *Chaetoceros calcitrans* (PLY537), at a ratio of 1:1:1. Veligers were reared until they reached competence to metamorphose. Competence of larval cultures to undertake metamorphosis was assessed based upon the development of pigmented eye-spots (Figure 3.3). Experiments were initiated with cultures once the eye-spot ratio reached approximately 35-50%, between 25 and 34 days post-fertilisation. The presence of early-stage, non-functional, gill structures was also noted (Figure 3.3).

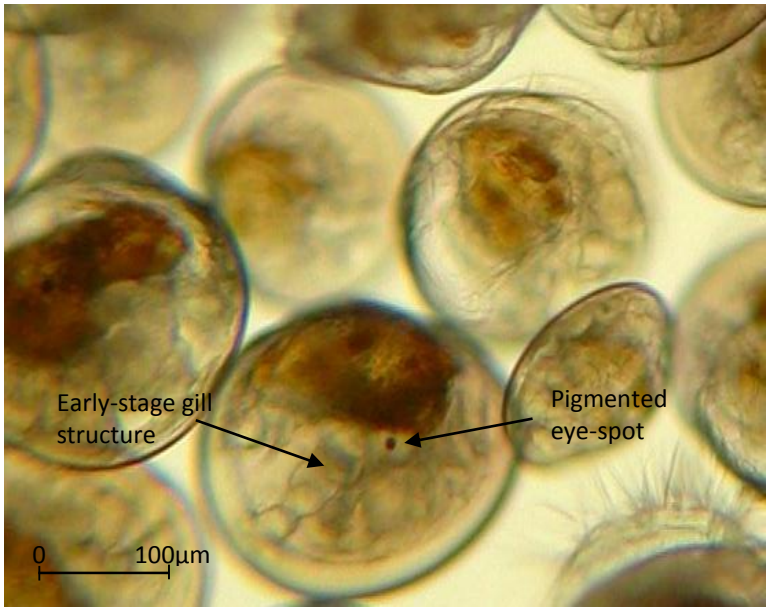

Figure 3.3: Veliger *Mytilus edulis* larvae with pigmented eye-spot and non-functional early-stage gill structure.

Chemical agents

All chemicals, potassium chloride (KCl), ammonium chloride (NH_4Cl), epinephrine and 3,4-Dihydroxy-L-phenylalanine (L-DOPA), were obtained from Sigma-Aldrich (Poole, UK). Immediately prior to use concentrated 1 molar stock solutions of KCl and NH_4Cl, and a 10^{-2}M stock solution of L-DOPA were prepared by dissolving the chemicals in FSW. A 10^{-2}M stock solution of epinephrine was prepared by dissolving in a solution of FSW containing 0.009N hydrochloric acid (HCl). Stock solutions were diluted into the FSW containing the larvae to achieve the required test concentrations. KCl was assayed at concentrations of 1.3, 13.4, 26.8 and 40.2×10^{-3}M; NH_4Cl at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}M; whilst epinephrine and L-DOPA were both assayed at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}M. For the experiment using epinephrine the concentration of HCl was adjusted to be equal in each treatment, including the control treatment. This equated to 0.0009N HCl in each replicate.

Chemical assays

To investigate the influence of potential chemical compounds on survival, metamorphosis and growth, experiments were carried out in 1-liter Pyrex-glass beakers containing up to 500ml of static FSW, stocked at a density of 10 larvae ml^{-1} and maintained at 16±1˚C (Figure 3.4). Each chemical assay was undertaken with a separate batch of larvae.

Prior to the chemical assays, in addition to determining metamorphic competence, assessment of larval size (µm), growth rate (µm day^{-1}) and percentage survival to the point of competence, was determined to provide additional reference indicators for the use of this species of bivalve. Initial veliger size and size at competence were based upon the shell length (µm) of approximately 30 larvae, with measurements made from digital analysis of photomicrographs using the image analysis software Image J. The difference between initial size and size at competence was used to estimate the growth of larvae. Survival prior to assays was estimated by comparing the initial stocked number of larvae and final number using replicate sub-sample counts. This showed that at the point of competence the mean shell length (µm) of larvae used for the L-DOPA assay was 227.5±32.8µm, with a mean veliger growth rate of 5.0µm day^{-1}. The mean shell length (µm) of larvae used for the epinephrine assay was 222.5±26.8µm, with a mean growth rate of 4.9µm day^{-1}. The mean shell length (µm) of larvae used for the KCl assay was 223.2±21.2µm, with a mean growth rate of 4.5µm day^{-1}. The mean shell length (µm) of larvae used for the NH_4Cl assay was 227.6±28.4µm, with a mean growth rate of 3.8µm day^{-1}. The slower growth seen in the larvae used of the NH_4Cl is due to a drop in culture temperature to 12±1°C over a 14 day period prior to competence. Survival rates in larval cultures from stocking were 88.0%, 97.4%, 82.0% and 87.2% for larvae to be used in the L-DOPA, epinephrine, KCl and NH_4Cl assays respectively. Cultures with greater larvae mortality where not used in assays. Larvae were considered to be suitable for use in assays with high survival and growth rates approximating those previously documented for *M. edulis* by Galley *et al.* (2010) of 2.7 to 8.0µm day^{-1}, under comparable conditions.

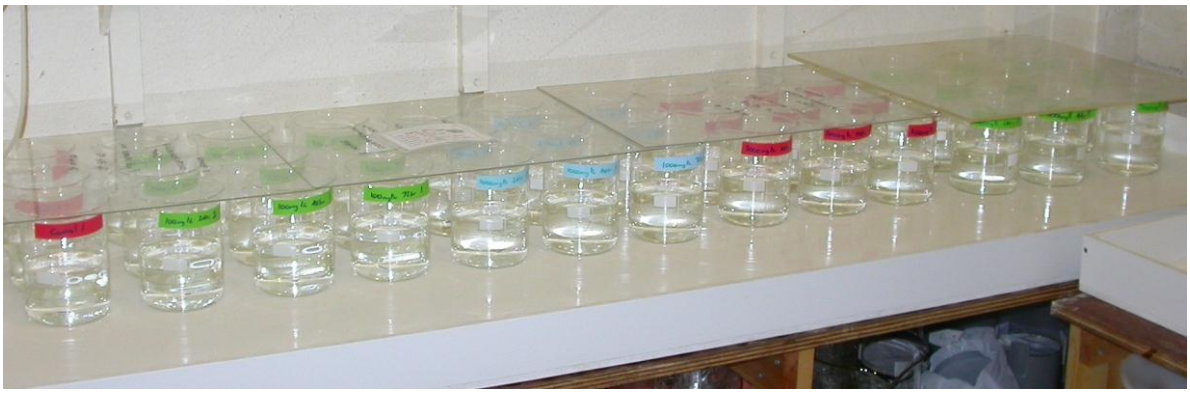

Figure 3.4: Chemical assay cultures of *Mytilus edulis* larvae comparing a range of potassium chloride (KCl) concentrations and exposure periods on metamorphosis, survival and growth.

During each assay chemicals were tested over three continuous exposure periods, 24, 48 and 72 hours, after which larvae were thoroughly rinsed on a 45µm mesh screen to remove residual chemicals and restocked in clean beakers of FSW. Further water changes were conducted every 2-3 days for all treatments. During the experiments larvae were fed with a mixed microalgae diet as

described for larval rearing. Separate batches of larvae were used for each chemical assay. All treatments were conducted in triplicate, including an untreated control group. All assays were conducted in the light, with the exception of epinephrine which was conducted in the dark during the initial treatment period to alleviate its rapid photo-oxidation.

Triplicate sub-samples were taken from each replicate after 72 hours and after a total experimental period of 7 days (5 days post-treatment). Immediately prior to sampling any attached post-larvae were dislodged by gentle agitation with jets of water in order to suspend all animals within the water column. The number of metamorphosed post-larvae was assessed utilising a Leica DM E binocular microscope, and the total remaining number of live individuals counted to determine survival. Percentage metamorphosis and percentage survival were both calculated relative to the initial stocking density. Larvae were considered to have metamorphosed if they possessed functional gill filaments (Figure 3.5). Growth under different treatments was assessed based upon final shell length (µm) of larvae of approximately 30 animals, taken from each replicate at the conclusion of each assay, and measured using Image J.

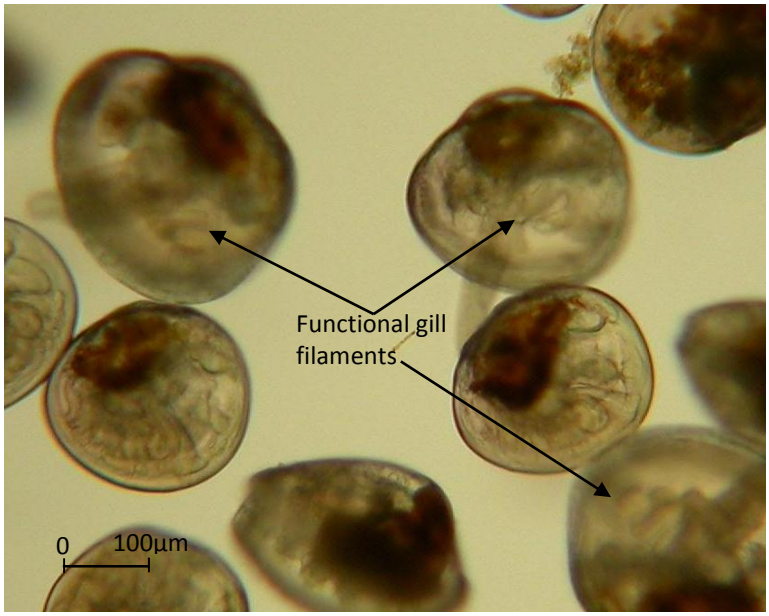

Figure 3.5: Post-larval *Mytilus edulis* possessing functional gill filaments.

Statistical analyses

All data sets are described as the percentage of metamorphosed larvae and percentage larval survival, whilst shell length is expressed in micrometres (µm). Before analysing, all percentage data sets were converted by arcsine square root transformation. The data presented in all figures is untransformed. Mean data sets were tested using the Anderson-Darling test to investigate

departure from normality and Bartlett's test to assess heteroscedasticity before applying any test of comparison (Sokal and Rohlf, 1995). ANOVA tests were used to determine if there was any significant difference among treatments, followed by pairwise comparisons using Fishers Least Significant Difference test (LSD). All results were considered to be significantly different when $P<0.05$. Analyses of data were undertaken using the statistical package Minitab®.

Results

Influence of L-3,4-dihydroxyphenylalanine

In response to testing a range of L-DOPA concentrations over exposure periods of 24, 48 and 72 hours Figure 3.6 and Figure 3.7 show the mean percentage of metamorphosed and surviving mussel larvae respectively, after culture periods of 72 hours and 1 week. Figure 3.8 shows the mean shell length (µm) of animals after 1 week. At the beginning of the trial 35.2±6.9% of larvae possessed eye-spots, with 7.1±4.3% showing some signs of early gill formation.

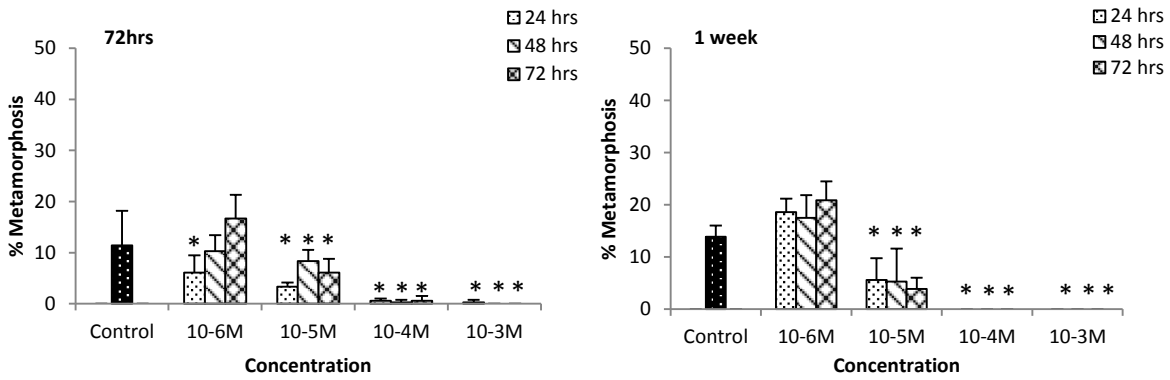

Figure 3.6: Mean percentage metamorphosis after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to L-DOPA at concentrations of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control ($P<0.05$).

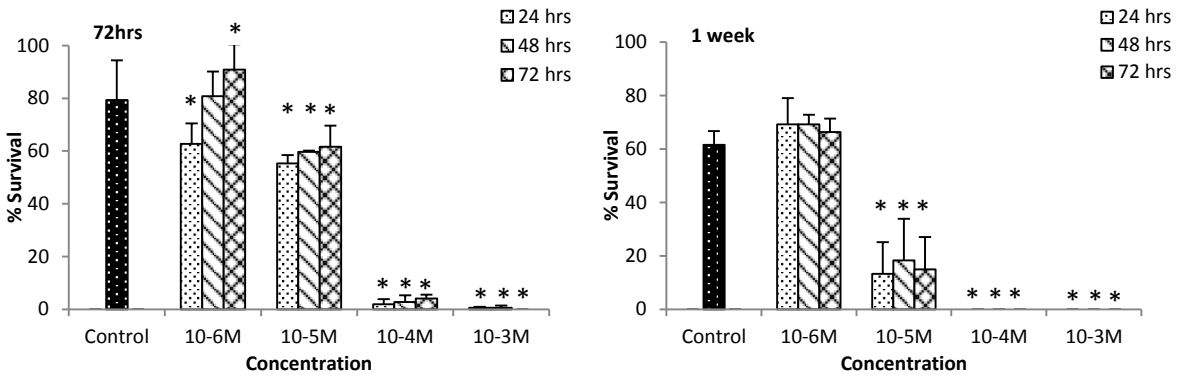

Figure 3.7: Mean percentage survival after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to L-DOPA at concentrations of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control (P<0.05).

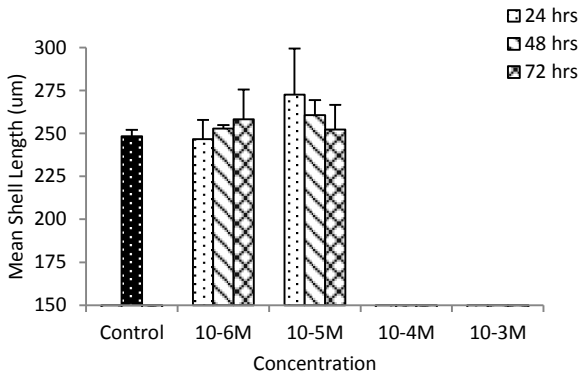

Figure 3.8: Mean shell length (µm) of *Mytilus edulis* larvae 1 week after commencement of exposure to a range of L-DOPA concentrations over exposure periods of 24, 48 and 72 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control (P<0.05).

L-DOPA treatments did not significantly improve metamorphosis, survival or growth of mussel larvae compared to the untreated control. Maximum metamorphosis was achieved at both 72 hours (16.7±4.6%) and after 1 week (20.8±3.6%) in the 10^{-6}M concentration over a 72 hour exposure period, however in neither case was the improvement over the control significant (Fisher's P>0.05). Metamorphosis was significantly lower than in the control (Fisher's P>0.05) at concentrations of 10^{-5}M and above, irrespective of exposure period by both 72 hours and 1 week (Figure 3.6). Concentrations of 10^{-5}M and above, irrespective of exposure period, proved toxic to larvae with survival significantly below that of the control (Fisher's P>0.05), particularly at 10^{-4}M and 10^{-3}M, at which few larvae survived by 72 hours and none after 1 week (Figure 3.7). A positive impact on survival was observed by 72 hours at a concentration of 10^{-6}M over an exposure of 72 hours, however by 1 week this advantage had disappeared. The mean size of larvae in several L-DOPA

treatments was greater than in the control after 1 week, though the difference was not significant (Figure 3.8).

Influence of epinephrine

At the beginning of the assay testing epinephrine, 38.6±3.2% of larvae possessed eye-spots, with 12.4±4.8% showing some signs of early gill formation. In response to testing a range of epinephrine concentrations over exposure periods of 24, 48 and 72 hours Figure 3.9 and Figure 3.10 show the mean percentage of metamorphosed and surviving mussel larvae respectively, after culture periods of 72 hours and 1 week. Figure 3.11 shows the mean shell length (µm) of animals after 1 week.

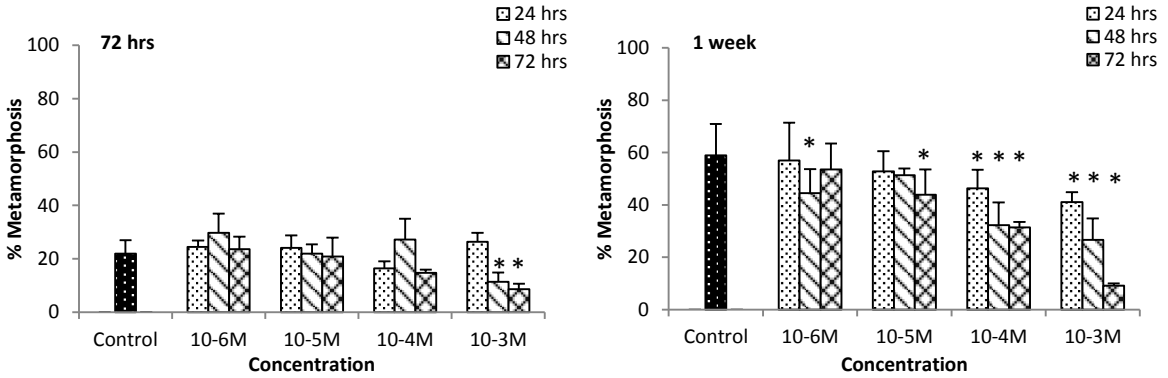

Figure 3.9: Mean percentage metamorphosis after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to epinephrine at concentrations of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly lower than the control (P<0.05).

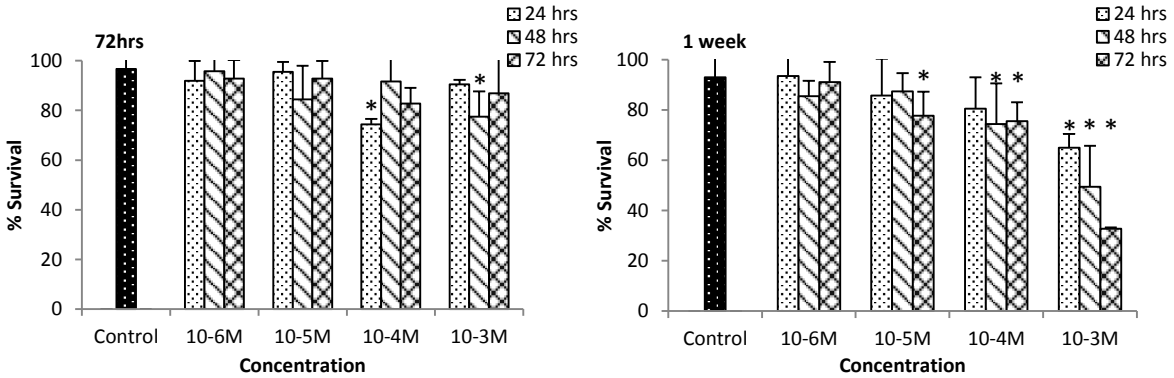

Figure 3.10: Mean percentage survival after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to epinephrine at concentrations of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly lower than the control (P<0.05).

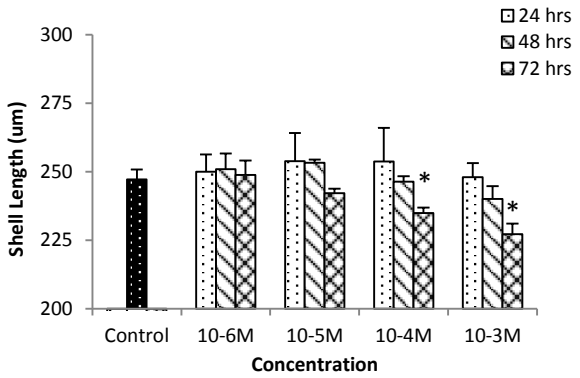

Figure 3.11: Mean shell length (µm) of *Mytilus edulis* larvae 1 week after commencement of exposure to a range of epinephrine concentrations over exposure periods of 24, 48 and 72 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control ($P<0.05$).

Epinephrine treatments did not significantly improve metamorphosis, survival or growth of mussel larvae compared to the untreated control. Analysis showed that by 72 hours only a concentration of 10^{-3}M over exposures of 48 and 72 hours suffered significantly reduced metamorphosis (Fisher's $P<0.05$) (Figure 3.9), and survival was generally unaffected by this point (Figure 3.10). However it is clear that epinephrine continues to act post-treatment, since by 1 week higher concentrations and longer exposures significantly reduced metamorphosis compared to the control (Figure 3.9), whilst the toxicity of epinephrine also increased with concentration and length of exposure (Figure 3.10). Analysis showed that extended exposure at high concentrations reduced the size of observed larval, potentially indicating a selection pressure against larger, more developed individuals (Figure 3.11).

Influence of potassium chloride

At the initiation of the trial, 35.8 ±8.4% of larvae possessed eye-spots, with 11.4 ±6.5% showing some signs of early gill formation. Figure 3.12 and Figure 3.13 show the mean percentage of metamorphosed and surviving mussel larvae respectively, after culture periods of 72 hours and 1 week in response to a range of KCl levels over exposure periods of 24, 48 and 72 hours. Figure 3.14 shows the mean shell length (µm) of animals after 1 week.

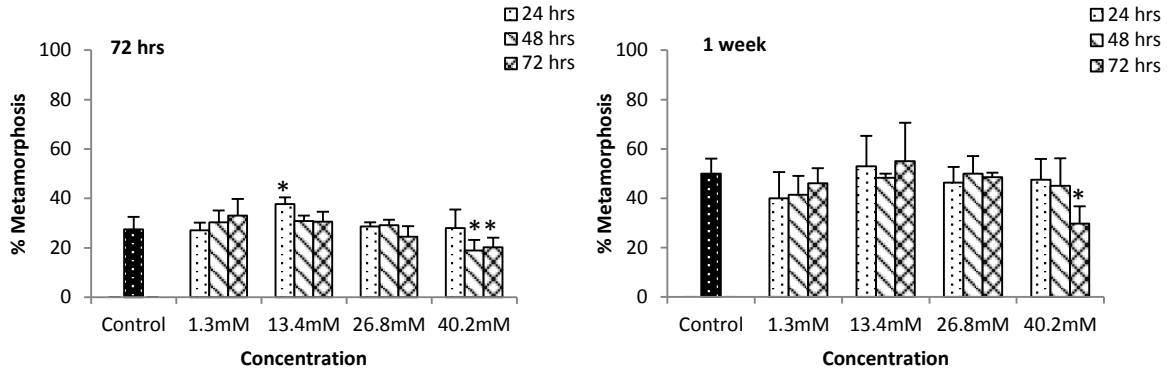

Figure 3.12: Mean percentage metamorphosis after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to KCl at concentrations of 1.3, 13.4, 26.8 and 40.2mM over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control (P<0.05).

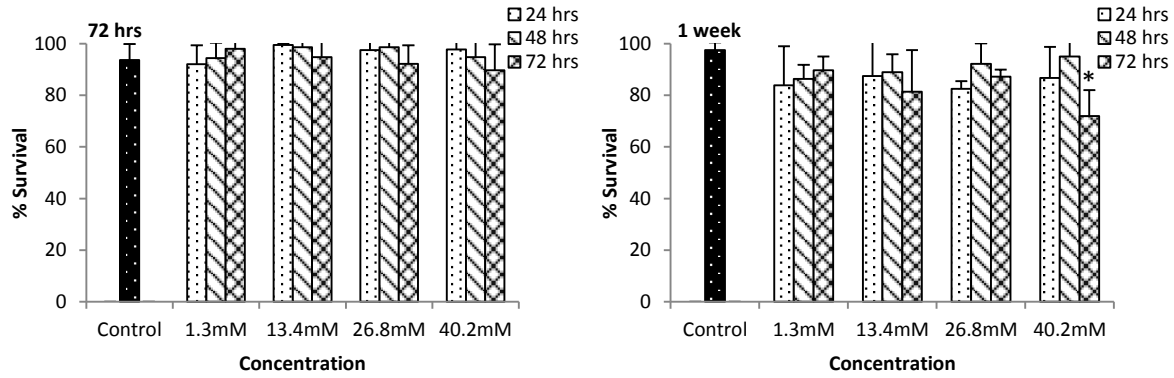

Figure 3.13: Mean percentage survival after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to KCl at concentrations of 1.3, 13.4, 26.8 and 40.2mM over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control (P<0.05).

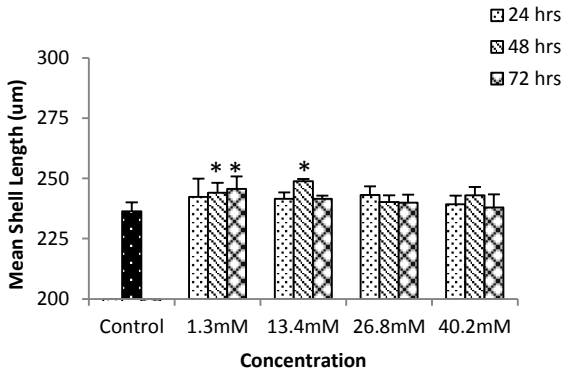

Figure 3.14: Mean shell length (µm) of *Mytilus edulis* larvae 1 week after commencement of exposure to a range of KCl concentrations over exposure periods of 24, 48 and 72 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the control (P<0.05).

Analyses showed that KCl had almost no impact upon metamorphosis or survival, positively or negatively. Only at the 72 hours sample point was a KCl treatment, 13.4mM over an exposure period of 24 hours, found to induce significantly higher metamorphosis than the control. However, by 1 week although maximum metamorphosis was achieved at a concentration of 13.4mM over a 72 hour exposure period (55.0±15.6%), no KCl treatment provided a significant improvement in metamorphic induction compared to the control (Fisher's $P>0.05$) (Figure 3.12). Analysis did show, that percentage metamorphosis could be significantly reduced, but only at the highest concentration over long exposure periods. No KCl treatment induced significantly lower mortality than the control at 72 hours (Figure 3.13, Fisher's $P<0.05$), and by 1 week only the highest treatment level, 40.2mM over 72 hours, resulted in a significant drop in survival compared to the control (Fisher's $P<0.05$). In terms of growth analysis showed that KCl treatments of 1.3mM after exposure for 48 and 72 hours, and 13.4mM after exposure for 48 hours conferred a significant improvement in the mean shell length of larvae compared to the controls (Fisher's $P>0.05$) (Figure 3.14). The level of improvement represents a 3.3 to 5.3% improvement, compared to the control.

Influence of ammonium chloride

At the beginning of the trial, 51.2 ±5.1% of larvae possessed eye-spots, with 15.5 ±7.1% showing some signs of early gill formation. In response to a range of NH_4Cl levels over exposure periods of 24, 48 and 72 hours Figure 3.15 and Figure 3.16 show the mean percentage of metamorphosed and surviving mussel larvae respectively, after culture periods of 72 hours and 1 week. Figure 3.17 shows the mean shell length (μm) of animals after 1 week.

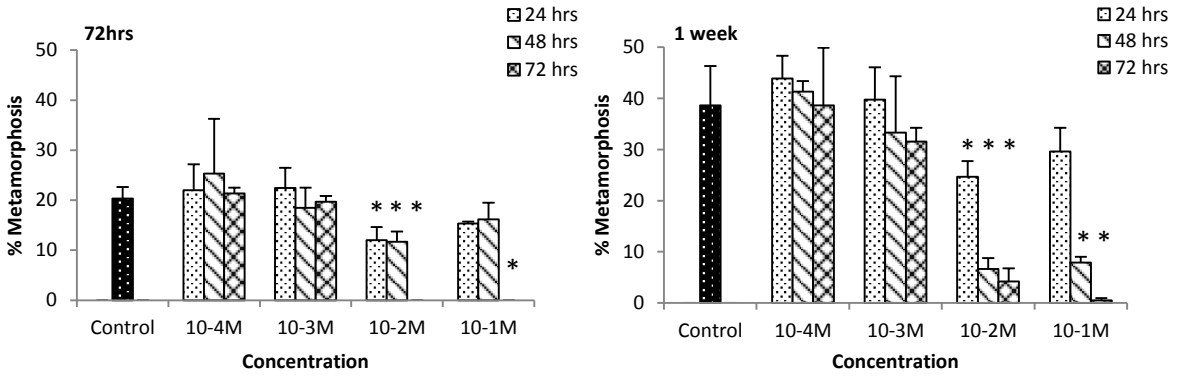

Figure 3.15: Mean percentage metamorphosis after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to NH_4Cl at concentrations of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} M over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different than the control ($P<0.05$).

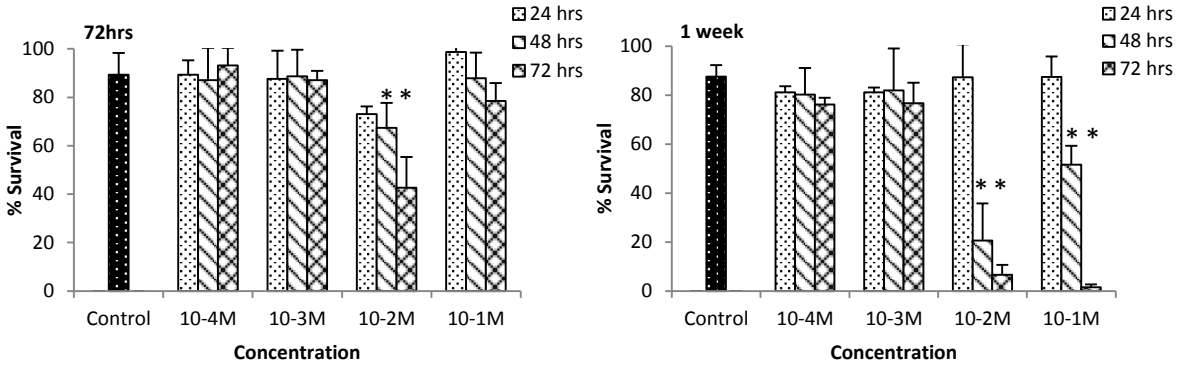

Figure 3.16: Mean percentage survival after culture periods of 72 hours and 1 week for *Mytilus edulis* larvae exposed to NH_4Cl at concentrations of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} M over periods of 24, 48 and 72 hours, compared to an untreated control. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different than the control ($P<0.05$).

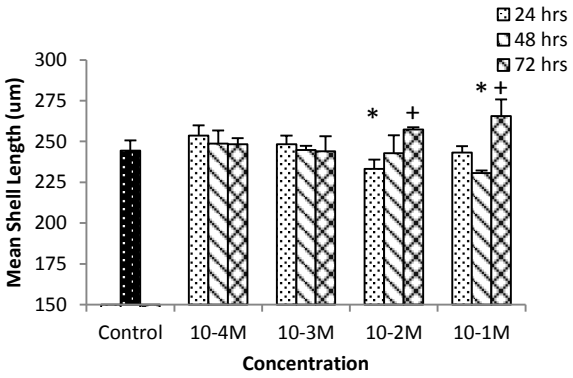

Figure 3.17: Mean shell length (µm) of *Mytilus edulis* larvae 1 week after commencement of exposure to a range of NH_4Cl concentrations over exposure periods of 24, 48 and 72 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly lower than the control, whilst a plus (+) represents a result significantly greater than the control ($P<0.05$).

NH_4Cl over the concentrations and exposures tested did not have a positive influence on either metamorphosis or survival of mussel larvae compared to the untreated control. Although maximum metamorphosis was achieved at a concentration of 10^{-4}M (Figure 3.15) at both 72 hours and after 1 week, with exposure periods of 48 hours and subsequently 24 hours being the most effective respectively, no NH_4Cl treatment significantly improved metamorphic induction. Concentrations up to 10^{-3}M at exposures up to 72 hours had no influence on metamorphosis or survival rates of larvae, however it is clear that after 1 week in most cases higher concentrations and exposure periods longer than 24 hours significantly reduced metamorphosis and survival (Figures 3.15 and 3.16). Larvae could however withstand concentrations as high as 10^{-1}M if exposed for only 24 hours. High concentrations combined with long exposures of 48 hours or more continued to influence larvae

post-treatment, with a clear decline in larval survival between 72 hours and 1 week (Figure 3.16). Analysis of shell length showed that at concentrations of 10^{-3}M and below, growth of larvae is unaffected by NH_4Cl. At 10^{-2}M and 10^{-1}M NH_4Cl results indicate that of surviving larvae exposed for 72 hours the mean shell length was significantly greater than in the control group (Fisher's $P<0.05$) (Figure 3.17). However, this corresponds with treatments with the greatest larval mortality, indicating a selection pressure with only the largest most well developed larvae surviving. However, the influence appears variable and unclear.

Discussion

Laboratory based study of the influence of chemical compounds on settlement and metamorphosis in marine invertebrates has been, and continues to be, a major tool in aquaculture and biofouling research (Dobretsov and Qian, 2003). A large number of compounds have been examined for a wide range of species, with responses varying from induction of normal settlement and metamorphosis, abnormal or partial development, mortality, no effect at all, as well as inhibition or amplification of other chemical agents (Morse *et al*., 1979; Hadfield, 1984; Yool *et al*., 1986; Baxter and Morse, 1987; Eyster and Pechenik, 1987; Pawlik, 1990; Pires and Hadfield, 1991; Boettcher and Targett, 1998; Satuito *et al*., 1999; García-Lavandeira *et al*., 2005). However, all responses, whether positive or negative, increase our understanding of the biochemical processes in action. This study assessed the influence of the compounds L-DOPA, epinephrine, KCl and NH_4Cl on the development and survival of the mussel *M. edulis*. These chemicals have previously been shown to induce metamorphosis in a number of bivalve species.

L-DOPA, the precursor to the catecholamine neurotransmitters, has proven to be an effective metamorphic induction agent in bivalves, including *C. gigas* (Coon *et al*., 1985; Nicholas *et al*., 1998), *Patinopecten yessoensis* (Kingzett *et al*., 1990) and *P. maximus* (Chevolot *et al*., 1991; Nicolas *et al*., 1998), with response concentration and exposure dependent. The mode of action remains unclear, although it has been linked to its conversion into dopamine (Bonar *et al*., 1990) and its oxidation into hydrogen peroxide (Pires and Hadfield, 1991; Boettcher and Targett, 1998). In *Mytilus* mussels, information on the influence of L-DOPA is limited. However, this genus has been shown to respond to L-DOPA, with *M. galloprovincialis* induced to metamorphose at a concentration of 10^{-5}M, although the level of induction was reportedly low (Satuito *et al*., 1999), and *M. edulis* induced to settlement at the same concentration (Dobretsov and Qian, 2003). In *M. edulis,* Cooper (1982)

reported a metamorphic response to L-DOPA at concentrations of 10^{-6}M (12.2% induction) and 10^{-5}M (36.9% induction), although experimental details are limited. This contrasts to the results of the present study, which found that L-DOPA, tested over a wide range of concentrations and exposures, including those stated by Cooper (1982), did not induce a significant increase in larval metamorphosis. Metamorphosis was higher than in the control after 1 week at a concentration of 10^{-6}M, particularly over a 72 hour exposure period, but not significantly. Metamorphosis in the present study however declined significantly at 10^{-5}M and above, compared to 10^{-6}M and the control. Furthermore whilst a slight improvement was seen in survival and growth, it was not significant. Although not stated by Cooper (1982) the current study does make it evident that above 10^{-6}M, irrespective of exposure period, survival was significantly reduced, with concentrations of 10^{-4}M and above proving 100% lethal. This corresponds with the observed formation of a black precipitate in cultures, with formation increasing with concentration of L-DOPA. This has previously been described as a heterogeneous polymeric melanin precipitate the presence of which has been associated with larval toxicity (Pawlik, 1990).

The difference between this study and Cooper's (1982) suggests many of the larvae in the L-DOPA assay may not have been competent to metamorphose. In this study the increase in metamorphosed larvae between 72 hours and 1 week is low. In the control the mean proportion of metamorphosed larvae rose from 11.4 ±6.8% to just 13.9 ±2.1%, whilst maximum metamorphosis reached only 20.8 ±3.6%. The values recorded in this assay are however not too dissimilar to those recorded by Cooper (1982). It is recognised that the effectiveness of an inducer is dependent upon the competence of larvae to undergo metamorphosis (Chevolot *et al*., 1991). However, there is often inherent variability in the sensitivity and threshold of larval responses to chemical inducers within any group as well as between different batches of larvae (Bonar, 1990). The outcome of induction with L-DOPA may therefore be different when applied to another batch of larvae. Additionally the larval response to alternative chemical agents is known to vary temporally, with *C. gigas* larvae displaying a settlement response to L-DOPA before showing a metamorphic response to epinephrine, which in turn precedes a metamorphic response to L-DOPA (Coon *et al*., 1990a). It appears that competence is a multi-step process developing through different mechanisms (Coon *et al*., 1990a). It would therefore be valuable to investigate the temporal relationship between larval stage and chemical inducers, including L-DOPA and others, in *M. edulis* to assess the onset of competence. As demonstrated by Coon *et al*. (1990a) the influence of chemicals could be assessed at regular intervals over an extended culture period with observations made of larval settlement and metamorphosis. This would provide a clear picture of the impact of the chemicals during

development. In *M. edulis* at 17 to 20°C 50% of larvae can reach the "eyed" stage in 21 days (Galley *et al*., 2010). It is therefore important that chemicals are assessed pre and post this point. Alternative related compounds may also prove beneficial. The catecholamine compound dopamine, formed from the decarboxylation of L-DOPA increased metamorphosis in *Mytilus coruscus* at concentrations between 10^{-6}M to 10^{-4}M (Yang *et al*., 2013), whilst the competitive inhibitor of L-DOPA, α-methyldopa, has proven effective with *M. galloprovincialis* (Yang *et al*., 2008).

The catecholamine hormonal neurotransmitter epinephrine, has proven to be an effective means of inducing larval metamorphosis in a large number of bivalve species, including *Mytilus* mussels (Coon *et al*., 1985; Satuito *et al*., 1999; García-Lavandeira *et al*., 2005; Teh *et al*., 2012; Yang *et al*., 2013). Previous studies with *M. galloprovincialis* have demonstrated that metamorphosis can be controlled through the application of adrenergic agonist and antagonist compounds (Satuito *et al*., 1999; Yang *et al*., 2008). Epinephrine, and other adrenergic agonists such as phenylephrine and clonidine stimulate metamorphosis, potentially through receptors similar to vertebrate type alpha$_{-1}$ adrenoreceptors (Yang *et al*., 2008) whilst antagonists such as phentolamine have been used to inhibit metamorphosis (Satuito *et al*., 1999). Epinephrine has been shown to induce metamorphosis in *M. galloprovincialis* (Satuito *et al*., 1999; García-Lavandeira *et al*., 2005; Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013) at concentrations from 10^{-4} to 10^{-6}M over exposures of 24 to 72 hours, although exposure for up to 72 hours appears to stunt induction in some cases. In contrast to the trial with L-DOPA, the level of metamorphosis seen in the epinephrine trial within the control after 1 week (58.9 ±12.1%) indicates that larvae were definitely ready to metamorphose. However, the results of the present study with *M. edulis* show that epinephrine had no positive inductive influence on larval metamorphosis. In *M. edulis* high concentrations and long exposures reduced metamorphosis and growth, and increase larval mortality. This consequence is not necessarily visible immediately after the treatment, only becoming apparent after a lag period of several days. The results of the present study suggest that the pathway of action within *M. edulis* is different to other species within the same genus.

Exposure to excess potassium ions (K^+) has been suggested as a general mechanism for eliciting a metamorphic response in invertebrate larvae (Baloun and Morse, 1984; Yool *et al*., 1986), potentially acting by depolarising excitable cells involved in the perception of an inductive stimulus (Baloun and Morse, 1984). The inductive influence of K^+ has been demonstrated in a number of mollusc groups including bivalves (Martinez *et al*., 1999; Yang *et al*., 2008; Yang *et al*., 2013) and gastropods (Baloun and Morse, 1984; Pechenik and Heyman, 1987; Gallardo and Sánchez, 2001), as

well as other marine invertebrates (Yool *et al*., 1986; Carpizo-Ituarte and Hadfield, 1998). In the *Mytilus* genus excess K^+ has been an effective metamorphic inducer in *M. galloprovincialis* at concentrations of $3x10^{-2}$M (Yang *et al*., 2008) and in *M. coruscus* at $5x10^{-2}$ (Yang *et al*., 2013) after exposure for 24 hours, although the level of response is considerably greater in *M. galloprovincialis* under similar experimental conditions. In both species the inductive response has a lag period of two days and outside specific treatment conditions based on concentration and exposure the response is limited or not stimulated (Yang *et al*., 2008; Yang *et al*., 2013). The results of the present study confirmed previous findings by Eyster and Pechenik (1987) that KCl is ineffective in *M. edulis*; excess K^+ did not stimulate a metamorphic response in this study, even when examined over an increased concentration range, an increased variety of exposure periods and over a longer time period than in the past study. The level of metamorphosis seen by the conclusion of the KCl trial in the control (up to 50.0 ±6.0%) indicates that larvae were however competent to metamorphose. This suggests that the route of metamorphic induction in *M. edulis* differs to other species within the same genus. In contrast to Eyster and Pechenik (1987) who reported that larvae failed to settle and metamorphose due to a lack of suitable settlement substrate or water agitation in treatments of 5 to 20mM for up to 48 hours, larvae in the present study continued to develop. K^+ treatments had almost no inhibitory influence on larval metamorphosis or survival in the present study. Excess K^+ was however found to have an influence on larval growth, with significantly improved growth (3.3 to 5.3%) over the control at concentrations of 1.3mM and 13.4mM ($1.3x10^{-3}$ and $1.3x10^{-2}$ respectively) after exposure for 24 and 48 hours. Within molluscs previous assessment of growth in the gastropod *Crepidula fornicata* following KCl treatment showed that growth was unaffected (Eyster and Pechenik, 1988), whilst increased growth was observed in the gastropod *Chorus giganteus* (Gallardo and Sánchez, 2001). Increased growth has been also been reported in *O. edulis* larvae exposed to the chemical γ-aminobutyric acid (GABA) at a concentration of 10^{-6}M for 4 hours (Mesías-Gansbiller *et al*., 2013). This treatment lead to settlement of 70.7% compared to 38.8% in the untreated controls after 4 days, with subsequent mean spat size by 15 days post-treatment 700µm in treated larvae compared to 425µm in the controls (Mesías-Gansbiller *et al*., 2013). Increased growth in induced post-larvae has been suggested as a consequence of increased availability of resources for growth by decreasing the energy costs associated with prolonged settlement and metamorphosis (Gallardo and Sánchez, 2001). Whether the increased growth observed in this study is a novel response in *M. edulis* or a consequence of biological variability warrants further investigation.

The influence of ammonium (NH_4^+) on settlement and metamorphosis in invertebrate larvae is species-specific. A response was induced in the bivalves *C. gigas* (Coon *et al*., 1990b), *P. yessoensis* (Kingzett *et al*., 1990), *M. galloprovincialis* (Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013), but not in *Pinctada maxima* (Zhao *et al*., 2003). In other marine invertebrates the response to NH_4^+ has also been mixed with induction in the bryozoan *Bugula neritina* (Yu *et al*., 2007) and the abalone *Haliotis diversicolor supertexta* (Yu *et al*., 2010), whilst in *C. fornicata* the response was limited (Pechenik and Heyman, 1987), with no response in *Hydroides elegans* (Bryan *et al*., 1997). The mode of action remains unclear, although NH_4^+, a weak acid, is known to play a role as a signalling agent within the nervous systems of vertebrates and invertebrates and may act by increasing intracellular pH (Coon *et al*., 1990b; Marcaggi and Coles, 2001). However, previous studies have raised questions over whether it is NH_4^+ or ammonia (NH_3), or possibly both, that acts to induce a response (Berking 1988; Coon *et al*., 1990b). In this study, the first known test of this compound with *M. edulis*, it is clear that NH_4Cl does not induce metamorphosis in this species. The level of metamorphosis seen by the conclusion of this trial (up to 43.9 ±4.5%) would indicate that larvae were however ready to metamorphose. However, whilst not monitored in the present study consideration in future should be given to culture pH, since if induction is influenced by intracellular alkalisation, a low culture pH may inhibit the process, with insufficient NH_3 formed from NH_4^+ for induction to occur (Yu *et al*., 2008). High concentrations ($\geq 10^{-2}$M) plus long exposures (≥ 48 hours) inhibited larval development and led to increased larval mortality, as has previously been demonstrated in other bivalves (Zhao *et al*., 2003; Yu *et al*., 2008). Both NH_4^+ and NH_3 play an important role in aquatic toxicity (Boardman *et al*., 2004) and it is feasible that the concentrations and exposures tested in the present study may be too extreme for larvae to endure, becoming inhibitory as a consequence of disturbing normal biochemical functions. In this study the detrimental influence was more noticeable several days post-treatment, indicating a continuing pressure. Improvements in growth were seen in the highest concentrations over the longest exposures (10^{-1}M and 10^{-2}M after 72 hours), though this is likely a consequence of the high levels of mortality affecting the smallest and least developed individuals, and is not seen as a positive influence. The lack of response by *M. edulis* contrasts to previous studies in other *Mytilus* species. Both *M. galloprovincialis* (Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013) responded to this compound at concentrations from $5x10^{-2}$M and 10^{-3}M after exposure periods of 3 to 24 hours with increased metamorphosis. The inductive response in their trials had a lag period of up to two days and extended exposure appeared to limit or prevent induction. The level of response and effective treatment level however differ noticeably between *M. galloprovincialis* (Yang *et al*., 2008) and *M. coruscus* (Yang *et al*., 2013). Almost 80% of *M. galloprovincialis* metamorphosed after exposure for 24 hours, whilst induction was maximised after

exposure for just 3 hours for *M. coruscus*, at almost 45%, with development stunted and mortality higher after exposure for 24 hours. This result combined with the results of our study implies that there may be a difference in the biochemical mechanisms controlling metamorphosis within this genus. Assessment of NH_4Cl over shorter exposure periods for *M. edulis* may prove beneficial, as seen in *M. coruscus* (Yang *et al*., 2013) and may warrant future consideration.

In conclusion the assessment and identification of suitable chemical cues, and application protocols, is a delicate process. It requires the careful refinement of concentration and length of exposure, as well as the identification of the optimum development stage for application, in order to optimise induction rates and maximise survival and growth. This study adopted an extended assessment period beyond the treatment window, with cultures containing thousands of larvae, in order to fully examine the impact of potential agents. Arising from this study it is recommended that future induction studies with *M. edulis*, and any other species, are conducted over sufficient time to observe the full effect of the treatments. In this case L-DOPA, epinephrine, KCl and NH_4Cl did not induce a positive metamorphic response in *M. edulis* larvae. The high concentrations and exposure periods tested were often detrimental to development and survival, with these negative impacts often observed or intensified only after a lag period post-treatment. However, whilst a suitable chemical agent and application protocol for *M. edulis* is still to be identified, the present results contribute to our expanding knowledge of metamorphic control in this species, and potentially the influence of chemical compounds in invertebrate larval as a whole. Comparison with studies in related *Mytilus* species indicates that metamorphic receptors and signalling pathways may be different in *M. edulis*, and the level of response in this genus is species-specific, however this requires verification. Whilst it may prove beneficial to explore the application of the chemicals used in this study over shorter exposure periods in *M. edulis,* there is an almost unlimited number of potential chemical compounds yet to test. Chemicals such as methoxyphenamine, clonidine, serotonin, as well as organic solvents including ethanol, methanol ethylene glycol and acetonitrile (Yang *et al*., 2008; Yang *et al*., 2013), have proven to be effective metamorphic inducers in other Mytilidae mussels and therefore should possibly be considered first for testing in future studies with *M. edulis*.

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Chapter 4

Investigation of mobile seed behaviour to increase the security of settlement and ease of management of the blue mussel *Mytilus edulis* (L.).

Abstract

The commercially important blue mussel, *Mytilus edulis*, is the focus of on-going research to optimise its culture and management. It is known to possess highly mobile early settlement stages able to relocate to new habitats. However the factors affecting retention and dispersal of farmed seed are yet to be fully understood. This study assessed the impact of substrate, attachment period, food availability, water agitation, and density on attachment, retention and mortality of seed mussel between 0.97 and 3.85mm in shell length. Attachment was affected by both substrate type and attachment period. Seed showed a clear preference for smooth glass and natural fibre wool felt, with attachment rates of up to 89.2% and 95.2% after 6 hours respectively, with seed requiring at least 3 hours to attach, after which there was no significant difference between attachment periods up to 48 hours long ($P>0.05$). Attachment to other natural fibre materials and slate was lower, although not necessarily significantly ($P>0.05$). Feeding, water agitation, density and substrate all significantly influenced seed retention, though not mortality, over the course of the study. Feeding seed significantly increased detachment and dispersal compared to seed receiving limited or no food ($P<0.05$). Fed seed were more likely to undertake active dispersal, with ≥75% of detachment associated with dispersal to alternative attachment sites. However, seed on restricted feeding regimes although less likely to detach, were more likely to remain *in situ*, since <40% of detached seed relocated. Seed may lack the energy reserves for active dispersal through crawling or byssus drifting, and may be reliant upon external forces, such as water currents, for dispersal. However, water agitation significantly increased the subsequent retention of individuals ($P<0.05$), and negated the influence of feeding seed. Compared to static conditions, water agitation decreased dispersal behaviour to alternative attachment sites by between 62 to 100% and total detachment by 55 to 100%. The stimulation of increased and stronger attachment by water movement dramatically increased the likelihood of retention. Seed detachment and dispersal was proportional to seed density, with detachment rates of between 0.067 to 0.158 seed per attached seed cm^{-2} and dispersal rates of 0.016 to 0.059 seed per attached seed cm^{-2}. However, the rate of detachment and active dispersal were significantly influenced by substrate type, with the rate of loss higher from the smooth glass than from the fibrous wool material ($P<0.05$). From an aquaculture perspective

substrate type has a significant impact upon stocking density with some substrates offering higher retention of more seed. The present study also demonstrated that although pedal crawling could not be used as a means of predicting either attachment or subsequent retention, a link between seed activity and seed mortality was identified. Seed displaying increased mobility exhibited higher survival, with 80 to 100% lower mortality over the study period than sedentary seed. This indicates that seed activity could be used as a simple predictor for seed performance, with activity related to level of fitness.

Keywords

Attachment, Detachment, Mortality, *Mytilus edulis,* Mussel, Seed

Introduction

In Europe production of the commercially important blue mussel *Mytilus edulis* is reliant upon the retention of collected seed. Collected seed are on-grown to a marketable size (>45mm) using either bottom culture, where seed are laid directly on benthic plots, or suspended culture utilising tubular stockings on bouchots, or attached to ropes suspended from rafts or long-line systems (Spencer, 2002; Maguire *et al*., 2007). However, many bivalve species including *M. edulis* display motile behaviour facilitating secondary dispersal post-metamorphosis (Sigurdsson *et al*., 1976; Sörlin, 1988; Beukema and de Vlas, 1989; Roper *et al*., 1995; Baker and Mann, 1997). In fact *M. edulis* is known to remain motile throughout its life cycle, employing several methods allowing them to disperse (Baker and Mann, 1997). As in many species, young post-larval *M. edulis,* up to at least 2.5mm in size, are able to sever their byssus attachment and secrete long monofilament drifting byssus (Sigurdsson *et al*., 1976). These threads, morphologically distinct and many times longer than attachment byssus, enable seed to enter a second post-larval pelagic phase (Lane *et al*, 1982; Lane *et al*., 1985). As seen in other species, seed up to at least 10mm in shell length may possess this ability (Beukema and de Vlas, 1989). However, whilst the ability to utilise byssus drifting is lost, *M. edulis* retains the abilities to secrete and sever attachment byssus, and use the muscular action of their foot organ to crawl across surfaces (Bayne, 1964a; Dare and Davies, 1975; Petraitis, 1987; Baker and Mann, 1997).

The causes of secondary dispersal behaviour in bivalves are unclear, though primary causes are thought to be in response to unfavourable conditions or a shift in habitat requirement (Sörlin, 1988; Beukema and de Vlas, 1989; Baker and Mann, 1997; Lundquist *et al*., 2004). This behaviour is important in the selection of a suitable habitat, however from an aquaculture perspective it represents a risk in the management of bivalves with mobile seed, especially since *M. edulis* seed supplies can be both unpredictable and inconsistent (Kamermans and Smaal, 2002; Spencer, 2002). Retention of seed is therefore a priority since the inefficient use of this valuable and finite resource makes production more costly and unpredictable, and can lead to large production and economic losses (Carton *et al*., 2007). The loss of *M. edulis* seed as a direct consequence of secondary dispersal behaviour from aquaculture sites has not been quantified, though the potential for enormous wastage has been highlighted. The loss of seed (1.5 to 5.0mm shell length) from rope cultures can be extremely high, with a 50% reduction in the first 2-4 months, though at high stocking densities losses can exceed 98% after 12 months (Dare and Davies, 1975), and loss from benthic sites, not associated with predation, may form part of the 17-41% attributed to mortality in the first

year after laying (Dare and Edwards, 1976). Even when initially confined, seed are known to migrate from bouchot stockings to cover the surface of the bouchot itself (Spencer, 2002).

Poor retention of bivalve seed has been linked to exposure to environmental change or stress, leading to increased detachment and motivation to undertake secondary dispersal. In the tellinid bivalve *Macoma balthica* dispersal behaviour has been linked to seasonal changes in temperature, increased current turbulence and changes in habitat requirement (Sörlin, 1988; Beukema and de Vlas, 1989). *Macomona liliana* employs pedal crawling and drifting in order to escape habitat contamination (Roper *et al*., 1995), and both the cockle *Austrovenus stutchburyi* and *M. liliana* are stimulated to undertake crawling and drifting in response to substrate type and water velocity (Lundquist *et al*., 2004). Byssus attachment is also known to be influenced by factors including animal size, temperature, salinity, hydrodynamic conditions and food availability (Allen *et al*., 1976; Dolmer and Svane, 1994; Paul, 1980; Price, 1982; Christophersen and Strand, 2003; Babarro *et al*., 2008; Lachance *et al*., 2008), with attachment strength in *M. edulis* showing seasonal variation (Price, 1982; Moeser and Carrington, 2006). These factors may stimulate dispersal or poor retention, since Carton *et al*. (2007) directly linked food availability and desiccation events to seed retention of the mussel *Perna canaliculus*. Conversely however, behavioural response by bivalve seed to environmental stimuli has been correlated to seed quality and fitness (Maguire *et al*., 1999a,b). Establishment of links between seed quality and seed behaviour through simple non-destructive tests is seen as offering the potential for predictive indicators of future performance (Paul, 1980; Carton *et al*., 2007), thereby providing aquaculture with further useful tools.

Ultimately however, seed that fail to attach or subsequently detach can be lost from production systems. Maximising retention of valuable seed animals is a priority and research to identify factors influential in the retention of *M. edulis* is essential in the development of effective management strategies. The aim of this study was to assess the influence of husbandry and environmental pressures on attachment, retention and mortality in *M. edulis* seed. Variables likely to be encountered during seed handling were examined including substrate type, attachment period, feeding level, water agitation and seed density. Notably a number of experimental substrates were examined during this study to reflect surfaces of varying rugosities on which to assess seed retention. Several of these were natural fibre materials which were assessed with a view to providing novel materials for deployment on benthic on-growing plots or suspended structures. Furthermore pedal crawling activity was assessed as a predictor of seed condition.

Materials and Methods

Source of mussel seed

Mussel broodstock maintenance, spawning, and fertilisation were as described in Chapter 3. Veliger larvae were reared at an initial density of 10 ml^{-1} in 65-litre polyethylene tanks in volumes up to 50-litres of 1µm filtered and UV-irradiated seawater (FSW) with a salinity of 33‰. Culture temperature was maintained at 16±1˚C. Three times a week the larvae were sieved onto a 45 µm mesh screen and inspected, the containers cleaned, the FSW replaced and the larvae fed. Larvae were fed a mixed microalgae diet equivalent to a cell concentration of 3.75 $x10^4$ cells ml^{-1} day^{-1} of *Pavlova lutheri* (PLY75), *Isochrysis* sp. (clone T-ISO) (PLY506A) and *Chaetoceros calcitrans* (PLY537), at a ratio of 1:1:1. Following metamorphosis, post-larvae were maintained for a further 7 weeks under the same regime, before transfer to an on-growing seed system.

Nursery on-growing system

Seed were maintained in a down-welling culture system (Figure 4.1). The system consisted of a central tank suspended in a 120-litre reservoir tank. Seed were held in cylindrical sieves (160mm dia.) with mesh diameters ranging from 125µm - 500µm, connected to the central tank. Juveniles were progressively transferred to sieves with larger mesh diameters as they increased in size. Water was pumped from the reservoir tank to the central tank, flowing back to the reservoir tank via the seed sieves.

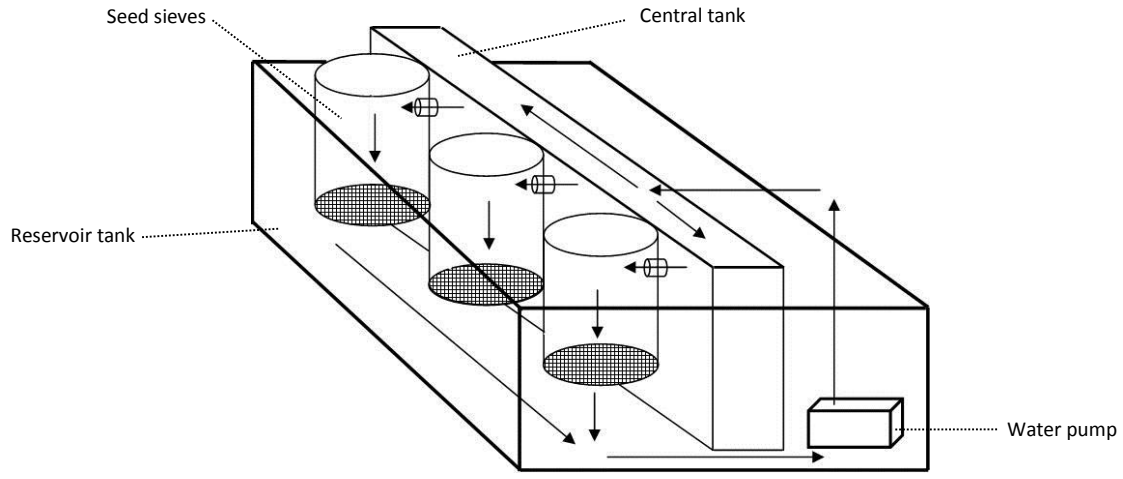

Figure 4.1: Nursery down-welling system. Arrows indicate direction of water flow.

The system was supplied with a constant inflow of seawater (0.28±0.06 litres minute^{-1}) filtered to 10µm and maintained at ambient temperature (ranged from 14 to 20˚C). The system was fed daily *ad libitum* a mixture of microalgae including *P. lutheri*, *Isochrysis* sp., *C. calcitrans*, *Rhinomonas reticulata* (CCAP 995/2) and *Tetraselmis chuii* (CCAP 8/6).

Seed conditioning

Prior to commencing experiments seed were "conditioned" for 6 days using a standardised husbandry protocol in an effort to ensure a comparable starting condition of seed used throughout the study, as this could not be guaranteed with seed taken directly from the nursery system. A random group of seed were removed from the nursery system and graded using a series of nylon mesh sieves to between 0.5-2.0mm. Size range (shell length in mm) of graded seed was determined from digital images taken using the Canon EOS 1000D, with images measured using the software Image J. The size range of mussel seed used for all experiments was between 0.97 and 3.85mm, with a mean size of 1.99±0.86mm (Figure 4.2). These were stocked in clean, static conditioning tanks at a density of 1.1±0.3g (wet weight) of seed litre^{-1} of FSW at 16±1˚C for 6 days. Tanks were aerated and 100% water changes conducted every 2-3 days. Seed were fed a mixed microalgae diet equivalent to a cell concentration of 7.5 x10^{4} cells ml^{-1} day^{-1} of *Isochrysis* sp., *P. lutheri* and *C. calcitr*ans, at a ratio of 1:1:1, unless otherwise stated.

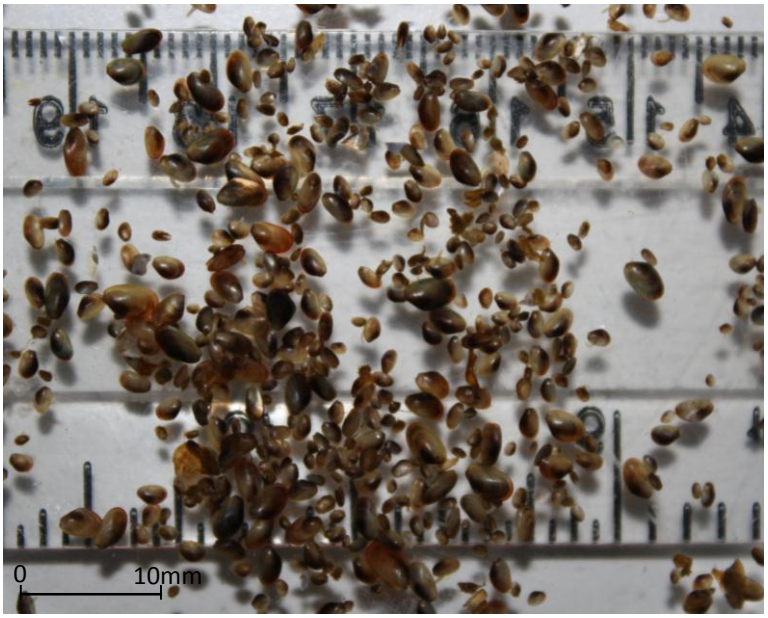

Figure 4.2: Conditioned *Mytilus edulis* seed.

Behavioural assays

The influence of environmental variables on mussel seed attachment, detachment and mortality was assessed through a series of five experiments. In all experiments, following conditioning seed were stocked at a known number or weight (g) directly onto individual test substrates set within plastic dishes filled with 50ml of FSW (Figure 4.3). Attachment was carried out in still water conditions, under constant illumination. Seed were fed as described for conditioning during attachment, unless otherwise stated, and the temperature was maintained at 15±2˚C throughout all experiments. Seed were allowed to attach, undisturbed for a defined attachment period before substrates were gently rinsed to remove any unattached seed. Details of experiments carried out are given in Table 4.1.

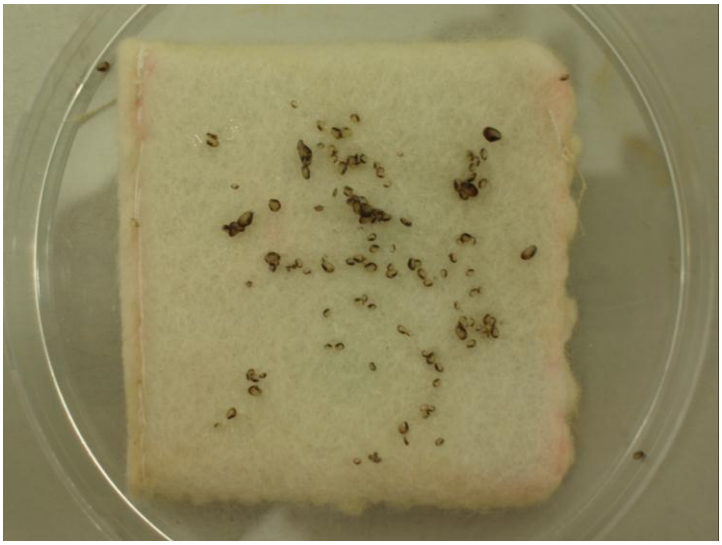

Figure 4.3: *Mytilus edulis* seed attached to an experimental wool substrate tile.

Table 4.1: Experiments carried out to assess larval behaviour.

Experiment	Stocked no. of seed per substrate replicate	Character tested	Treatments	Duration	Replicates
1	49±3	Attachment	Time: 1, 3, 6, 12, 24 and 48 hours. Substrate: glass, slate and wool	Up to 48 hr	6
2	44±5	Attachment	Substrate: glass, slate, wool, cotton, soy and hemp	6 hr	6
3	100±6	Attachment, Detachment and Mortality	Feeding: fed during and after conditioning, starved after attachment, starved from 3 days before attachment, and starved from 6 days before attachment Water condition: static and agitated.	Attachment: 6 hr Detachment: 20 days	5
4	-	Attachment and Detachment	Seed density: 1 to 200 seed cm^{-2} Substrate: glass and wool.	Attachment: 6 hr Detachment: 20 days	- -
5	46±3	Attachment, Detachment and Mortality	Mobility: Pedal crawling seed and static seed. Substrate: glass and wool.	Attachment: 6 hr Detachment: 22 days	5

Experiment 1 assessed attachment over time to three different substrates. All substrates were formed into small tiles of similar dimensions. The glass substrate had mean dimensions of 50.4 x 50.2 x 3.9mm (width x length x height), the slate 50.6 x 50.6 x 3.5mm, and the wool felt substrate 56.6 x 56.9 x 7.4mm (Figure 4.4). The wool was wrapped round a core of slate to provide a rigid structure.

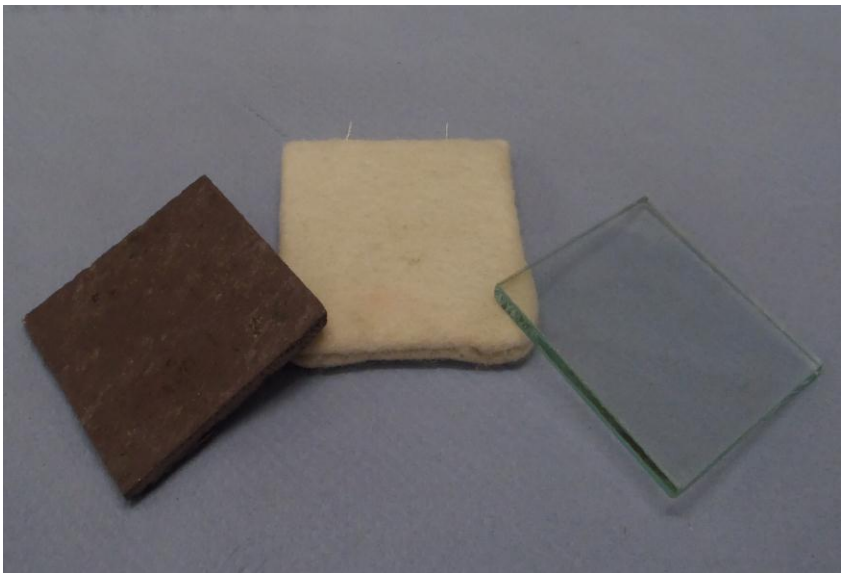

Figure 4.4: Slate, wool and glass tile experimental substrates.

Experiment 2 assessed alternative substrate materials. Seed were placed on glass, slate and wool as described for experiment 1, plus a cotton fabric (54.3 x 53.9 x 5.2mm), a soy based fabric (54.1 x 53.3 x 5.1mm), and a hemp fabric (55.4 x 55.8 x 4.9mm), all wrapped around a core of slate. Attachment was conducted over a 6 hour period, based upon its suitability as determined in experiment 1. Experimental substrates were sourced from Hughes Glass & Glazing Ltd (Llandegai, North Wales) for the glass, Huws Grays Building and Timber Merchants (Llandegai, North Wales) for the slate, Handmade Presents for the wool felt (www.handmadepresents.co.uk), Greenfibres for the cotton (www.greenfibres.com), and the soy and hemp fabrics were from Hemp Fabric UK (www.hempfabric.co.uk). Experiment 3 assessed the impact of feeding and levels of starvation. Seed were exposed to different feeding levels equating to fed throughout (during and after conditioning), starved after attachment, starved from 3 days before attachment, and starved from 6 days before attachment. Seed were placed onto glass substrates to attach, before transferring substrates with attached seed to separate 1-litre glass dishes containing 500ml FSW to monitor detachment. Glass substrates were used for this experiment as based on earlier experiment seed readily attached to it, but importantly it was the same material as the surrounding glass detachment dish, thereby limiting substrate preference of seed as a potential dispersal or detachment factor.

Each feeding treatment was further sub-divided into two sub-treatments of static and agitated water conditions. Water agitation was generated by vigorous aeration through a 3mm airline, whilst the still treatment was undisturbed. In experiments 4 and 5 glass and wool substrates were used as based on earlier experiment seed attachment was maximised on these surfaces. They reflected comparable opposites in terms of texture; with the glass a smooth, hard surface compared to the soft, dense fibrous wool felt material. Experiment 4 assessed the impact of seed density on different substrate surfaces. Seed were stocked at 0.02 to 4.02g wet weight on glass substrates, and 0.03 to 5.67g on wool substrates, equivalent to 1 to 200 seed cm^{-2}. The relationship between wet weight (g) and seed number was determined prior to the start of the experiment from sub-samples of conditioned mussel seed, allowing wet weight to be directly related to stocking number (Figure 4.5). After attachment substrates were transferred to aerated detachment dishes to monitor detachment. Experiment 5 assessed seed activity as a determinant of seed fitness. Seed were separated into two groups; those which had crawled up the sides of conditioning tanks and those that had remained in the original position on the tank floor. Seed from each behavioural group were placed onto glass and wool substrates to attach for 6 hours before transfer to aerated detachment dishes to monitor detachment. In all detachment experiments 50% water changes were conducted every 2-3 days for all detachment dishes, with seed fed as described for conditioning, unless otherwise stated.

In experiments 1 and 2 percentage attachment was determined from direct counts of the number attached to the substrate. In experiment 3 and 5 initial attachment was determined from the number of seed remaining attached at the end of the experiment, those which had detached over the course of the experiment and those which did not initially attach. Attachment in experiment 4 was determined from the estimated number stocked, calculated from the relationship between wet weight and seed number (Figure 4.5), and the number which failed to attach. Direct counts of attached seed on substrates in experiments 3, 4 and 5 were not made after attachment in order to reduce anthropogenic disturbance and influence detachment. In experiments 3, 4 and 5 the number of seed severing attachment from the substrate, including those that remained in situ and those that dispersed, were recorded to determine the total seed detachment, whilst the number of seed dispersing to alternative attachment sites away from the substrate were recorded to determine active dispersal. These are presented either as a percentage or as number of seed per attached seed cm^{-2}. Detachment was assessed by gently agitating seed with a jet of water. In experiment 3 and 5 counts of the number of animal mortalities following attachment were made over the course of the

experiments. Detachment and mortality was monitored every 1 to 3 days, with all detached, dispersed and dead seed removed.

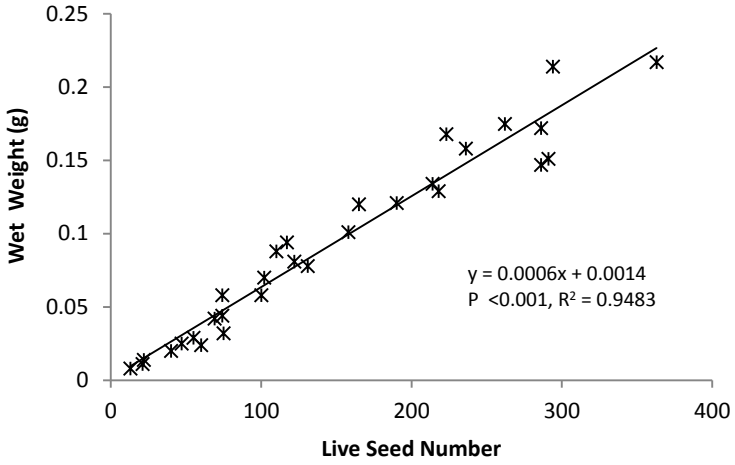

Figure 4.5: Relationship between wet weight (g) and number of seed animals of the mussel *Mytilus edulis* over the size classes of 0.97 to 3.85mm.

Statistical analyses

Data for experiments 1 to 4 are described as the percentage of seed or as the number of seed per attached seed cm^{-2}. Prior to analysing, all percentage data sets were converted by arcsine square root transformation. All data sets were tested using the Anderson-Darling test to investigate departure from normality and Bartlett's or Levene's test to assess heteroscedasticity before applying any test of comparison (Sokal and Rohlf, 1995). In experiments 1, 2, 3 and 5 ANOVA (parametric data), Kruskal-Wallis or Moods Median (non-parametric data) tests were used to determine if there was any significant difference in percentage attachment, total detachment, active dispersal and mortality. This was followed by pairwise comparisons between treatments using Bonferroni Method (parametric data) or Dunns Method (non-parametric data). In experiment 4 the relationship between attachment density and total detachment and active dispersal was assessed using Pearson's Correlation Coefficient before employing comparative linear regression to define the identified relationships. Relationships were compared using ANOVA, with stocking density of attached seed as a covariate. All results were considered to be significantly different when $P<0.05$. Analyses were undertaken using the statistical package Minitab®.

Results

Seed behavioural observations

Seed held in the nursery system were observed crawling over and attaching to all surfaces within down-welling sieves, including the vertical sides. Seed were also observed floating at the water surface away from sites of attachment. These seed had extended their foot and secreted a byssus thread that could be caught by passing a needle around the seed. In experiments seed were also observed to crawl from substrates, whilst a small number were observed floating at the surface.

Influence of substrate type and attachment period

Experiment 1 was a preliminary assessment of the effect of experimental substrate and the length of attachment period before disturbance on *M. edulis* seed attachment. Figure 4.6 shows the mean percentage of seed attaching to smooth glass, textured slate and to the fibrous wool substrates over time periods between 1 and 48 hours.

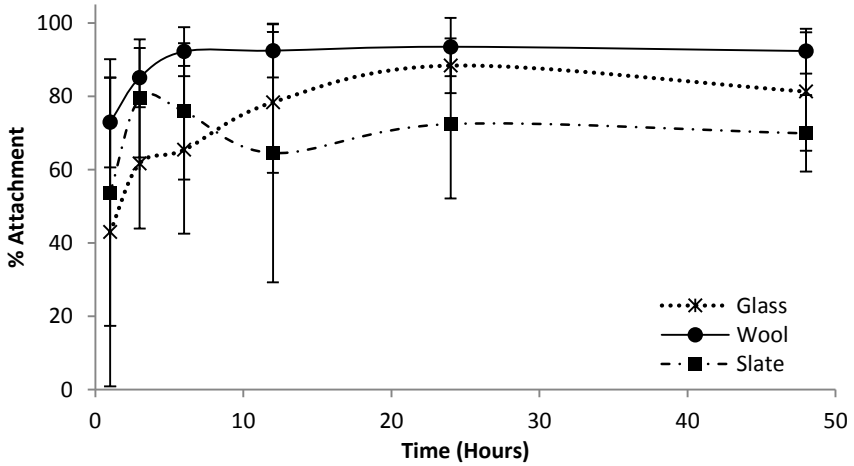

Figure 4.6: Relationship between mean percentage attachment (± standard deviation) of *Mytilus edulis* seed to glass, wool and slate substrates over time (hours).

Seed attached to smooth surfaces, as well as to uneven and fibrous materials, with attachment generally increasing to a plateau after 6 to 12 hours. Maximum attachment was 93.4±7.9% to wool, 88.3±7.4% to glass and 79.4±16.1% to slate. Analysis showed that attachment was significantly influenced by both the length of attachment period (Kruskal-Wallis $H=14.13$, $P=0.015$, $DF=5$) and by the type of substrate (Kruskal-Wallis $H=18.19$, $P<0.001$, $DF=2$). Attachment was significantly lower after 1 hour compared to 24 hours (Dunns $P<0.05$), whilst attachment at all other time periods was

approximately equal (Dunns P>0.05). Comparisons of the substrates showed that attachment to the wool substrate was significantly greater (Dunns P<0.05) than to either of the glass or slate substrates, between which there was no significant difference (Dunns P>0.05). Variability within the experiment was clearly high, although this reduced over longer attachment periods. Based upon this experiment an attachment period of 6 hours was chosen for subsequent experiments, allowing sufficient attachment whilst limiting the time required for this phase.

Experiment 2 assessed the influence of novel fabric substrates, compared to texture and smooth hard surfaces on *M. edulis* seed attachment over a six hour period. Table 4.2 shows the mean percentage attachment was significantly influenced by substrate type (ANOVA F= 4.74, P=0.004, DF=5). Highest attachment was exhibited on the smooth glass substrate, although attachment was not significantly different to any of the natural fibre materials (Table 4.2). Attachment was lowest on the slate substrate, but was only significantly lower than the glass and wool substrates (Bonferroni P<0.05). Additionally, attachment to slate was considerably lower than in experiment 1 at the same time point, whilst attachment to the wool was slightly lower and glass performed better.

Table 4.2: Mean attachment, total detachment and active dispersal of *Mytilus edulis* seed as a consequence of the variables substrate, feeding regime and seed behaviour.

Experiment	Treatment	% Attachment (±SD)	Detachment				% Mortality (±SD)	
			Total % Detachment (±SD)		% Active Dispersed (±SD)			
2	Wool	78.4 (±11.1) [a]	-		-		-	
	Hemp	59.0 (±24.6) [a, b]	-		-		-	
	Soy	73.1.0(±3.5) [a, b]	-		-		-	
	Cotton	75.1(±21.3) [a, b]	-		-		-	
	Slate	40.3(±15.6) [b]	-		-		-	
	Glass	85.3(±10.1) [a]	-		-		-	
			Static	Agitated	Static	Agitated	Static	Agitated
3	Fed during and after conditioning	81.6(±6.1)	49.0(±9.1)	7.7(±3.3)	36.6(±7.8)	7.0(±3.5)	4.3(±1.0)	1.4(±2.2)
	Starved after attachment	73.4(±13.1)	16.0(±10.5)	4.5(±3.7)	6.3(±2.5)	1.1(±1.4)	1.7(±2.3)	7.5(±5.2)
	Starved from 3 days before attachment	66.2(±21.9)	19.1(±9.8)	0.0	2.6(±3.0)	0.0	5.9(±0.9)	4.9(±2.2)
	Starved from 6 days before attachment	67.8(±25.0)	25.4(±17.0)	11.3(±10.1)	7.1(±5.8)	2.7(±2.8)	4.5(±3.5),	2.3(±2.0)
5	Glass / Static seed	89.2(±2.9)	17.7(±8.5)		17.7(±8.5)		5.6(±3.4)	
	Glass / Crawled seed	70.9(±16.9)	9.9(±8.5)		9.9(±8.5)		1.1(±1.5)	
	Wool / Static seed	91.4(±9.1)	6.3(±2.6)		5.2(±2.2)		3.4(±3.9)	
	Wool / Crawled seed	89.2(±13.0)	16.2(±6.4)		11.5(±6.7)		0.0	

Within each experiment mean values with different superscripts are significantly different ($P<0.05$). SD = standard deviation.

Influence of feeding level and water condition

Experiment 3 assessed the impact of feeding level pre- and post-attachment on the attachment of seed and subsequent total detachment, active dispersal and mortality in static and agitated water conditions. Table 4.2 shows the mean percentage values of the test treatments in experiment 3. Results show that although percentage attachment was higher by seed fed during the conditioning period, variability within treatments was high and the different feeding regimes had no significant influence on mean attachment (Kruskal-Wallis H = 2.78, P=0.427, DF = 3) (Table 4.2).

Total detachment percentage was found to be significantly influenced by both water condition (ANOVA F=50.71, P<0.001, DF=1) and feeding level (ANOVA F=7.65, P=0.001, DF=3), although there was no interaction between these two variables (ANOVA F=2.77, P=0.060, DF=3). Agitation of culture water significantly reduced the level of seed detachment compared to static water conditions. Detachment was reduced by 55 to 100% (Table 4.2). However, feeding seed pre- and post-attachment significantly increased total detachment compared to starvation following attachment and starvation from 3 days before attachment (Bonferroni P<0.05). Total detachment in seed starved throughout the experiment (from 6 days before attachment) was intermediate, and did not significantly differ from any of the other feeding levels (Bonferroni P>0.05). Comparing total detachment between feeding treatments within separate water conditions determined that in static conditions detachment by seed fed pre- and post-attachment was significantly higher than by seed starved after attachment (Bonferroni P<0.05). Whilst detachment by seed starved from 3 days and those starved from 6 days before attachment was approximately equal to all other treatments (Bonferroni P>0.05). In contrast in agitated conditions detachment was approximately equal in all feeding treatments (Bonferroni P>0.05).

Percentage active dispersal of seed was significantly influenced by both water condition (ANOVA F=41.24, P<0.001, DF=1) and feeding level (ANOVA F=28.83, P<0.001, DF=3). Agitation of culture water significantly reduced the level of active dispersal compared to static water conditions. However, active dispersal was significantly higher in fed seed than in all treatments with restricted feeding levels (Bonferroni P<0.05) and seed starved throughout were also found to have significantly higher dispersal than seed starved after 3 days (Bonferroni P<0.05). Lowest dispersal was seen in seed starved from 3 days before attachment (Table 4.2). Comparing active dispersal between feeding treatments within separate water conditions showed that in static conditions dispersal by seed fed pre- and post-attachment was significantly higher than all other feeding treatments

(Bonferroni P<0.05), whilst all other treatments were approximately equal (Bonferroni P>0.05). In contrast in agitated conditions active dispersal was only higher by seed fed pre- and post-attachment than seed starved from 3 days pre-attachment (Bonferroni P<0.05), with all other treatments approximately equal (Bonferroni P>0.05). There was also a significant interaction between water condition and feeding level (ANOVA F=4.19, P=0.014, DF=3). This is potentially because of the greater impact of water agitation on reducing active dispersal in fed seed compared to other treatments.

Assessment of the proportion of active dispersal to total detachment showed that in the fed treatments 75% and 91% of total detachment was attributable to active dispersal from the substrate in static and agitated treatments respectively, whist for the starved treatment it was just 28% and 24%. In the treatment starved after attachment, 39% and 24% of total detachment was due to active dispersal in static and agitated treatments respectively, whilst in the treatment starved after 3 days, 14% was attributable to active dispersal in static treatments, with no detachment in agitated conditions. In treatments with reduced feeding, seed appear more likely to detach but remain in place, whilst fed seed are more likely to crawl away.

Percentage mortality was not significantly influenced by either feeding regime (ANOVA F=1.75, P=0.180, DF=3) or water condition (ANOVA F=0.16, P=0.691, DF=1). However, it was determined that there was a significant combined effect of these two variables (ANOVA F=5.15, P=0.006, DF=3) as mortality was lower in the agitated treatments compared to the static treatments at all feeding levels except in the treatment that was starved after attachment (Table 4.2). In fact in static conditions lowest mortality was in seed starved after attachment, but this feeding treatment had the highest mortality in agitated conditions.

Influence of seed density

Experiment 4 assessed the influence of seed density on attachment, detachment and dispersal of seed from wool and glass substrates after 20 days. Attachment to the wool substrate was uniformly high, ranging from 90.3 to 97.3% at stocking densities from 1 to 200 seed cm^{-2} with a mean attachment rate of 95.2±1.5%. Attachment to the glass substrate was more variable, ranging from 32.1 to 99.3%, with a mean rate of 81.9±21.4%. However, irrespective of density, there was no significant difference in attachment to the two substrates (Moods median test: $Chi^2 = 3.60$, P=0.058, DF=1).

Figure 4.7 shows the total detachment of attached seed and loss as a consequence of dispersal behaviour from the glass and wool substrates. On both substrates the total detachment of mussel seed and dispersal cm^{-2} increased with density of attached seed mussels. The loss of attached seed was correlated to initial attachment density on each substrate and was significant for both total detachment (Pearson's Correlation Glass: r=0.898, P<0.001; Wool: r=0.721, P<0.001) and for active dispersal (Pearson's Correlation Glass: r=0.958, P<0.001; Wool: r=0.872, P<0.001).

Multiple linear regression analysis for total detachment showed that the intercepts of the relationships (Intercepts: Glass=-2.176±0.812 total detached seed cm^{-2}; Wool=0.103±0.812 total detached seed cm^{-2}) were not significantly different from zero (ANOVA F=1.97, P=0.169, DF=1). Total detachment was significantly influenced by initial attachment density (ANOVA F=87.77, P<0.001, DF=1), and the rate of loss differed significantly between the two substrates (ANOVA F=14.36, P=0.001, DF=1), with a detachment rate of 0.158±0.017 seed per attached seed cm^{-2} from the glass substrate and 0.067±0.02 seed per attached seed cm^{-2} from the wool substrate. This equates to a 2.36 times greater rate of detachment of seed from glass than from wool.

Analysis of active seed dispersal showed that the intercepts of the relationships (Intercepts: Glass=-0.231±0.157 dispersed seed cm^{-2}; Wool=-0.129±0.157 dispersed seed cm^{-2}) were not significantly different from zero (ANOVA F=0.10, P=0749, DF=1). Active dispersal was significantly influenced by initial attachment density (ANOVA F=254.65, P<0.001, DF=1), with the dispersal rate differing significantly between the two substrates (ANOVA F=85.38, P<0.001, DF=1). The rate of dispersal was 3.69 times greater from glass than from wool, at 0.059±0.003 seed per attached seed cm^{-2} from the glass substrate and just 0.016±0.003 seed per attached seed cm^{-2} from the wool substrate.

For seed attached to the glass substrate, the rate of active dispersal accounted for 37.1% of the rate of total detachment, whilst for seed on the wool substrate the rate of active dispersal accounted for 23.3% of the rate of total detachment.

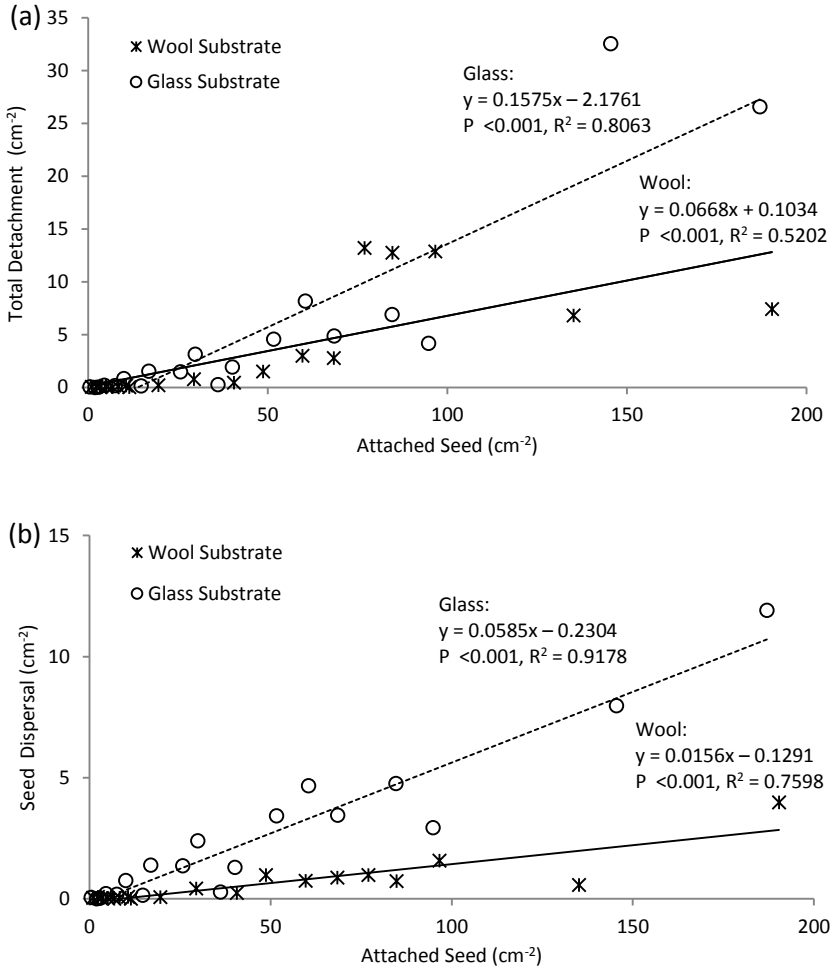

Figure 4.7: Relationship between (a) the total detachment of *Mytilus edulis* seed (seed cm^{-2}) and (b) active dispersal (seed cm^{-2}) to the density of attached seed (cm^{-2}) on glass and wool substrates.

Influence of pedal crawling behaviour

Experiment 5 assessed the influence of seed pedal crawling activity on attachment and subsequent total detachment, active dispersal and mortality on wool and glass substrates. Table 4.2 shows the mean percentage values of each of the test treatments in experiment 5. Analysis show that there was no significant difference in seed attachment, which was high in all treatments (ANOVA F=2.64, P=0. 093, DF = 3) (Table 4.2).

Total detachment was low, at less than 20% in each treatment, and was not significantly influenced by either seed activity (ANOVA F=0.15, P=0.709, DF=1) or substrate type (ANOVA F=0.51, P=0.486, DF=1). However, it was determined that there was a significant combined effect of these two variables (ANOVA F=7.87, P=0.015, DF=1) as detachment was higher from the glass substrate in the static group, though on the wool substrate detachment was higher from the active group (Table 4.2).

Whilst not significant, lowest detachment was seen in the static seed on the wool substrate (Table 4.2). Notably, there was higher detachment from glass substrates in experiment 5 than in experiment 3 under the same conditions.

Percentage active dispersal of seed was not influenced by either seed activity (ANOVA F=0.02, P=0.892, DF=1) or substrate type (ANOVA F=2.52, P=0.136, DF=1), neither was there an interaction effect between the variables (ANOVA F=4.61, P=0.051, DF=1). Although not significant lowest dispersal was seen in static seed attached to the wool substrate. Active dispersal of seed accounted for 100% of total detachment in both static and active seed on the glass substrate, and 71 and 83 % in static and active seed respectively on the wool substrate (Table 4.2).

Analysis showed that seed activity had a significant influence on the level of seed mortality recorded (ANOVA F=9.96, P=0.008, DF=1). Mortality was significantly lower in seed displaying increased crawling activity prior to attachment (Bonferroni $P<0.05$) (Table 4.2). Substrate type did not influence mortality (ANOVA F=3.15, P=0.099, DF=1), nor was there a significant interaction between the factors (ANOVA F=0.12, P=0.740, DF=1).

Discussion

Maximising the retention of valuable seed animals is a priority for bivalve aquaculture, since dispersal is potentially more important than mortality for the population dynamics of juvenile bivalves over small and meso spatial-time scales (Norkko *et al*., 2001). This study provides an important insight into the influence of factors including substrate, attachment period, feeding level, water agitation, and density on attachment and detachment, as well as seed activity as a predictor of seed condition for the mussel *M. edulis*.

Influence of substrate type and attachment period

The ability of bivalve seed to attach is the first hurdle to address in the assessment of any novel substrate. In the wild young juvenile *M. edulis* will typically attach to filamentous objects, such as algae and hydroids, before dispersing to a permanent settlement site , typically established mussel beds, stones, rocks, moorings and other hard surfaces (Bayne, 1964b; Spencer, 2002). Commercial collection of mussel seed is undertaken using a variety of materials, including natural fibres such as

coconut and sisal, and synthetic materials such as polypropylene and polyethylene (Dare and Davies, 1975; Spencer, 2002). However, the effectiveness of artificial and natural substrates as bivalve spat collectors is influenced by the substrates properties (Pearce and Bourget, 1996; Devakie and Ali, 2002; Saucedo *et al*., 2005; Brenner and Buck, 2010). Pearce and Bourget (1996) found that spat of the scallop *Placopecten magellanicus* showed a preference for polyester filter wool over substrates including nylon monofilament, polyethylene onion bag, polyethylene astroturf, and smooth and rough acrylic. Spat also showed a preference for substrates with a natural biofilm, and polyethylene mesh with a 3.0mm mesh diameter over mesh of other diameters. Surface properties of substrates have also been found to affect attachment by *M. edulis*, with the spatial structure of the substrate influencing the build-up of mussel/substrate conglomerates, with the most suitable substrates formed from nylon and natural fibre materials (Brenner and Buck, 2010). However, a one size fits all substrate may not exist, as Brenner and Buck (2010) also determined that substrate preferences change as mussels progress through their life cycle stages, with *M. edulis* showing a changing preference from microfiber fleece to filamentous substrates with increasing size, thereby impacting upon seed retention. In addition to the suitability of material as collectors and sites of settlement, choice of material is also based upon availability, durability and cost (Spencer, 2002). In this study, experimental substrates were not chosen to imitate natural substrates but instead to reflect a range of alternative textures on which to assess the influence of seed retention. The behaviour of seed in relation to their environment and handling is important to future hatchery operations for this species. Furthermore the natural fibre materials were seen as offering a novel substrate, with hatchery produced seed settled upon them before either being laid out on benthic on-growing plots or deployed from suspended structures such as being wound round bouchots or ropes. Our results show that attachment of *M. edulis* seed was significantly influenced by substrate type, as well as by attachment period, though variability was generally high. Seed attached to all tested substrates examined during the study, however there was a preference for the natural fibre wool material with attachment up to 95.2% after 6 hours in experiment 4, followed by smooth glass, with attachment up to 89.2% after 6 hours in experiment 5, although attachment to both substrates did vary. In contrast attachment to the hard slate was generally lower and more variable, with mean attachment ranging from 40.3% to 75.9% after 6 hours over the course of the study. This may be related to the life stage of the seed, however this does not explain the preference for glass. Whilst glass is unsuitable for large scale application in a hatchery or in open water, the wool felt offers a potentially attractive mechanism that is both an abundant and renewable material. However, testing of its continued suitability for larger seed and juveniles would be beneficial (Brenner and Buck, 2010). Both glass and wool were used in subsequent experiments. In addition, seed allowed longer to

attach had increased attachment; peak attachment was from approximately 6 hours, and this period was chosen for subsequent experiments, although 3 hours was generally sufficient. Previously longer attachment periods in bivalves have been associated with increased retention and ability to resist higher drag forces exerted by water currents (Gagné *et al*., 2012).

Influence of food availability

Food availability is believed to influence bivalve seed attachment and retention. There is a potential link between nutritionally compromised seed and their ability to remain attached due to a lack of energy reserves to undertake byssus production (Carton *et al*., 2007). In some cases food availability may not necessarily influence attachment in natural environments (Price, 1982), with Lachance *et al*. (2008) showing that for *M. edulis* in the Gulf of St Lawrence, Canada, variations in attachment strength were independent of variations in natural food availability. However, the metabolic requirement for byssus formation has been estimated to be up to 8% of monthly energy expenditure in mussels and must compete with the energetic demands of other biological activities (Hawkins and Bayne, 1985; Carrington, 2002). In natural mussel populations, seasonal variations in attachment tenacity have been linked to reproductive cycles, with tenacity low during periods of high reproductive development, as byssal production and quality is compromised by the reduced availability of energetic resources (Carrington, 2002; Moeser and Carrington, 2006). Whilst the energy reserves of spat stage animals will not be compromised by the necessity to partition energy towards reproductive efforts, it is still feasible that conflicting demands for limited resources, such as for growth, may impact on byssus production in spat. However, Clarke (1999) has demonstrated that starved mussels continue to partition resources to the production of fewer threads, whilst limited food availability leads to the sole transfer of resources to byssus production, as opposed to soft tissues. Similarly Babarro *et al*. (2008) showed that in juvenile *Mytilus galloprovincialis* (26-30mm shell length) a significant drop in byssus secretion and attachment force was observed following starvation for seven days, with simultaneous reduction in condition and glycogen stores, suggesting a constant transfer between soft tissues and byssus under stress. Periods of low tenacity are known to place mussel populations at increased risk of dislodgement by excessive hydrodynamic forces (Carrington, 2002). Carton *et al*. (2007) suggested that secretion of fewer or mechanically weaker byssus threads would lead to lower retention, or alternatively low food availability and diminishing seed condition may trigger secondary dispersal behaviour to sites with improved feeding conditions. Carton *et al*. (2007) showed that byssus detachment in seed of the mussel *P. canaliculus* increases significantly as a consequence of starvation, for periods of 4 days or more. However

retention can be substantially increased in nutritionally compromised seed by artificially feeding prior to transfer to on-growing sites. In the present study, as found by Carton *et al*. (2007), attachment was unaffected by starvation of seed for up to 6 days, and mortality was unaffected by feeding levels. However, in contrast to Carton *et al*. (2007) our results show that feeding seed pre- and post-attachment significantly reduced seed retention by increasing detachment. Whilst this behaviour may increase the potential for dispersal by water currents the results presented here indicate that fed seed are more likely to undertake active dispersal, since ≥75% of detachment was associated with dispersal to alternative attachment sites. Seed on restricted feeding regimes were less likely to detach, however they are more likely to remain *in situ* if they do; <40% of detached seed in this group moved off the substrates. In food compromised seed a slightly higher rate of detachment and active dispersal was registered in *M. edulis* seed starved for 6 days pre-attachment, although this was not always significant. This suggests that longer starvation events may decrease attachment, although this would require further investigation to confirm. Whilst food limitation is unsuitable for seed culture, the results suggest that food limited seed may lack the energy reserves for active dispersal either through pedal crawling or byssus drifting unlike their fed counterparts, and may instead be reliant upon external forces, such as water currents, for dispersal to more suitable areas.

Influence of water condition

Hydrodynamic conditions are known to have a strong influence on bivalve dispersal and play a major role in recruitment and population structure (Martel and Chia, 1991; Commito *et al*., 1995; Knight *et al*., 2006; Hunt *et al*., 2007; Jennings and Hunt, 2009). Young bivalves of many species utilise water currents to facilitate secondary pelagic dispersal to new recruitment areas through byssus drifting (Bayne, 1964b; Sigurdsson, 1976; Beukema and de Vlas, 1989; Armonies, 1992). This dispersal occurs on a population-wide scale and has been associated with seasonal, diurnal and lunar cycles (Beukema and de Vlas, 1989; Armonies, 1992), and factors including temperature (Sörlin, 1988). There is also a correlation between post-settlement transport with rates of sediment transport and current velocity, with many species, particularly from soft-bottom habitats, such as *Mya arenaria*, *Mercenaria mercenaria* and *Gemma gemma* utilising bedload transport as a means of dispersal (Commito *et al*., 1995; Hunt *et al*., 2007; Jennings and Hunt, 2009). However, active processes involving a behavioural response by bivalves commonly play a role in either promoting or preventing the chance of dispersal by water currents (Sörlin, 1988; Roper *et al*., 1995; Lundquist *et al*., 2004; Jennings and Hunt, 2009). It is logical to think that detachment of byssus could be considered as a

means of promoting dispersal. Laboratory studies have also shown that bivalve behavioural responses to unsuitable habitats can be influenced and modified by changing water velocities, with incidence of pedal crawling or byssus drifting increasing in conjunction with water velocity (Roper *et al*., 1995; Lundquist *et al*., 2004). Hydrodynamic conditions also play a significant role in stimulating byssus attachment in *M. edulis*, thereby influencing mussel retention. It has been repeatedly demonstrated that attachment by *M. edulis,* in terms of both byssus production and strength, increases in response to increasing water agitation, turbulence or flow velocity, with attachment significantly reduced in static water environments (Glaus, 1968; Price, 1982; Dolmer and Svane, 1994; Lachance *et al*., 2008). Dolmer and Svane (1994) found that in still water attachment strength was estimated to be just 21% of potential strength, whilst at a water velocity of 7.7cm $second^{-1}$ measured strength constituted 40%, and at a velocity of 19.4cm $second^{-1}$ measured strength constituted 81%. In natural assemblages, decreased byssus strength in *M. edulis* has been associated with a reduction in wave action (Price, 1982). Work by Young (1985) suggests that increased byssus formation is stimulated by direct agitation of mussels, with formation reduced in undisturbed individuals. The results of the present study shows that static conditions are sub-optimal as water agitation increased the subsequent retention of individuals. This is in line with the findings of previous studies. Water agitation decreased dispersal behaviour to alternative attachment sites by between 62 to 100% and total detachment by 55 to 100%, without affecting seed survival. In particular water agitation heavily reduced the influence of food in fed seed, with total detachment reduced by 84%. The stimulation of increased and stronger attachment by water movement decreases the likelihood of detachment and dispersal, a situation beneficial to future culture efforts for this species. It should therefore be considered that the provision of sufficient water agitation is essential for hatchery systems and even sheltered environments to reduce detachment and dispersal. However, it must be considered that whilst water agitation in this study was sufficient to encourage retention, it was insufficient in strength to provide an effective means of dispersal. As found by Lundquist *et al*. (2004) the assessment of critical culture and environmental factors such as substrate properties, food availability and seed density under flowing water conditions may provide important insights into the relationships acting on post-settlement dispersal behaviour.

Influence of seed density

Density is known to play a significant role in the regulation of growth, reproductive effort and mortality in bivalve populations (Josefson, 1982; Okamura, 1986; McGrorty *et al*., 1990; Jensen,

1993; Lauzon-Guay *et al*., 2005). It is also an influential factor on bivalve recruitment and dispersal patterns (Commito *et al*., 1995; Turner *et al*., 1997; Hunt and Mullineaux, 2002). However, dispersal may be species and location specific, since Hunt *et al*. (2007) suggests that sediment transport is more important than local density in driving juvenile bivalve dispersal. In the case of *M. edulis,* they are adapted to living in dense aggregations with more mussels producing byssus threads when clumped together than when maintained as individuals, possibly due to tactile stimulation of the foot in contact with other mussels (Martella, 1974; Gosling, 2003). They also show little tendency to move within established groups (Okamura, 1986), indicating increased retention of mussels in aggregations. However, populations of benthic suspension feeders, including bivalves will naturally undertake self-thinning as a consequence of space availability (Fréchette and Lefaivre, 1990), and high seed losses of *M. edulis* in the first year after settlement have been correlated with density (McGrorty *et al.*, 1990). For highly mobile species, particularly seed stage animals, it is logical to think that a density-dependent response could be to increase dispersal. High rates of juvenile bivalve dispersal from sandflat habitats has been reported by Norkko *et al.* (2001), with a 50% turnover of post-larvae <1mm within 17.4 hours and 50% of 1-4mm juveniles in 31.5 hours from an area of 0.25m^2, with active dispersal playing a significant role as dispersal rates could be uncoupled from sediment transport. Powers and Petersen (2000) demonstrated that at high water velocities, up to 0.28m $second^{-1}$, dispersal of the scallop *Argopecten irredians concentricus* increased with density of scallop, with 0% at 12 scallop m^2, 60% at 25m^2 and 71% at 62m^2. Hunt and Mullineaux (2002) also found that the number of transported post-larvae of the clam *Mya arenaria* was related to density, with higher densities leading to higher transport. Equally in the deposit-feeding gastropod *Hydrobia ventrosa,* whilst movement was impeded with increasing density, floating behaviour was increased, providing a mechanism for dispersal from dense populations, as the animals become susceptible to water currents (Levinton, 1979). The results of the present study show that mussel seed detachment and dispersal is proportional to seed density, with total detachment rates of 0.067 to 0.158 seed per attached seed cm^{-2} and dispersal rates of 0.016 to 0.059 seed per attached seed cm^{-2}, at densities up to 200 seed cm^{-2} on glass and wool fibre substrates. On each substrate seed detached and remained *in situ* or dispersed to alternative attachment sites. The rate of detachment and active dispersal was also significantly influenced by the type of substrate, with both being lower on the fibrous wool than from the smooth glass. The results indicate that the choice of substrate has a major impact upon seed behaviours. From an aquaculture perspective substrate type has a significant impact upon stocking density with some substrates offering higher retention of more seed. In this study there was little detachment from the wool substrate up to approximately 50 seed cm^{-2}.

Predicting future performance

The ability to predict future performance of bivalve seed by determining seed quality and fitness represents a useful tool for bivalve aquaculture. Previous work has demonstrated that simple and practical assessments can be applied. Webb and Heasman (2006) showed that the uptake of fast green dye by the mussel *P. canaliculus* was inversely proportional to spat health and fitness. Staining increased in spat exposed to stressors, with lethally stressed spat showing the greatest staining. Whilst righting and recessing behaviour of *Pecten maximus* has also been found to be directly proportional to length of desiccation event and the density of individual seed, with recessing speed decreasing with increase in stressor (Maguire *et al*., 1999a,b). Carton *et al*. (2007) demonstrated that seed activity and behaviour could be used as a measure of future retention performance, since *P. canaliculus* seed that rapidly attached in a flume tank had significantly higher retention rates on ropes than seed which failed to attach in the flume. In the present study, as found by Carton *et al*. (2007), pedal crawling could not be used as a means of predicting either initial attachment to substrates or subsequent retention. However, a direct link between seed activity and seed mortality was identified, with seed showing increased mobility displaying on average 80 to 100% lower mortality over the study. This indicates that seed activity could be used as a predictor for seed survival, with seed displaying increased activity having a higher level of fitness.

Conclusions

In conclusion attachment and retention of the blue mussel *M. edulis* is clearly affected by factors including substrate characteristics, attachment period, food availability, water agitation and seed density. In this study attachment was highest to glass and wool substrates, however highest retention of seed was in agitated water conditions on wool substrates, with wool able to support higher stocking densities. Whilst limiting food availability also increased retention, this would understandably be detrimental to aquaculture efforts, and the impact of feeding can be reduced by the application of water agitation. The results of this study contribute to our increasing knowledge of seed behaviour in this species and the influence of environmental factors. However, it is clear that influential factors should not be considered in isolation, since multiple variables can operate collectively to influence bivalve behaviour and the effects of one factor can modify and be modified by another (Paul, 1980; Lundquist *et al*., 2004). The natural fibre wool stands out as a substrate material warranting further investigation, with high rates of attachment and retention, whilst being a renewable natural resource. Testing this material in the field is recommended. Seed activity, in

this case pedal crawling, can also be used as a simple measure of seed quality, since seed displaying increased crawling behaviour prior to attachment show decreased mortality over seed showing little or no movement.

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Chapter 5

Impact of environmental conditions on the attachment and detachment of juvenile *Pecten maximus* (L.).

Abstract

Environmental factors play a key role in the control of byssus attachment and detachment of many bivalve species. Identifying and understanding the impact of influential factors is essential in order to optimise the management and culture of commercially important species, such as the scallop *Pecten maximus.* This study assessed the impact of substrate type, substrate pre-conditioning, attachment period, feeding ration, agitation and illumination on attachment of juvenile scallops between 1.9 and 7.2mm in shell length. Testing a variety of substrates including slate, nylon mesh, glass, and the natural fibre materials, wool felt, hemp, cotton and soy, showed that scallop juveniles have a preference for the textured slate surface, with mean attachment rates up to 75.6±14.4% after 24 hours. Attachment periods longer than 1 hour are essential to allow juveniles sufficient time to attach, as significantly higher attachment to all substrate material occurred after a time period of 24 hours (ANOVA $P<0.05$). Attachment was boosted by pre-conditioning substrates in flow-through tanks of unfiltered seawater for 1 to 2 weeks, with significant improvements seen on substrates conditioned for 2 weeks over clean substrates (Tukey $P<0.05$). However, parameters including agitation (static vs agitated conditions), illumination (24 hours illumination vs 24 hours darkness) and feeding regime (starvation, 0.025g, 0.05g and 0.15g organic weight of microalgae g^{-1} spat (live weight) $week^{-1}$) had no impact on juvenile attachment. The impact of substrate type, substrate pre-conditioning and feeding ration on detachment of juvenile seed was also examined in a benthic water flume over increasing velocities (0, 7.2±0.1, 10.1±0.2, 12.2±0.1 and 12.6±0.2 cm $second^{-1}$). In each experiment detachment increased with increasing water velocity. However of the substrates examined (wool, nylon and slate) although retention was variable, over the course of the study slate ensured the greatest chance of retention, with attachment up to 100% at 12.6±0.2 cm $second^{-1}$. In contrast to attachment, comparison of substrate pre-conditioning showed that under increasing water velocities conditioning for 2 weeks significantly compromises juvenile retention (Tukey $P<0.05$). Feeding regime also impacted upon retention with detachment highest by starved animals, with retention significantly higher in juveniles fed 0.025g microalgae. Feeding higher rations showed no improvement in retention over juveniles fed just 0.025g or starved juveniles. The results

of this study contribute to our expanding knowledge of juvenile attachment and detachment in this species. Based upon the current findings recommendable parameters for maximising juvenile *P. maximus* attachment and retention in water velocities up to 12.6±0.2cm second^{-1}, include utilising a slate type substrate, pre-conditioned for 1 week, with juveniles having been fed a diet of at least 0.025g microalgae.g^{-1} juveniles.week^{-1}, and allowed to attach for 24 hours.

Keywords

Attachment, Detachment, Juveniles, Scallop, *Pecten maximus*

Abbreviations

*Um*ax	Maximum water velocity
Uav	Depth-average water velocity
*U**	Shear water velocity

Introduction

Most pectinid scallops begin their settlement stage attached by thin byssal threads to benthic surfaces, enabling them to withstand hydrodynamic pressures and avoid dislodgement from their chosen environment (Beninger and Le Pennec, 1991; Minchin, 1992). It has repeatedly been demonstrated that byssus production, strength and attachment are a reflection of prevailing environmental conditions. In bivalves, temperature, salinity (Allen *et al*., 1976; Paul, 1980a,b; Christophersen and Strand, 2003), agitation (van Winkle, 1970; Young, 1985), desiccation (Carton *et al*., 2007), diet (Carton *et al*., 2007; Babarro *et al*., 2008; Gagné *et al*., 2012), substrate type and condition (Pearce and Bourget, 1996; Brenner and Buck, 2010), as well as hydrodynamic forces (Dolmer and Svane, 1994; Lachance *et al*., 2008; Gagné *et al*., 2012) all play an influential role on attachment and retention. Attachment loss has been associated with exposure to sub-optimal and stressful conditions (Carton *et al*., 2007), which has been linked to secondary dispersal behaviour, including pedal crawling and byssal drifting (Roper *et al*., 1995; Lundquist *et al*., 2004).

In the wild, young post-settlement individuals of the commercially important scallop *Pecten maximus* are generally found in shallow and sheltered bays attached to surfaces free of silt, including algae, invertebrates and materials such as coconut fibre, polypropylene rope, monofilament netting and pebbles, among others (Minchin, 1992). Attachment is only temporary with most remaining attached up to 4-13mm in shell length, with few attached greater than 15mm, after which they recess into sediments and are subject to dispersal by water turbulence and swimming (Beninger and Le Pennec, 1991; Brand, 1991; Minchin, 1992). Although brief, attachment represents a significant life stage with previous studies in pectinids associating rapid attachment, and high and stable attachment rates with high growth and survival (Paul, 1980a,b; Christophersen and Strand, 2003). In pectinids, including *P. maximus,* byssus attachment has been found to decrease with salinity (Paul, 1980a; Christophersen and Strand, 2003) and increase with temperature (Paul, 1980b; Christophersen and Strand, 2003), whilst short attachment periods and sub-optimal nutrition compromise retention, with detachment increasing with shear velocity (Gagné *et al*., 2012). Locomotion is also not restricted to unattached juveniles, as attached juveniles are able to separate the proximal end of the byssus thread from the stem of the foot by relaxing numerous muscle fibres, detaching the animal from its connection to the substrate (Beninger and Le Pennec, 1991). This enables juveniles to disperse, with early post-larvae plantigrades alternating between periods of attachment and periods of pedal crawling (Gruffydd and Beaumont, 1972). In addition juveniles less than 500µm in shell length are also able to employ byssal drift, a widespread and effective

mechanism employed by a large number of bivalve species. This involves the secretion of long threads that increase the viscous drag exerted upon the young animals, enabling them to disperse on relatively weak currents (Sigurdsson *et al*., 1976; Lane *et al*., 1985; Beaumont and Barnes, 1992). Like most pectinids, *P. maximus* is also able to swim, with juveniles as small as 3mm, but usually greater than 5mm, actively swimming or jumping by ejecting water from the mantle cavity to propel them through the water column, an ability retained throughout their benthic existence (Brand, 1991; Minchin, 1992). *P. maximus* is an emerging aquaculture species (Spencer, 2002; Andersen *et al*., 2011), and the influence of many pre- and post-attachment factors on juveniles under different conditions remains to be determined. Animals that are attaching, detaching or actively dispersing are diverting valuable resources away from somatic growth. Furthermore both wild collected and cultured spat typically have to be detached from initial settlement materials, typically mesh bags or screens, before transfer to new equipment, such as mesh trays and pearl nets for subsequent culture stages (Millican, 1997; Andersen *et al*., 2011), it is therefore important that re-attachment is promoted. Understanding how environmental factors affect seed both in terms of attachment and dispersal will allow effective management strategies to be adopted in hatcheries, supporting the improvement of scallop culture by innovating juvenile nursery systems (Andersen *et al*., 2011), whilst providing increased knowledge on the natural ecology of this species.

In this series of experiments we examined the influence of different husbandry and environmental conditions on promoting attachment and retention of *P. maximus* seed animals. Our specific goals were (1) to assess the impact of variables likely to be encountered during hatchery culture on seed attachment, including substrate type and pre-condition, attachment period, illumination level, water agitation and feeding level, and (2) to measure the impact of water flow velocity on the level of seed detachment and retention, and the level of interaction with other environmental conditions.

Materials and Methods

Scallop source

Veliger *P. maximus* larvae (202 ±19µm in shell length) were obtained from the Scalpro AS hatchery (Rong, Norway) and transported by air freight to the marine laboratory at the Centre for Marine Sciences, Bangor University (Anglesey, Wales). On arrival imported larvae were assessed, before stocking at a density of 5 larvae ml^{-1} in 65-litre static polyethylene tanks, filled to a volume of up to

45-litres with 1µm filtered, UV-light irradiated seawater (FSW), at a salinity of 33‰. Culture temperature was maintained at 16±1˚C. Three times a week the larvae were sieved onto a 45µm mesh screen and inspected, and the containers cleaned before the larvae were restocked. Larvae were fed with a mixed microalgae diet equivalent to 3.75 cells $x10^4$ cells ml^{-1} day^{-1} consisting of *Pavlova lutheri* (PLY75), *Isochrysis* sp. (clone T-ISO) (PLY506A) and *Chaetoceros calcitrans* (PLY537) at a ratio of 1:1:1. Following metamorphosis post-larvae were transferred to an on-growing system.

Juvenile on-growing

Juvenile scallops were maintained in a down-welling culture system. The system consisted of a narrow central tank suspended in a large 120 litre reservoir tank. Scallops were held in cylindrical sieves (160mm dia.) with mesh diameters ranging from 180µm - 500µm, connected to the central tank. Water was pumped from the reservoir to the central tank which flowed back to the reservoir through the sieves. Juveniles were progressively transferred to sieves with larger mesh diameters as they increased in size. The system was supplied with a constant trickle inflow (0.28±0.06 litres $minute^{-1}$) of seawater filtered to 10µm and maintained at ambient temperature (ranged from 10 to 20˚C). The system was fed daily a mixture of microalgae including *P. lutheri*, *Isochrysis* sp., *C. calcitrans*, *Rhinomonas reticulata* (CCAP 995/2), and *Tetraselmis chuii* (CCAP 8/6).

Seed conditioning

Prior to commencing experiments juveniles were "conditioned" for 6 days using a standardised husbandry protocol in an effort to ensure a comparable starting condition used throughout the study, as this could not be guaranteed with juveniles taken directly from the nursery system. A random group of juveniles were removed from the nursery system and graded using a series of nylon mesh sieves to between 2.0-5.0mm. These were conditioned for six days in clean static tanks of FSW at volume of 4 to 8-litres, at a temperature of 12±1˚C. In experiments 1 to 5, juveniles were stocked at a density of 1.0g (wet weight) juvenile scallops $litre^{-1}$. In experiment 6, juveniles were stocked at a density of 31 juvenile scallops $litre^{-1}$. Tanks were aerated and 100% water changes conducted every 2-3 days. Juveniles were fed a mixed microalgae diet equivalent to a cell concentration of 7.5 $x10^4$ cells ml^{-1} day^{-1} of *Isochrysis* sp., *P. lutheri* and *C. calcitr*ans, at a ratio of 1:1:1, unless otherwise stated. Measurements of shell height were made to determine the experimental size range after juvenile conditioning (Figure 5.1). Juveniles were digitally

photographed using the Canon EOS 1000D, and images measured using the image analysis software Image J.

Figure 5.1: Conditioned *Pecten maximus* juveniles.

Behavioural experiments

Six experiments were carried out to assess the influence of environmental variables on juvenile scallop attachment and detachment. Experiments 1 to 3 focussed on assessing attachment rates, experiment 4 assessed dispersal of unattached juveniles in flowing water conditions, whilst experiments 5 and 6 examined both attachment and the influence of variables on detachment in flowing water conditions. In all experiments juveniles were stocked at 0.5±0.05 juveniles cm^{-2} directly onto substrates (Figure 5.2). In experiments 1, 3, 5 and 6 attachment was carried out in static plastic dishes filled with 50ml of FSW, whist in experiment 2 substrates were set within a 1-litre glass dish filled with 250ml FSW. Juveniles in experiment 4 underwent no initial attachment period. Water temperature was maintained at 12±1˚C and attachment dishes were fed as described for conditioning. Dishes were undisturbed for the duration of the attachment period, and under constant illumination, unless otherwise stated. In all experiments attachment percentage was determined from the number attached to each substrate, after defined time periods. Substrates were gently rinsed to remove any unattached juveniles.

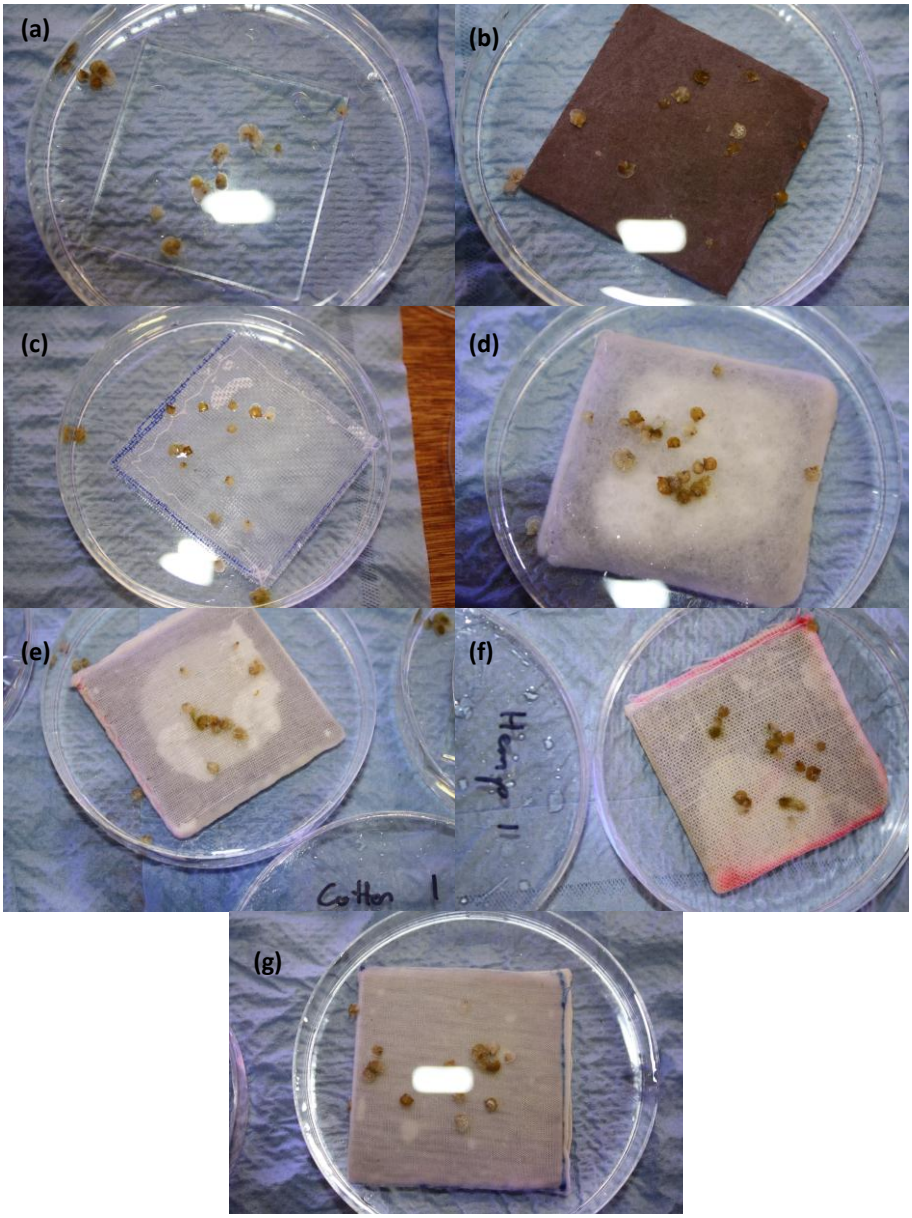

Figure 5.2: Juvenile *Pecten maximus* on (a) glass, (b) slate, (c) nylon mesh, (d) wool felt, (e) cotton fabric, (f) hemp fabric, and (g) soy fabric substrate tiles during attachment period.

Influence of substrate and length of attachment period

Experiment 1 tested attachment to seven substrates: glass, slate, nylon mesh with a 500µm diameter weave, and the natural fibre materials of wool felt, cotton, hemp and soy , over attachment periods of 1 hour and 24 hours (Figure 5.2). Experimental substrates were sourced from Hughes Glass & Glazing Ltd (Llandegai, North Wales) for glass, Huws Grays Building and Timber Merchants (Llandegai, North Wales) for slate, Clarcor UK for the nylon mesh (www.clarcoruk.com), Handmade Presents for wool felt (www.handmadepresents.co.uk), Greenfibres for cotton (www.greenfibres.com), and the soy and hemp fabrics were from Hemp Fabric UK

(www.hempfabric.co.uk). All substrates were formed into small tiles of similar dimensions. The glass was cut from standard glazing panes and the slate from roofing tiles. The glass substrate had mean dimensions of 50.4 x 50.2 x 3.9mm (width x length x height), the slate 50.6 x 50.6 x 3.5mm, the nylon mesh 53.9 x 53.9 x 1.5mm, the wool substrate 56.6 x 56.9 x 7.4mm, the cotton 54.3 x 53.9 x 5.2mm, the soy fabric 54.1 x 53.3 x 5.1mm, and the hemp 55.4 x 55.8 x 4.9mm. All fabric substrates were wrapped round a core of slate to provide a rigid structure. Five replicates of each substrate were tested over both time periods. Size range of juvenile scallops was 1.9mm to 5.5mm shell height (mean shell height 3.3 ±0.8mm).

Influence of water agitation on attachment

Experiment 2 compared juvenile attachment to wool, slate and nylon substrates in still and agitated water conditions. The water agitation treatment was generated by vigorous aeration through a 3mm airline, whilst the still water treatment was undisturbed. Five replicates for each substrate per water condition over a 24 hour period were carried out. Size range of juvenile scallops was 2.6 to 6.2mm shell height (mean shell height 3.4 ±0.9mm).

Influence of illumination on attachment

Experiment 3 assessed attachment under light levels of 24 hours illumination and 24 hours darkness on wool, slate and nylon substrates. Three replicates per substrate were conducted in 24 hours of darkness, and three replicates per substrate in 24 hours light. Size range of juvenile scallops was 2.7 to 5.7mm shell height (mean shell height 3.6 ±0.8mm).

Influence of substrate on the passive retention of unattached juveniles

Experiment 4 assessed the influence of substrate structure on passive juvenile retention with a group of unattached juveniles. These were placed directly onto nylon, slate and wool substrates set in a benthic water flume (Figure 5.3) at a density of 0.5 juveniles cm^{-2} with detachment recorded over increasing mean maximum water velocities of 0.0, 7.2±0.1, 10.1±0.2, 12.2±0.1 and 12.6±0.2 cm $second^{-1}$ for 10 minutes at each velocity. Each substrate was conducted in triplicate at each velocity. Water velocity is expressed as the mean (±standard deviation) maximum water velocity (mean *Umax*) across the three substrate types. In all flume experiments detachment was quantified from counts of juveniles at the beginning and end of each velocity period, with dispersed seed removed.

Influence of substrate and surface pre-conditioning on attachment and detachment

Experiment 5 compared juvenile attachment to wool, slate and nylon substrates pre-conditioned for 0, 1 and 2 weeks in a flow-through tank, with fresh seawater supplied via a settlement tank. In this experiment "pre-conditioning" refers to the length of time that substrates were exposed in seawater, prior to the transfer of juvenile scallops to the substrates, and is not to be confused with the 6 day conditioning period which juvenile scallops underwent before each experiment. The substrate pre-conditioning tank was a 120-litre polypropylene tank maintained at 12±1°C and supplied with an inflow of approximately 0.42±0.12 litres minute^{-1}. Seed attachment was over 24 hours, with each treatment conducted in triplicate per substrate, per pre-conditioning period. Size range of juvenile scallops was between 1.9 and 5.9mm (mean shell height 4.0 ±1.0mm). The impact of water velocity on attached juveniles was assessed by transferring substrates with attached juveniles to a benthic water flume. Attached juveniles were exposed to increasing mean *Umax* of 0.0, 7.2±0.1, 10.1±0.2, 12.2±0.1 and 12.6±0.2 cm second^{-1} for 10 minutes at each velocity.

Influence of feeding regime and substrate on attachment and detachment

Experiment 6 assessed the influence of four dietary rations, 0 (starved), 0.025g, 0.05g and 0.15g organic weight of microalgae g^{-1} spat (live weight) week^{-1} fed during the 6 day juvenile conditioning period, on attachment and detachment to wool, slate and nylon substrates. A ration of 0.15g microalgae g^{-1} spat (live weight) week^{-1} is recommended for optimum growth and maintenance of highest condition in juvenile *P. maximus* (Laing, 2000) Substrates were also pre-conditioned in seawater flow-through tanks for 1 week. Initial measurements of shell height of scallops were made prior to conditioning, to estimate mean live weight based on the formula: live weight (mg) = 0.0732 x shell height (mm)$^{2.89}$ (Laing and Psimopoulous, 1998). Scallops were fed daily a mixed microalgae diet of *Isochrysis* sp., *P. lutheri* and *C. calcitr*ans, at a ratio of 1:1:1 based upon organic weight (Table 5.1), equating to cell concentrations of 55 to 350 cells µl^{-1}.

Table 5.1: Organic weights of study microalgae (Helm and Bourne, 2004).

Microalgae species	organic wt (mg per 10^6 cells)
T-iso	0.02
Pavlova lutheri	0.02
Chaetoceros calcitrans	0.007

Three replicates of each substrate were assessed for each feeding level. Attachment was assessed after 24 hours. The size range of juvenile scallops was between 3.4 and 7.2mm, with a mean shell height 5.2±0.9mm. The impact of water velocity on attached juveniles was then assessed in the benthic flume at mean *Umax* of 0.0, 7.2±0.1, 10.1±0.2, 12.2±0.1 and 12.6±0.2 cm second^{-1} for 10 minutes at each water velocity.

Benthic water flume

Detachment of juvenile scallops was examined in a laminar flow re-circulating benthic water flume at the Centre for Applied Marine Sciences (Figure 5.3). The flume consisted of a 4m long and 0.4m wide fibre glass raceway tank with PVC bottom plate. A 40mm diameter return pipe running below the flume and connected to an Aqua-Medic Eco Runner 12000 circulation pump provided the water current. Water velocity was controlled by adjusting the diameter of the return pipe from the circulation pump between diameters of 15, 20, 30 and 40mm, corresponding to settings 1, 2, 3 and 4 respectively. Within 100cm of the flume entrance were positioned flow straighteners, consisting of a wooden baffle plate flush with the water surface, followed by a horizontal stack of pipes (diameter 15mm each).

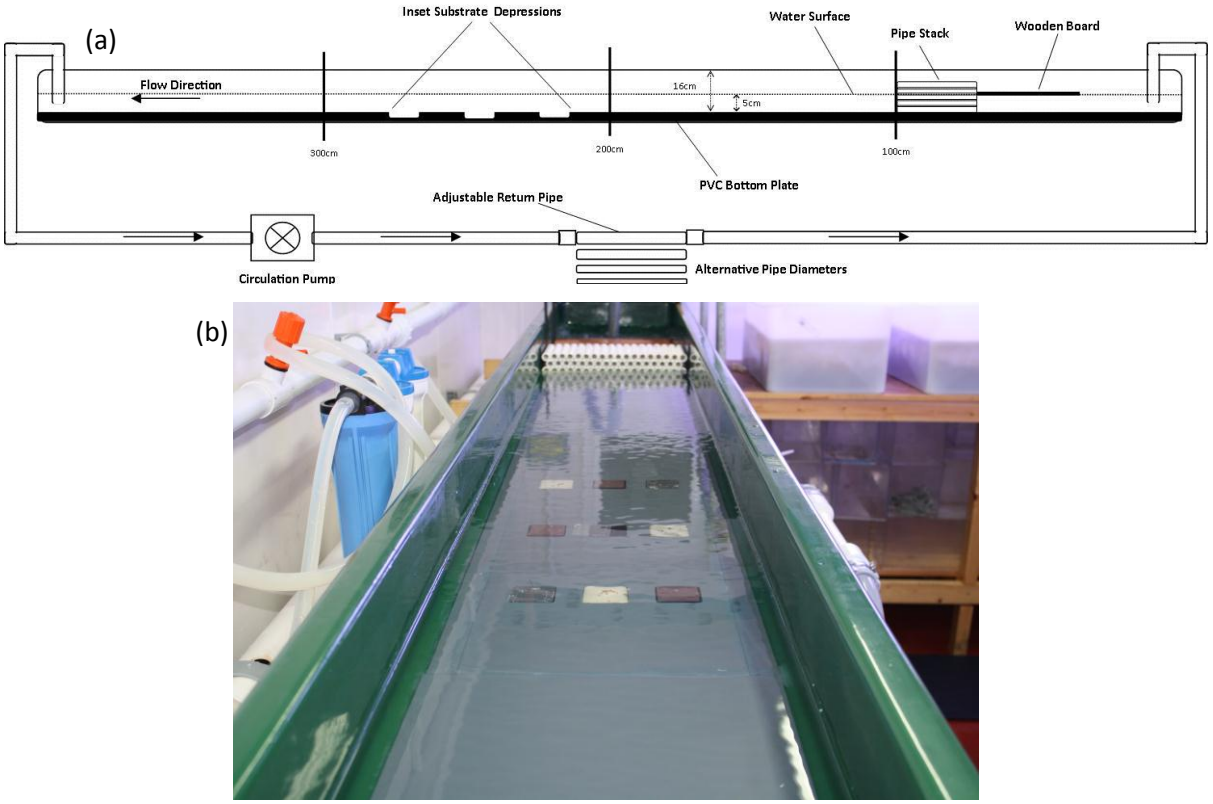

Figure 5.3: (a) Diagram of the benthic flume constructed to test the influence of water velocity on juvenile scallops; (b) photo of the internal layout of the flume.

Between 1.15m and 1.75m downstream of the pipe stack, in a section of the PVC bottom plate, nine 5.5x5.5cm depressions were distributed perpendicular and parallel to water flow in a 3x3 configuration (Figure 5.4). Each depression allowed a single substrate tile to be set flush with the flume floor. In each flume experiment nine substrate tiles were tested at once, corresponding to three replicates of each substrate from the same treatment. All tested substrates were evenly placed across the width of the flume.

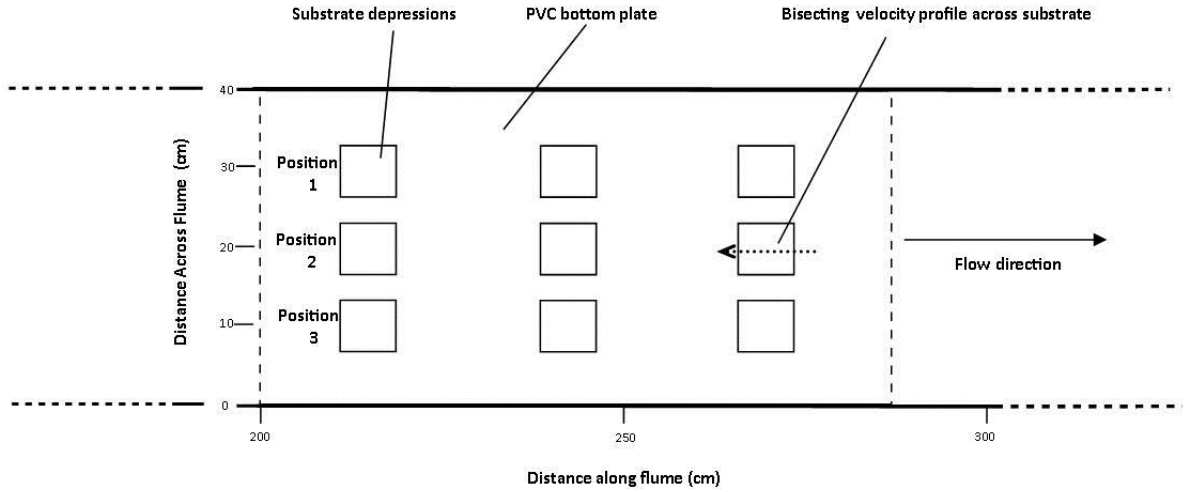

Figure 5.4: Plan view of the substrate section of the flume.

The flume was filled with FSW to a constant depth of 5cm, with an approximate total volume of 90 litres. Water temperature was maintained at 12±1˚C and experiments were carried out in constant illumination. In order to assess variations in flow conditions, velocity profiles were made across the flume stream bisecting each substrate for each flume velocity setting using a 4 MHz Ultrasonic Doppler Velocity Profiler (UDVP) with a diameter of 0.8cm (UVP-Duo Model by MET-FLOW). The UDVP quantified the velocity by determining the Doppler shift in ultrasound frequencies as small particles passed through the measurement volume. Particles derived from amino plastic media (Avialite Type 2, Mac'Ants Group, UK) were used as tracer particles for the UDVP. A single UDVP, set within a movable vertical array was employed to calibrate the flume. Profile measurements were made at heights of 0.5, 1.0, 1.8, 3.0 and 4.5cm above the flume floor. The UDVP acquired velocity data on a profile of up to 128 points along the axis of the ultrasound beam which extended to 15.1cm from the probe head, with a channel distance of 1.11mm and width of 0.74mm. Maximum water velocity (*Umax*), depth-average water velocity (*Uav*) and shear water velocity (*U**) were determined. *Umax* is the highest recorded velocity across each substrate at each flume setting and *Uav* is based upon logarithmic velocity distributions. Estimates of shear velocity (*U**) were derived from regression of velocity versus the natural log height above the flume bed. However, as the flow

in the flume was laminar this causes the profile to diverge from logarithmic, therefore *U** is only suitable as an indicator of difference between substrates.

Statistical analyses

All attachment data sets are described as the percentage of attached seed, whilst flume velocities are described as cm second^{-1}. Prior to analysing, all percentage data were converted by arcsine square root transformation. Data presented in all figures is untransformed. Data sets were tested using the Anderson-Darling test to investigate departure from normality and Bartlett's test to assess heteroscedasticity before applying any test of comparison (Sokal and Rohlf, 1995). ANOVA tests were used to determine if there was any significant difference in percentage attachment, followed by pairwise comparisons between treatments using Tukey's comparison test. All results were considered to be significantly different when $P<0.05$. Analyses were undertaken using the statistical package Minitab®.

Results

Influence of substrate, attachment period, agitation and illumination on attachment

The mean percentage attachment of juvenile scallops to seven experimental substrate materials after 1 and 24 hours in experiment 1 is shown in Figure 5.5, whilst Table 5.2 details the results of comparative analysis. The results show that the ability of scallops to attach was significantly influenced by both substrate type and length of attachment period (Table 5.2), although variability within treatments is noticeably high (Figure 5.5). Attachment after 1 hour was limited, with zero juveniles attaching on soy and cotton, however by 24 hours significantly more juveniles had attached (ANOVA $P<0.001$), with juveniles attached to all substrate types. Highest attachment was exhibited by juveniles to the slate substrate, with attachment significantly higher than on all substrates (Tukey $P<0.05$), except wool and glass (Tukey $P>0.05$). Attachment was lowest to the cotton substrate, but was only significantly lower than the slate, wool and glass substrates (Tukey $P<0.05$). All other substrates had approximately equal attachment (Tukey $P>0.05$). The high variability is likely to account for the limited difference seen between substrates. No combined effect of the two variables was observed (Table 5.2).

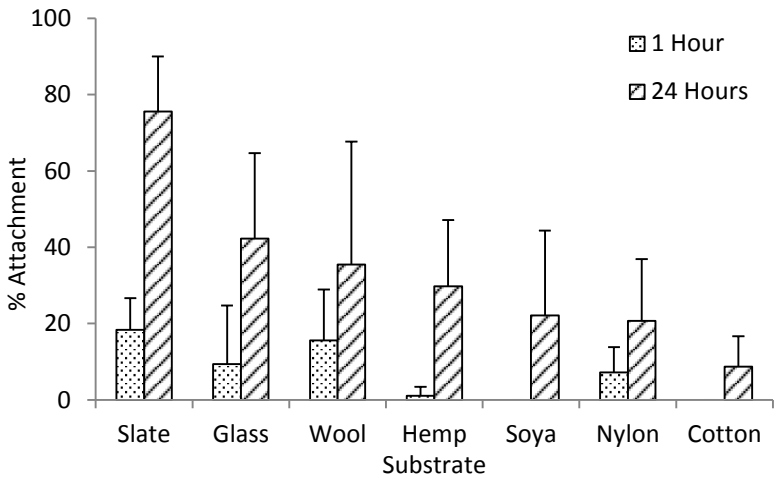

Figure 5.5: Mean percentage attachment (± standard deviation) of juvenile *Pecten maximus* (shell height 3.3±0.8mm) to seven different substrates over attachment periods of 1 and 24 hours.

Table 5.2: Two-way analysis of variance on the effect of different substrates and length of attachment period on the percentage attachment of juvenile *Pecten maximus* scallops. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	6	8543.2	8543.2	1423.9	8.80	<0.001*
Attachment period	1	9021.2	9021.2	9021.2	55.73	<0.001*
Interaction	6	1370.3	1370.3	228.4	1.41	0.227
Error	56	9064.7	9064.7	161.9		
Total	69	27999.5				

Neither water agitation in experiment 2 (Table 5.3) nor the light levels tested in experiment 3 (Table 5.4) significantly affected the levels of scallop attachment. Scallop attachment in agitated conditions was marginally higher, although not significantly, with 20.2±14.1% attachment on slate, 24.6±14.7% on wool and 11.3±12.0% on nylon, whilst in static conditions attachment was 17.2±11.8% on slate, 15.4±10.8% on wool and 10.3±9.8% on nylon. However, neither was there a significant difference in attachment between the three different substrates in this experiment, nor was there a combined effect. With the exception of the nylon substrate, attachment to slate and wool substrates was lower than in experiment 1.

Table 5.3: Two-way analysis of variance of the effect of agitated and static water conditions on the percentage attachment of juvenile *Pecten maximus* scallops to different substrate surfaces. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	519.9	519.9	259.9	1.87	0.177
Water condition	1	73.9	73.9	73.9	0.53	0.474
Interaction	2	14.4	14.4	7.2	0.05	0.950
Error	24	3344.1	3344.1	139.3		
Total	29	3952.2				

In experiment 3, scallop attachment in illuminated conditions was 16.8±12.8% on slate, 30.4±7.5% on wool and 19.0±5.8% on nylon, whilst in dark conditions attachment was 29.2±7.2% on slate, 45.0±10.8% on wool and 20.8±15.7% on nylon. Attachment was higher under dark conditions although the increase was not significant (Table 5.4). Substrate type did however have a significant influence in this experiment, with attachment significantly higher to wool than to nylon (Tukey $P<0.05$), whilst there was no difference between slate and either of the other substrates (Tukey $P>0.05$). There was no significant interaction between the variables.

Table 5.4: Two-way analysis of variance of the effect of light and dark conditions on the percentage attachment of juvenile *Pecten maximus* scallops to different substrate surfaces. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	484.49	484.49	242.25	4.38	0.037*
Light/Dark	1	168.83	168.83	168.83	3.05	0.106
Interaction	2	77.33	77.33	38.67	0.70	0.516
Error	12	664.00	664.00	55.33		
Total	17	1394.66				

Benthic flume conditions

The mean maximum flow velocities (mean *Umax*) for the flume, 7.2±0.1, 10.1±0.2, 12.2±0.1 and 12.6±0.2cm second^{-1} at inflow diameters of 15, 20, 30 and 40mm respectively, were used to classify operational water velocities for the flume, in addition to the static setting of 0.0cm second^{-1}. Table 5.5 summarises maximum water velocity (*Um*ax), average water velocity (*Uav*) and shear water velocity (*U*)* operational velocities across each substrate type.

Table 5.5: Maximum (*Umax*), average (*Uav*) and shear velocities (*U**) in cm second^{-1} (± standard deviation) recorded across three different substrates in a variable speed benthic water flume.

Substrate	Velocity measurement	Flume setting			
	(cm second^{-1})	1	2	3	4
Wool	*Umax*	7.1±0.3	10.1±1.2	12.3±1.3	12.9±0.6
	Uav	5.3±0.1	8.2±0.9	9.9±0.8	10.7±0.5
	*U**	3.3±1.1	5.9±2.2	9.9±2.5	9.0±3.5
Slate	*Umax*	7.3±0.6	10.4±0.7	12.2±0.9	12.5±0.6
	Uav	5.7±0.2	8.9±1.2	10.7±1.0	11.1±0.5
	*U**	0.0±0.9	3.4±0.2	4.7±0.8	4.5±0.3
Nylon	*Umax*	7.2±0.5	9.9±1.0	12.1±1.3	12.6±0.8
	Uav	5.7±0.5	8.7±0.9	10.7±0.8	11.1±0.5
	*U**	1.6±1.0	2.9±1.2	5.2±1.6	4.8±1.9

Analyses of flow velocity determined that for both *Umax* and *Uav* there was no significant difference between mean velocities over the wool, slate and nylon substrates or between the three positions across the flume (ANOVA $P>0.05$). However, for both *Umax* and *Uav* measurements of velocity increased significantly between flume settings up to setting 3 (Tukey $P<0.05$), whilst there was no difference between velocities recorded at setting 3 and setting 4 (Tukey $P>0.05$). Analysis of *U** showed that whilst lateral position across the flume had no influence (ANOVA $F=0.81$, $P=0.456$, $DF=2$), substrate type significantly affected velocity (ANOVA $F=8.51$, $P=0.001$, $DF=2$), with mean *U** on wool significantly greater than across both of the other substrates (Tukey $P<0.05$). *U** was approximately twice as high on wool compared to both nylon and slate, at each flume setting (Table 5.5). *U** increased with flume velocity setting (ANOVA $F=7.74$, $P=0.001$, $DF=3$), with settings 3 and 4 significantly higher than flume setting 1 (Tukey $P<0.05$), all other settings were approximately equal.

Influence of substrate and water velocity on unattached juveniles

In experiment 4 water velocity and substrate influenced the passive retention of juvenile *P. maximus*. Figure 5.6 shows the percentage decrease of unattached juvenile scallops retained on nylon, slate and wool substrates with increasing water velocities (cm second^{-1}), although results are variable. Table 5.6 summarises the statistical comparisons. The dispersal of unattached individuals from all substrates significantly increased with increasing water velocity (Figure 5.6, Table 5.6), with dispersal at the highest flow speed, 12.6±0.2cm second^{-1}, significantly higher than all other water velocities (Tukey $P<0.05$), except 12.2±0.1cm second^{-1} (Tukey $P>0.05$). Dispersal at all water

velocities was significantly greater than at 0.0cm second^{-1} (Tukey P<0.05), whilst dispersal at 7.2±0.1cm second^{-1} was less than at 10.1±0.2cm second^{-1}, which was approximately equal to 12.2±0.1cm second^{-1}. However, significantly more individuals were displaced from the slate and the nylon substrates than from the wool substrate in all flow treatments above 0.0cm second^{-1} (Tukey P<0.05), whilst there was no difference between slate and nylon. There was no significant interaction between water velocity and substrate on the dispersal of unattached juveniles.

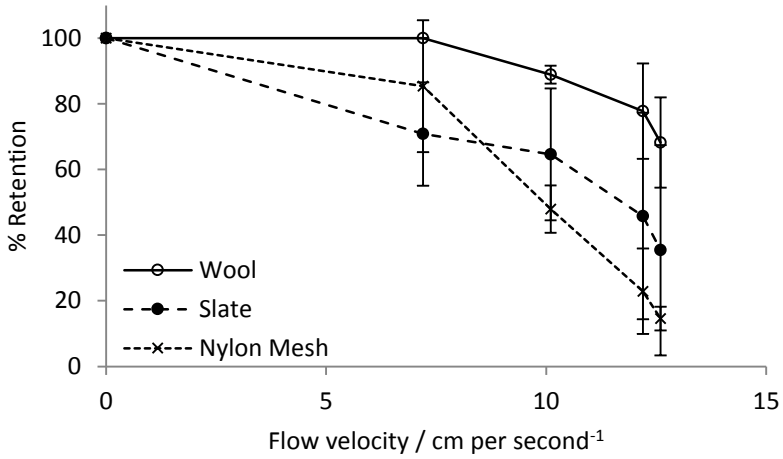

Figure 5.6: Mean percentage retention (± standard deviation) of unattached juvenile scallops (shell height 4.0±1.0mm) on wool, slate and nylon substrates in a benthic water flume over a series of increasing water velocities.

Table 5.6: Two-way analysis of variance of the influence of substrate and water velocity on the passive retention of juvenile *Pecten maximus* scallops on three different substrate surfaces. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	4363.2	4363.2	2181.6	15.74	<0.001*
Water Velocity	4	17078.5	17078.5	4269.6	30.81	<0.001*
Interaction	8	1921.4	1921.4	240.2	1.73	0.131
Error	30	4158.0	4158.0			
Total	44	27521.2				

Influence of substrate and surface pre-conditioning on attachment and detachment

Figure 5.7 shows the percentage attachment of juvenile scallops after 24 hours to slate, wool and nylon pre-conditioned in seawater for 1 and 2 weeks compared to unconditioned substrates in experiment 5. The results in several treatments show a high degree of variability. Table 5.7

summarises the statistical comparisons. There was no significant effect of substrate type on attachment of juvenile scallops in this experiment, however there was a difference between substrate pre-conditioning treatments. Attachment to substrates pre-conditioned for 2 weeks significantly higher than unconditioned substrates (Tukey P<0.05). This is likely a consequence of the disparity in attachment to unconditioned nylon mesh compared to pre-conditioned nylon, since for wool and slate although mean attachment increased with pre-conditioning period the increase was within observed levels of variability. No significant interaction between the variables was observed.

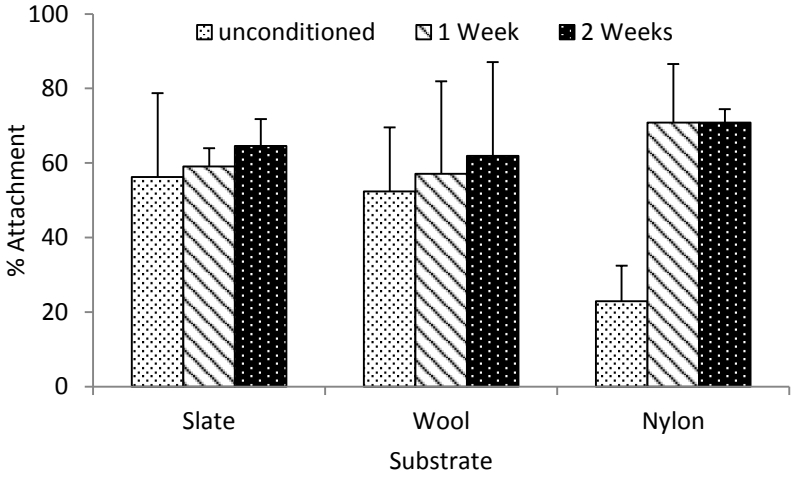

Figure 5.7: Mean percentage attachment (± standard deviation) of juvenile *Pecten maximus* (shell height 4.0±1.0mm) to three substrate types pre-conditioned in sea water for 0, 1 and 2 weeks after 24 hours.

Table 5.7: Two-way analysis of variance of the influence of substrate pre-conditioning on the percentage attachment of juvenile *Pecten maximus* scallops to three different substrate surfaces. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	41.7	41.7	20.8	0.19	0.828
Conditioning	2	958.4	958.4	479.2	4.37	0.028*
Interaction	4	875.3	875.3	218.8	2.00	0.138
Error	18	1972.0	1972.0	109.6		
Total	26	3847.4				

Figure 5.8 shows the mean percentage retention of juvenile scallops on slate, wool and nylon substrates pre-conditioned for 0, 1 and 2 weeks under different water velocities in experiment 5. The results show a high degree of variability. Table 5.8 summarises the statistical comparisons from a 3-way ANOVA. Analysis showed that the level of substrate pre-conditioning and water velocity

significantly influenced juvenile retention, whilst substrate type had no influence (Table 5.8). In almost all cases retention of juvenile scallops was highest to substrates pre-conditioned for 1 week, followed by unconditioned substrates, with retention lowest on substrates pre-conditioned for 2 weeks particularly at higher flow velocities (Figure 5.8). Analysis showed that mean retention on substrates pre-conditioned for two weeks was significantly lower than on both unconditioned and 1 week pre-conditioned treatments (Tukey $P<0.05$). Maximum retention was on substrates pre-conditioned for 1 week, although retention on unconditioned substrates was approximately equal (Tukey $P>0.05$). The ability of juvenile scallops to remain attached to all substrates decreased with increasing velocity (Figure 5.8), with highest mean dispersal at the highest velocity (12.6±0.2cm second^{-1}). Dispersal at 12.6±0.2cm second^{-1} was significantly higher than at all other velocities (Tukey $P<0.05$), except 12.2±0.1cm second^{-1}. However, velocities up to 10.1±0.2cm second^{-1} did not induce dispersal significantly higher than in static conditions (Tukey $P>0.05$). Velocities of 7.2±0.1 and 10.1±0.2cm second^{-1}, and 10.1±0.2 and 12.2±0.1cm second^{-1} displayed approximately equal retention. A combined effect of substrate pre-conditioning and water velocity was also found (Table 5.8), as the magnitude of loss from substrates pre-conditioned for 2 weeks increased at a greater rate with increasing velocity, than on substrates pre-conditioned for 0 or 1 week, with larger differences observed at 12.2±0.1cm second^{-1} and above.

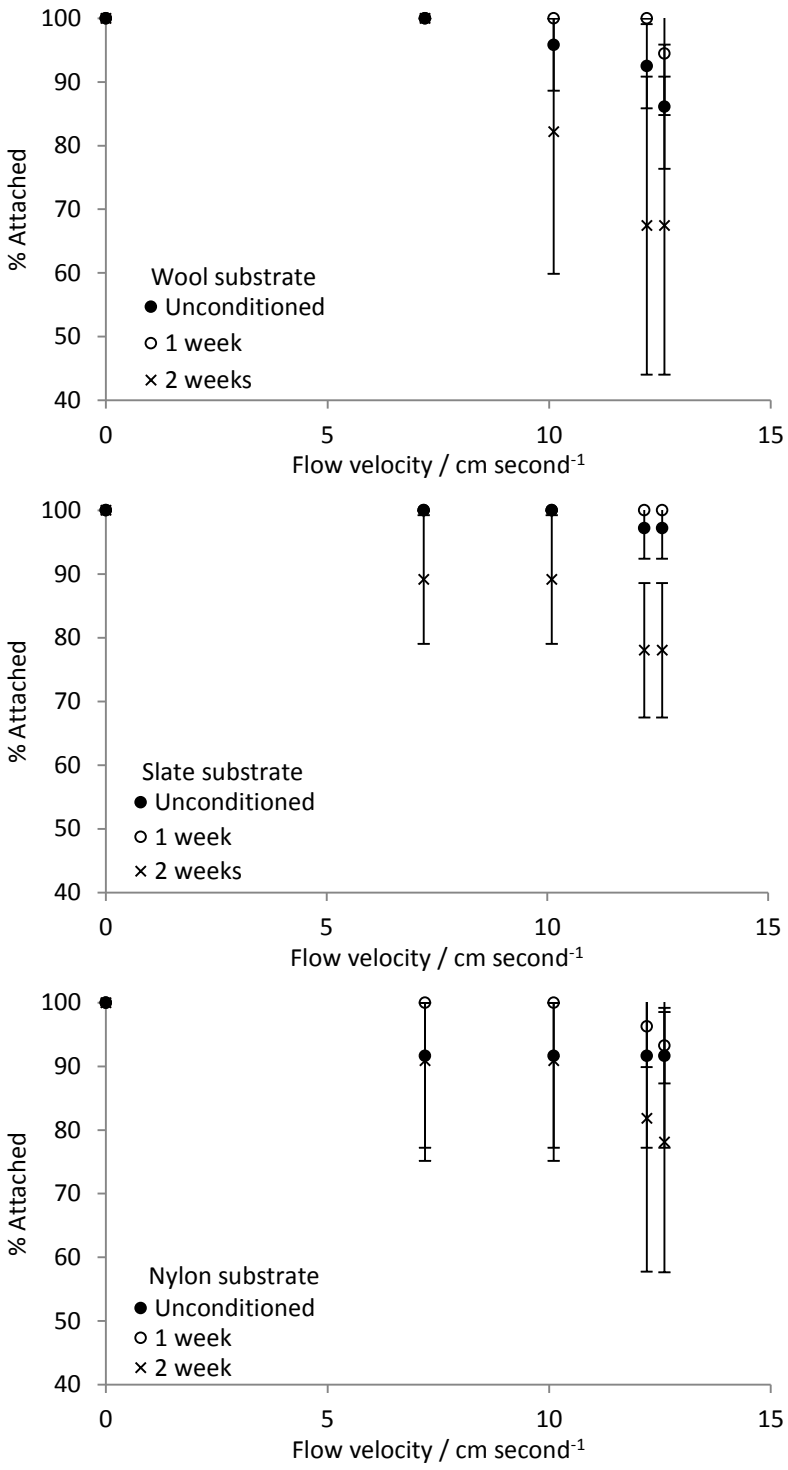

Figure 5.8: Mean percentage retention (± standard deviation) of attached juvenile scallops (shell height 4.0±1.0mm) on wool, slate and nylon substrates pre-conditioned for 0, 1 or 2 weeks over a series of water velocities generated in a benthic water flume.

Table 5.8: Three-way analysis of variance of the influence of substrate type, substrate pre-conditioning and water velocity on the percentage retention of attached juvenile *Pecten maximus* scallops within a benthic water flume. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	161.9	161.9	81.0	0.75	0.474
Pre-conditioning	2	4837.1	4837.1	2418.6	22.52	<0.001*
Velocity	4	4604.7	4604.7	1151.2	10.72	<0.001*
Substrate x Pre-conditioning	4	443.4	443.4	110.8	1.03	0.395
Substrate x Velocity	8	676.8	676.8	84.6	0.79	0.615
Pre-conditioning x Velocity	8	2023.6	2023.6	253.0	2.36	0.024*
Substrate x Pre-conditioning x Velocity	16	762.7	762.7	47.7	0.44	0.966
Error	90	9667.0	9667.0	107.4		
Total	134	23177.1				

Influence of feeding regime and substrate on attachment and detachment

The results of experiment 6 showing the percentage attachment of juvenile scallops to slate, wool and nylon substrates pre-conditioned for 1 week after feeding a range of microalgae rations (g (organic weight of algae) g^{-1} (live weight of scallops) $week^{-1}$) is displayed in Figure 5.9. Table 5.9 summarises the statistical comparisons. The results displayed a high degree of variability and analysis indicated that there was no significant effect of feeding ration on attachment of juvenile scallops. However substrate type did affect attachment, with attachment to slate significantly higher than to wool (Tukey P<0.05). No significant interaction between the variables was observed.

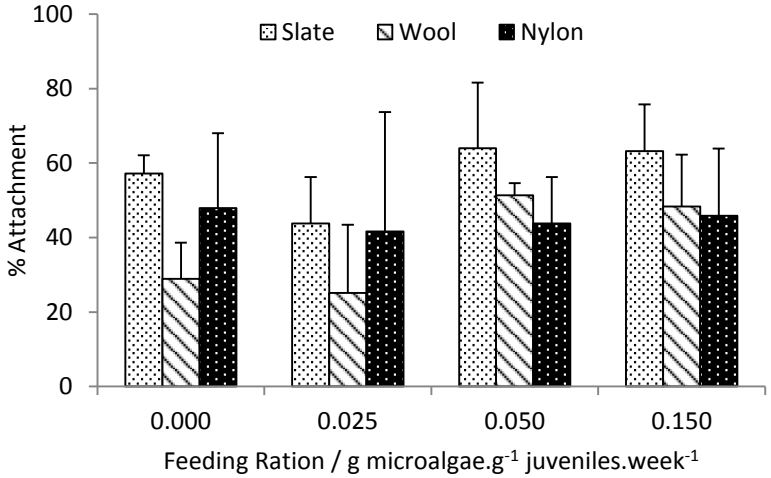

Figure 5.9: Mean percentage attachment (± standard deviation) of juvenile *Pecten maximus* (shell height 5.2±0.9mm) to pre-conditioned slate, wool and nylon substrate after 24 hours, following feeding on a range of microalgae rations (g (organic weight of algae) g^{-1} (live weight of scallops) $week^{-1}$).

Table 5.9: Two-way analysis of variance of the influence of feeding ration on the percentage attachment of juvenile *Pecten maximus* scallops to three different substrate surfaces. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	832.7	832.7	416.3	3.81	0.036*
Feeding Ration	3	685.7	685.7	228.6	2.09	0.128
Interaction	6	301.9	301.9	50.3	0.46	0.830
Error	24	2620.9	2620.9	109.2		
Total	35	4441.1				

The influence of microalgae ration (g (organic weight of algae) g^{-1} (live weight of scallops) $week^{-1}$) on the retention of attached juvenile scallops in experiment 6 to slate, wool and nylon substrates over a range of water velocities is displayed in Figure 5.10. Table 5.10 summarises the statistical comparisons from a 3-way ANOVA. In general attachment declined with increasing velocity, although there was a high degree of variability. Analysis showed that substrate type, feeding level and water velocity all significantly influenced the retention of juvenile scallops (Table 5.10). In this experiment mean retention was highest on slate substrates, and was significantly higher than on wool (Tukey $P<0.05$). Retention by nylon was not significantly different from either of the other substrate treatments (Tukey $P>0.05$). The influence of feeding ration is unclear, however analysis showed that maximum dispersal was by juveniles in the starved treatment, with significantly higher retention achieved by juveniles fed a ration of 0.025g microalgae.g^{-1} juveniles.$week^{-1}$ (Tukey $P<0.05$). Feeding juveniles greater rations (0.05g and 0.15g) did not improve retention compared to starved individuals or those fed the smallest ration (Tukey $P>0.05$). As in the previous experiment the ability of juvenile scallops to remain attached to all substrates decreased with velocity (Figure 5.10), with highest mean dispersal at the highest velocity (12.6±0.2cm $second^{-1}$). However, dispersal at 12.6±0.2cm $second^{-1}$ was only significantly higher than velocities of 7.2±0.1cm $second^{-1}$ and below (Tukey $P<0.05$). Velocities of 10.1±0.2cm $second^{-1}$ and above induced significantly higher dispersal than observed in static conditions (Tukey $P<0.05$). Velocities of 10.1±0.2 to 12.6±0.2cm $second^{-1}$ displayed approximately equal retention, as did zero and 7.2±0.1cm $second^{-1}$. Importantly however a combined effect of substrate type and feeding ration was also found (Table 5.10). It is noticeable from the assessment of the interaction that on nylon, starved juveniles have a much lower retention rate than juveniles on slate and wool. Whilst retention on slate and nylon shows a general increase with feeding ration, particularly at high water velocities, juveniles on the wool show the converse with lower retention at higher feeding levels.

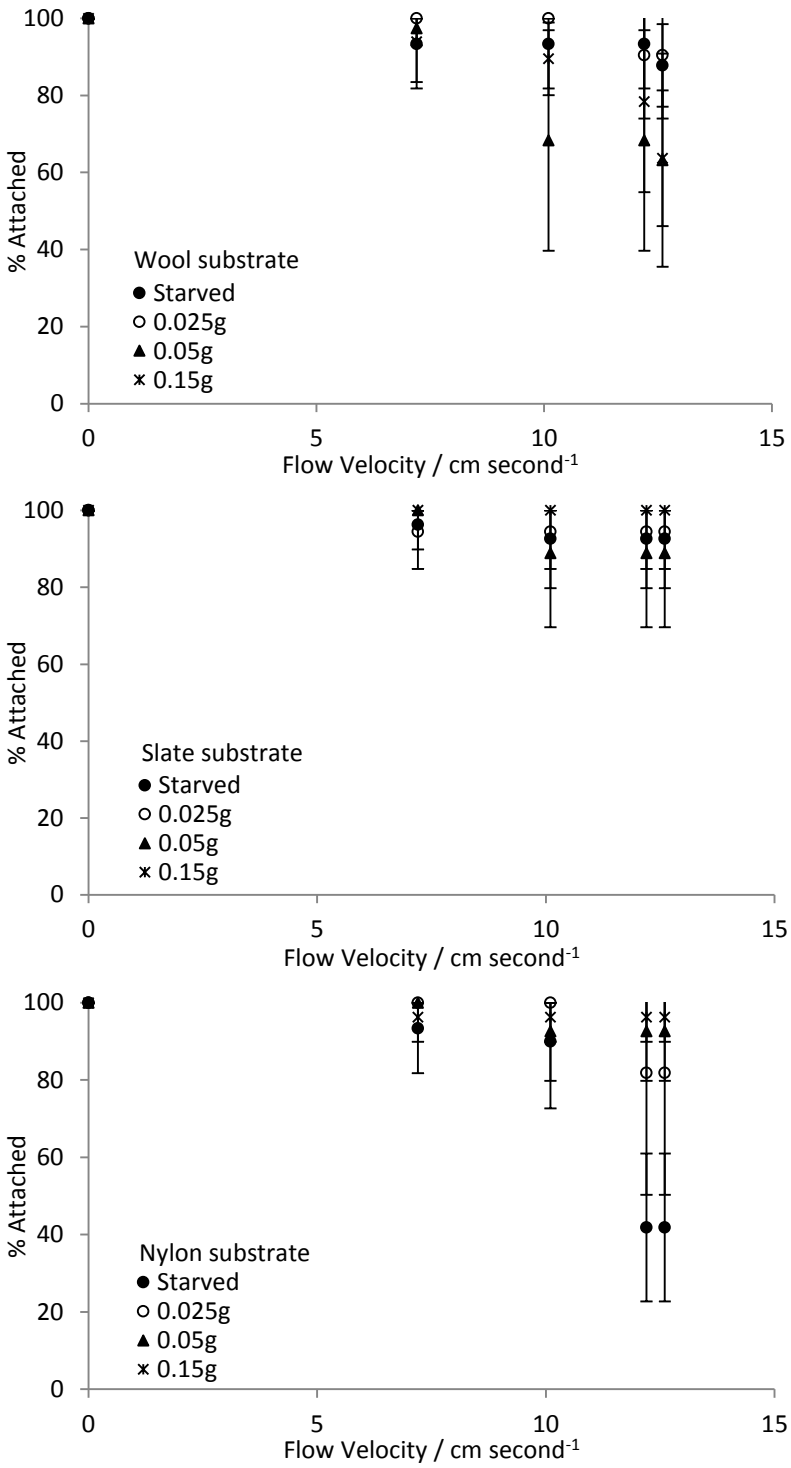

Figure 5.10: Mean percentage retention (± standard deviation) of attached juvenile *Pecten maximus* (shell height 5.2±0.9mm) on pre-conditioned wool, slate and nylon substrates following feeding on a range of microalgae rations (g (organic weight of algae) g^{-1} (live weight of scallops) $week^{-1}$), over a series of water velocities generated in a benthic water flume.

Table 5.10: Three-way analysis of variance of the influence of substrate type, feeding ration and water velocity on the percentage retention of attached juvenile *Pecten maximus* scallops within a benthic water flume. * indicates a significant difference at the 95% confidence interval.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Substrate	2	1837.7	1837.7	918.8	5.34	0.006*
Feeding Ration	3	1644.4	1644.4	548.1	3.19	0.026*
Velocity	4	7860.7	7860.7	1965.2	11.43	<0.001*
Substrate x Feeding Ration	6	5323.7	5323.7	887.3	5.16	<0.001*
Substrate x Velocity	8	1897.0	1897.0	237.1	1.38	0.212
Pre-conditioning x Velocity	12	1638.6	1638.6	136.6	0.79	0.656
Substrate x Feeding Ration x Velocity	24	4538.5	4538.5	189.1	1.10	0.354
Error	120	20629.0	20629.0	171.9		
Total	179	45369.6				

Discussion

Maximising the retention of valuable seed animals is a priority for bivalve aquaculture, however many environmental stimuli have been identified as factors influencing attachment and detachment in juvenile bivalves. This study provides important insight into the influence of multiple factors on the scallop *P. maximus*.

Substrate type

The culture of this species typically involves transferring settled spat, between 1-5mm in size, from indoor nurseries to outdoor on-shore or off-shore nursery systems for on-growing to 15-30mm in size (Andersen *et al*., 2011). In some cases, where spat have initially settled on collector bags or screens within the hatchery these can be transferred directly to outside nurseries (Magnesen and Christophersen, 2007), however in other cases spat must be removed from the mesh or substrate they attached to, before placement on or in new equipment (Millican, 1997; Andersen *et al*., 2011), it is therefore important that re-attachment is promoted. This is also applicable to those collected from the wild. In the wild young *P. maximus* are found settled on a variety of silt-free surfaces raised above the seabed including both natural and artificial surfaces, before they lose the ability to produce byssus (Brand *et al*., 1980; Minchin, 1992). Plastic mesh bags filled with plastic and nylon material have been used as spat collectors, with effective collectors incorporating fillings of Netlon and polyethylene onion bags, although numbers collected can be low and variable (Brand et al.,

1980; Paul et al., 1981). In the hatchery and laboratory nylon mesh (Millican, 1997) and PVC surfaces (Gagné *et al*., 2012) have proven suitable attachment materials. However, the effectiveness of artificial and natural substrates as sites of bivalve attachment in other species has proven to be dependent upon the properties of the substrate (Pearce and Bourget, 1996; Devakie and Ali, 2002; Saucedo *et al*., 2005; Brenner and Buck, 2010). Pearce and Bourget (1996) found that spat of the scallop *Placopecten magellanicus* showed a preference for polyester filter wool over nylon monofilament, polyethylene onion bag, polyethylene astroturf, and smooth and rough acrylic, whilst they also preferred polyethylene mesh with a 3.0mm mesh diameter over mesh of other diameters. Similarly Devakie and Ali (2002) demonstrated the preference of *Crassostrea iredalei* for substrates with rough textured surfaces over smooth. This study analysed alternative substrate materials offering a range of properties including hard surfaces in the form of smooth glass and textured slate, soft natural fibrous materials, as well as nylon mesh as a representative of material used within the bivalve hatchery industry. Substrate type did influence re-attachment of juvenile *P. maximus* in several experiments, however variability within treatments was high, limiting statistically significant differences. In experiment 1, juveniles attached to all tested materials, after 24 hours, but showed a clear preference for slate, with 75.9±14.4% attachment after 24 hours, compared to most other materials. This was followed by glass (42.2±22.4%) and wool (35.5±32.2%) which were not statistically different to slate as a consequence of the high variability. Cotton proved to be the most unsuitable material, however with the exception of slate and cotton all other substrates had approximately equal attachment after 24 hours. In the following experiments the slate, wool and nylon mesh were used in order to represent a range of substrate characteristics on which to assess additional environmental variables. During these experiments mean attachment under similar conditions to experiment 1 varied greatly on wool (15.4 to 56.3%) and on slate (17.2 to 52.4%). Only on nylon did attachment remain relatively consistent, 10.3 to 22.9% compared to 20.7% in experiment 1. The influence of substrate type between slate, wool and nylon was inconsistent, as attachment varied between being statistically equal on all three substrates, to wool outperforming slate or slate outperforming wool, whilst nylon was always equal to slate and wool. Based upon this study slate, wool and nylon are all effective substrates, however attachment is variable. A more suitable and reliable substrate material remains to be identified, although it is likely that other environmental factors act in concert with substrate properties. Orientation and design shape of attachment structures have also been suggested as important factors for study (Paul *et al*., 1981).

In many cases the retention of attached bivalves based upon variables such as substrate properties, is also dependent upon the relationship with hydrodynamic pressures. Hydrodynamics play a strong

role in bivalve dispersal, recruitment and population structure (Eckman, 1987; Martel and Chia, 1991; Commito *et al*., 1995; Hunt *et al*., 2007; Jennings and Hunt, 2009). In the pectinid *Argopecten irradians* survival of young recruits has been found to be compromised in areas of high flow, where attachment to eelgrass blades may be broken (Eckman, 1987), whilst post-settlement transport in several soft-sediment species has been correlated with rates of sediment transport and current velocity (Commito *et al*., 1995; Hunt *et al*., 2007; Jennings and Hunt, 2009). Previous studies have demonstrated that the influence of substrate properties can be modified by changing water velocities, with incidence of pedal crawling or byssus drifting increasing in conjunction with water velocity (Roper *et al*., 1995; Lundquist *et al*., 2004). In flume experiments Lundquist *et al*. (2004) demonstrated that juveniles (<8mm in length) of the bivalves *Macomona liliana* and *Austrovenus stutchburyi* undertook increasing active dispersal behaviour, in response to unsuitable substrate type and increasing flow velocity. In our study the test substrates had no influence on mean (*Uav*) and maximum (*Umax*) velocities, however there was a profound difference in the estimated shear velocities (*U**). As expected the wool substrate had a consistently higher *U*,* approximately twice as high compared to nylon and slate, in line with rougher surfaces having a higher shear velocity (Kirkgöz, 1989). The substrate properties had a significant impact upon passive juvenile retention, with the increased roughness and complexity of the wool increasing retention of unattached juveniles. Dispersal of all unattached individuals increased with water velocity for each substrate, however more individuals were displaced from the slate and nylon substrates than from the wool substrate. At the highest velocity (12.6±0.2 cm second^{-1}) dispersal was on average 32.8 to 53.7% lower on wool than on slate and nylon respectively. In all flume experiments with attached juveniles, detachment increased with water velocity, although juvenile detachment varied. Gagné *et al*. (2012) also demonstrated the increase in dispersal of smaller *P. maximus* juveniles (<1.2 to >1.5mm in length) with increasing water velocity. In our study juveniles between 1.9 and 7.2mm could however resist significant dispersal at *Umax* velocities from 7.2±0.1 to 10.1±0.2cm second^{-1}, at least over short periods. Whilst this is greater than the velocities applied in the on-shore nursery systems developed in Norway, fed with natural seawater at a flow rate of 2 to 4cm second^{-1} (Magnesen and Christophersen, 2007), it demonstrates that juvenile scallops can resist dispersal by greater velocities, including those that may be experienced in off-shore systems. It also indicates that flow rates in on-shore systems could be increased, even over short periods of time, in order to increase food availability, preventing potential reductions in juvenile growth due to food depletion (Magnesen and Christophersen, 2007). It is recognised that water velocity affects scallop filtration and feeding behaviour, with excessive velocities leading to stress and growth inhibition. Flow speeds between 0.2 and 6cm second^{-1} have been recommended for different species, as reviewed

by Magnesen and Christophersen (2007). However, it is notable that increasing velocities to increase food availability could be possible without necessarily causing detachment and dispersal.

Future investigations to assess the impact of extending the study period would prove beneficial. However, the influence of the tested substrate types on detachment of juvenile *P. maximus* in flowing water is less clear. In experiment 5 assessing substrate pre-conditioning the actual substrate material had no influence on juvenile dispersal. Whilst in experiment 6 assessing the influence of dietary level, the type of substrate had a profound influence on dispersal. Detachment of attached juveniles was greater from wool than from slate, with mean proportion of remaining attached seed at the highest velocity (12.6±0.2cm second^{-1}) 94.0±4.6% on slate compared to 76.3±14.9% on wool. The impact of the test substrates on detachment is therefore variable, and may be a consequence of interrelated factors. Further investigation of substrate materials is required, however from the results of this study slate appears to be the best choice.

Attachment period

The length of attachment period prior to disturbance also plays a crucial role (Paul, 1980b). Drag forces associated with detachment have been significantly and positively correlated with the duration of attachment in cyprids of the barnacle *Balanus Amphitrite* (Eckman *et al*., 1990). It is therefore essential that sufficient time to attach is allowed. In bivalves, attachment can be rapid, as seen in the juveniles of the scallop *Chlamys opercularis* (5-10mm length) with 80% attachment within approximately 25 minutes at 18°C, with attachment rate being temperature dependent (Paul, 1980b). However, adequate anchoring to resist environmental disturbances can take time. In the mussel *Mytilus edulis* rate of thread production ranges from approximately 15 to 35 threads per 24 hours, equating to <2 threads per hour, and is dependent upon animal size and environmental salinity (Allen *et al*., 1976). In *P. maximus* Gagné *et al*. (2012) demonstrated that in juveniles, ranging from <1.2 to >1.5mm in length, higher detachment was exhibited following an attachment period of just 30 minutes compared to a 12 hour attachment period when subjected to shear velocities of 1.42 cm second^{-1} and over. The results of this study concur with this assessment in larger *P. maximus* juveniles (1.9 to 5.5mm shell height), with attachment after 1 hour significantly lower than after 24 hours on all tested substrates. In particular attachment increased by over 300% on slate after 24 hours. The implication for culture activities is that *P. maximus* are slow to form attachments and require sufficient time following detachment, such as when being moved between rearing stages, to reform secure byssus attachment before being subjected to hydrodynamic

pressures. Assessment of a wide range of attachment periods could be beneficial to optimise handling protocols.

Agitation

Environmental agitation generated by hydrodynamic pressures has been found to play a significant role in determining byssogenesis response in bivalves. In several studies increased byssogenesis has been associated with increasing water agitation, turbulence or flow velocity, including in bivalves such as *M. edulis* (Young, 1985; Dolmer and Svane, 1994; Lachance *et al*., 2008) and *Perna canaliculus* (Alfaro, 2006). However, other studies show that decreasing byssogenesis can be linked to progressive increases in agitation (van Winkle, 1970; Clarke and McMahon, 1996). The influence of environmental agitation may be linked to evolutionary traits and adaptations associated with the respective habitat of a species (Clarke and McMahon, 1996; Seed and Richardson, 1999). The impact of agitation in experiment 2 on *P. maximus* in the present study was not significant and may be a reflection of the temporary nature of byssus production in this species or the environments it inhabits. This may however be a consequence of the failure to accurately simulate a natural stimulus, as Seed and Richardson (1999) suggested that laboratory generated uniform turbulence does not replicate the severe and irregular nature of conditions on wave-exposed shores. Notably however, in juveniles (3-5mm in length) of the mussel *P. canaliculus* air bubble agitation failed to induce higher spat attachment, but did increase subsequent retention of attached spat in flowing water conditions (Alfaro, 2006). Indicating it acts as a mechanical stimulus to increase byssus production and develop a stronger attachment (Alfaro, 2006). Providing simple air driven agitation in juvenile culture systems for *P. maximus* may encourage retention and should be investigated further.

Illumination

Illumination is known to play a significant role in the recruitment and dispersal of marine invertebrates, influencing larval and juvenile locomotion and attachment (Bayne, 1964; Thorson, 1964; Maldonado and Young, 1996). The ability to respond to light is seen as an important survival tool, as light usually indicates sites exposed to open water, close to surface waters, or both, where threats of desiccation and predation are more likely (Kobak, 2006). In the mussel *Dreissena polymorpha,* individuals <10mm and >10mm actively migrated to shadowed sites and avoided illuminated sites, with attachment increased in dark conditions (Kobak, 2001). Similarly in the

mussel *M. edulis,* as larvae become competent to settle they become photonegative with crawling pediveligers attaching to algal substrates more readily in the dark, with attachment inversely proportion to light intensity (Bayne, 1964). In the present study attachment of juvenile scallops (2.7 to 5.7mm shell height) is size to all tested substrates (slate, wool and nylon) was higher in dark conditions, however the increase was not significant. This may be a consequence of the high variability seen in the study. On reflection further examination of the influence of illumination on *P. maximus* behaviour is advisable, including intensity and stimulus direction.

Surface pre-conditioning

Pre-conditioning of attachment substrates with natural biofilms and other biological material has been shown to increase the proportion of attaching bivalves (Pearce and Bourget, 1996; Devakie and Ali, 2002). In the scallop *P. magellanicus* spat showed an attachment preference for both artificial and natural substrates covered with a natural biofilm, created by running in unfiltered seawater for 16 days prior to use, compared to non-filmed counterparts (Pearce and Bourget, 1996). However, it is reported that biofilm conditioning does not necessarily improve upon optimum substrate characteristics nor does it necessarily fully compensate for unsuitable substrate characteristics (Devakie and Ali, 2002). Since *C. iredalei* were found to settle preferentially on rough plastic without a biofilm (37.6±2.2%), followed by rough plastic with a biofilm (27.1±1.8%), then smooth plastic with a biofilm (22.0±2.1%), with lowest settlement on smooth plastic without a biofilm (11.0±1.2%) (Devakie and Ali, 2002). In the present study a marginal improvement in attachment on slate and wool was seen with increased length of pre-conditioning, whilst a dramatic increase was observed between nylon mesh conditioned for 1 week or more over clean unconditioned nylon. Overall pre-conditioning substrates for 2 weeks significantly improved attachment over clean substrates, whilst pre-conditioning for 1 week resulted in attachment that was neither significantly higher nor lower than either other treatment. Whilst not directly assessed in this study natural biofilms are known to play an important role in the stimulation of invertebrate larvae settlement. Biofilm establishment on artificial surfaces is a progressive process, with rapid bacterial colonisation followed by a succession of different diatom species, and a build-up of detrital material (Hudon and Bourget, 1981). It is accepted that biofilm coverings on substrates serve as an attraction for bivalve settlement (Fritt *et al*., 1990; Pearce and Bourget, 1996; Devakie and Ali, 2002; Zhao *et al*., 2003) and settlement of other marine molluscs (Slattery, 1992; Daume *et al*., 1999; Zhao and Qian, 2002). This attraction has been associated with the release of chemical stimuli from biofilm components, including bacteria and diatoms (Bonar *et al*., 1990; Fritt *et al*., 1990; Satuito *et al*., 1995; Zhao and Qian, 2002; Zhao *et*

al., 2003). It may be that the properties that act on larval settlement also trigger a response in older juveniles to some degree. The present study showed that substrate pre-conditioning also directly influences the retention of juvenile animals. In flume experiments retention was higher, with increasing velocity, on substrates pre-conditioned for 1 week, although this was not significantly higher than unconditioned substrates. In contrast to the results seen for attachment it is clear that pre-conditioning substrates for too long, i.e. 2 weeks, compromised the mean retention of juvenile *P. maximus* to artificial substrates. The cause of this requires verification. However, the shearing away or erosion of biofilms is related to hydrodynamic factors, with material detachment increasing with fluid velocity (Trulear and Characklis, 1982). Therefore excess biofilm material may not provide a stable attachment structure, with erosion leading to a loss of attachment. In addition the results also indicate that there is an interaction between substrate pre-condition and water velocity. This is because not only was the detachment higher from substrates conditioned for 2 weeks, but the magnitude of loss from substrates conditioned for 2 weeks increased at a greater rate with increasing velocity, than on substrates conditioned for 0 or 1 week. The detrimental nature of excess biofilm was magnified with increasing velocity. It is therefore clearly important to establish the impact of substrate condition both on attachment and retention. In this case a moderate conditioning period of 1 week optimises attachment whilst maximising the retention of those that attached. In a subsequent experiment retention on slate conditioned for 1 week was up to 100% at water velocities up to 12.6±0.2cm second^{-1}.

Feeding regime

Food availability has been linked to bivalve seed attachment and retention. The metabolic requirement for byssus formation represents a significant expenditure estimated to be up to 8% of total monthly energy expenditure in *M. edulis* (Hawkins and Bayne, 1985) and 4 to 14% of the energy budget related to somatic growth in the scallop *Chlamys islandica* (Vahl, 1981). This requirement must compete with the energetic demands of other biological activities. Babarro *et al*. (2008) showed that in juvenile *Mytilus galloprovincialis* (26-30mm shell length) a significant drop in byssus secretion and attachment force was observed following starvation for seven days, with simultaneous reduction in condition and glycogen stores, suggesting a constant transfer between soft tissues and byssus under stress. It is therefore essential that cultured animals be provided with adequate nutrition not only to ensure growth but to undertake all essential biological activities, including byssogenesis. In our study the feeding levels tested had no impact upon attachment of juvenile *P. maximus*, with juveniles able to withstand starvation for up to 6 days. In fact no feeding

regime had any significant influence. Examination of higher feeding levels may be beneficial. Similarly Carton *et al*. (2007) found that the mussel *P. canaliculus* could also tolerate starvation for at least 6 days without affecting attachment. However in our study, and as found by Carton *et al*. (2007), dietary regime can affect retention of attached juveniles. Carton *et al*. (2007) reported significant seed detachment after starvation periods of 4 days or more. Under flume conditions in the present study, detachment showed a general increase with water velocity in all feeding regimes, however highest detachment from substrates was by starved animals, with retention significantly higher by juveniles fed 0.025g microalgae.g^{-1} juveniles.$week^{-1}$. Starved mussels are known to continue to partition resources to the production of fewer threads, whilst limited food availability leads to the sole transfer of resources to byssus production, as opposed to soft tissues (Clarke, 1999). The secretion of fewer or mechanically weaker byssus threads will lead to lower retention, or may trigger secondary dispersal behaviour to sites with improved feeding conditions (Carton *et al*., 2007). In *P. maximus* Laing (2000) determined that optimum feeding ration was relative to environmental temperature, with a ration of 0.15g organic weight of microalgae g^{-1} juveniles $week^{-1}$ recommended for maintaining highest condition and best growth at an optimum temperature of 17°C. Notably at 12°C, a ration of 1.0g appears adequate, although growth was lower. At both 12°C and 17°C lower rations supported lower growth rates (Laing, 2000). Interestingly in this study *P. maximus* juveniles provided with microalgae rations greater than 0.025g showed no improvement in mean retention compared to juveniles fed 0.025g or those starved. Notably however at the highest velocities maximum retention on nylon and slate was achieved at the highest rations. The impact of feeding rations on juvenile *P. maximus* retention is a topic for further investigation. At present it appears that available energy, at rations of 0.025g and above, is not a limiting factor on retention, although providing excess food did not convey a benefit. Future studies incorporating longer experimental periods may be able to ascertain whether the excess resources were available for other biological processes, such as growth Tailoring feeding levels is essential to optimize production costs since microalgae production alone has been estimated to represent approximately 30% of bivalve seed production costs (Coutteau and Sorgeloos, 1992). In relation to the interaction between diet level and substrate type, this may indicate that the lower retention on nylon at higher velocities by starved juveniles may be the first indication of these being nutritionally compromised, potentially requiring greater energy to remain attached to this surface. However, it is unclear why feeding rations higher than 0.025g would cause juveniles attached to wool to display increased detachment compared to juveniles fed less or those attached on slate or nylon.

However, whilst food quantity is therefore important, the quality of available food is equally as important. Previous work has shown that *P. maximus* spat growth is dependent upon the type and combination of available microalgae species (Laing and Psimopoulous, 1998), however it also influences attachment. Gagné *et al*. (2012) found that post-larval *P. maximus* fed a microalgae diet including *Rhodomonas salina* in combination with *P. lutheri, Isochrysis galbana* and *C. calcitrans* showed higher detachment (57%) at shear flow velocities between 1.42 and 2.45cm second^{-1} than post-larvae fed a diet that that did not include *R. salina* (36% detachment). It was speculated that increased lipid accumulation and brassicasterol enrichment of sterol negatively affected attachment. Further testing of the influence of diet is recommendable, but it is clearly essential that *P. maximus* is provided with suitable levels of appropriate nutrition to maximize retention in the presence of hydrodynamic forces.

Conclusions

This study examined the impact of a wide range of culture and environmental factors on the attachment and retention of juvenile *P. maximus* and demonstrated that factors including substrate type, substrate pre-conditioning, attachment period, dietary ration and water velocity all play an important role. Based upon the results, recommended parameters for maximising the chance of juvenile *P. maximus* attachment and retention in water velocities up to 12.6±0.2cm second^{-1}, is to use a slate substrate, pre-conditioned for 1 week, with juveniles having been fed a diet of at least 0.025g microalgae.g^{-1} juveniles.week^{-1}, and allowed to attach for at least 24 hours. The results of this broad study provide additional insight into life stages key to the effective culture and management of this commercially important species. However, it is clear that influential factors should not be considered in isolation, since multiple variables can operate collectively to influence bivalve response and the effects of one factor can modify and be modified by another (Paul, 1980a).

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Chapter Six

General Discussion

Introduction

A significant problem facing the production of the commercially important king scallop *Pecten maximus* and blue mussel *Mytilus edulis* is a current over-dependence on natural sources of seed animals. Natural variability in spat fall and limited windows of availability, conservation legislation, plus increasing demand, places a growing burden on the industry (Millican, 1997; Kamermans and Smaal, 2002; Spencer, 2002; Maguire *et al*., 2007). The obvious solution to meet demand and ensure a reliable supply of seed is to establish hatchery production as a source of high quality seed (Millican, 1997; Utting and Spencer, 1991; Spencer, 2002; Maguire *et al*., 2007).

Many forms of aquaculture globally are still dependent upon harvesting wild resources, either through the collection of seed, broodstock or food for cultured animals; however, more countries are now adopting hatchery culture for seed production (Davenport *et al*., 2009). Traditionally bivalve culture in general has relied upon the collection of naturally sourced spat, however limited natural breeding areas, or insufficient production and erratic breeding by adult populations means seed sources cannot be guaranteed. Furthermore growers may desire to focus on particular strains and species, or even non-native species that suit their needs but may not be available locally (Helm and Bourne, 2004). Bivalve hatcheries have existed for over half a century and are now well established in many European countries, producing or having produced a number of commercially important species including the grooved carpet shell clam *Ruditapes decussates,* the Manila clam *Ruditapes philippinarum*, pullet carpet shell clam *Venerupis pullastra,* the hard clam *Mercenaria mercenaria,* European flat oyster *Ostrea edulis,* the Pacific oyster *Crassostrea gigas,* the king scallop *P. maximus,* the Mediterranean mussel *Mytilus galloprovincialis* and the common cockle *Cerastoderma edule* (Helm and Bourne, 2004; REPROSEED, 2014). Whilst culture protocols are available, the establishment of commercial hatcheries for *M. edulis* have yet to be realised, with production limited to experimental quantities due to the low unit value of the animal (P. Kamermans personal communication; Brake *et al*., 2001) However effective protocols have led to hatchery activities been carried out for *P. maximus* in many European countries, notably Norway, although production continues to face a number of challenges (Millican, 1997; Bergh and Strand, 2001; Spencer, 2002; Torkildsen and Magnesen, 2004; Robert and Nicolas, 2000; REPROSEED, 2014). Until

recently bivalve cultivation simply involved growing the bivalves to a marketable size, particularly when utilising natural seed, however with the development of bivalve hatcheries it has become possible to achieve more (Helm and Bourne, 2004). Hatchery culture enables the careful control of the culture environment and animal husbandry through the establishment of suitable facilities and rearing protocols (Utting and Spencer 1991; Millican, 1997; Helm and Bourne, 2004). Research and development is continuing to be made in fields such as triploid production (Ruiz-Verdugo *et al*., 2000), improved larval rearing systems, including the development of flow-through systems to replace traditional batch culture for some species (Helm and Bourne, 2004; Torkildsen and Magnesen, 2004), improved nursery systems (Magnesen and Christophersen, 2007), improvements in spat and seed handling (Bergh and Strand, 2001), and continued assessment of nutritional and dietary requirements, with a growing importance for essential fatty acids (Pernet and Tremblay, 2004; Tremblay *et al*., 2007; Pettersen *et al*., 2010; Andersen *et al*., 2013). This is in addition to continuing work on settlement, metamorphosis and post-larval behaviour of bivalves (Carton *et al*., 2007; Yu *et al*., 2008; Gagné *et al*., 2012; Yang *et al*, 2013). In particular, hatchery production for the pacific oyster *Crassostrea gigas* has led to the ability to directly control development and produce triploid animals that benefit from reduced reproductive development, increased size, increased survival and improved flesh quality (Allen and Downing, 1985; Dunham, 2012). Triploid induction was initially achieved through the application of chemical or physical stress to inhibit meiosis I or meiosis II after fertilisation, but has since been achieved through the use of tetraploid males to fertilise eggs from diploids to produce batches of 100% triploids (Beaumont and Hoare, 2003; Nell, 2002). Triploid *C. gigas* are considered by some to be a commercial success, since diploid "normal" *C. gigas* become unmarketable as they reach sexual maturity in the summer with reproductive tissue ramifying and glycogen stores converted to gametes, resulting in the loss of flavour and reduced quality. Triploids do not however suffer the same fate, as reproductive development is retarded, providing a year-round product (Beaumont and Hoare, 2003; Gosling, 2003). As reviewed by Gosling (2003) triploid production has been achieved in many other bivalve species as well, including *M. edulis*, *R. Philippinarum*, *P. maximus, O. edulis,* the Virginia oyster *Crassostrea virginica*, the variegated scallop *Chlamys varia*, the pearl oyster *Pinctada martensii*, and the Atlantic bay scallop *Argopectin irradians*, among others, with increased growth following reproductive maturity and higher genetic heterozygosity. It has been alleged the higher heterozygosity has enabled some meiosis-I induced triploids, such as *O. edulis*, to sustain basal metabolism with lower energy expenditure thereby allowing faster growth (Gosling, 2003). It is believed that where *C. gigas* production is based upon the hatchery supply of seed it is likely that with the use of tetraploid oysters the farming of triploid oysters will increase, as seen with the enthusiastic adoption of

triploids by commercial operations on the West Coast of North America (Nell, 2002). Hatcheries are also able to utilise chemical agents, in particular epinephrine, to produce unattached "cultchless" oyster spat that do not require an attachment substrate (Coon *et al*., 1985), which is a recognised hatchery technique for both *C. gigas* (Wallace *et al*., 2008) and the Sydney rock oyster *Saccostrea glomerata* (O'Connor *et al*., 2008).

Research and development is continuing to refine and improve bivalve hatchery technology making it more efficient and profitable (Helm and Bourne, 2004), this includes the mussel *M. edulis* (Galley *et al*., 2010) and the scallop *P. maximus* (Andersen *et al*., 2011), in order to ensure the high densities and fast growth rates needed for commercial production.

In an effort to address major challenges continuing to face bivalve hatchery culture this thesis investigated the control of timing of metamorphosis in *P. maximus* and *M. edulis* using exogenous chemicals. It also investigated the influence of exogenous factors on attachment and dispersal behaviour in their motile post-larvae. It is hoped that this study furthers our understanding of bivalve biology and behaviour, and aims to provide useful tools, techniques and strategies designed to benefit commercial culture efforts.

Chemical control of metamorphosis

The ability to control settlement and metamorphosis in many marine bivalves using exogenously applied chemical agents has been repeatedly demonstrated (Coon *et al*., 1985; Nicholas *et a*l., 1998; Dobretsov and Qian, 2003; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005; Mesías-Gansbillar *et al*., 2008). Some chemicals offer a routine, inexpensive and effective method for increasing larval development and synchronicity, suitable for deployment in bivalve hatcheries (Coon *et al*., 1985; García-Lavandeira *et al*., 2005). However, effectiveness of inducers has proven to be species-specific and dependent upon concentration and length of exposure, with many chemicals proving toxic at high concentrations and over long exposures (Kingzett *et al*., 1990; Martinez *et al*., 1999; Dobretsov and Qian, 2003; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005). Chemical agents can also affect subsequent post-larval growth, as Dobretsov and Qian (2003) found that whilst isobutlmethylxanthine (IBMX) was an effective settlement inducer at a concentration of 10^{-3}M in *M. edulis* it also completely inhibited larval growth compared to both an untreated control and lower IBMX concentrations. It is therefore critical to rigorously assess the influence of potential chemical agents with any selected bivalve species.

Induction in *Pecten maximus*

Assessment was made of the influence of the chemicals potassium chloride (KCl), ammonium chloride (NH_4Cl), acetylcholine chloride, γ-aminobutyric acid (GABA), 3,4-dihydroxyphenylalanine (L-DOPA) and epinephrine on the induction of larval metamorphosis and larval toxicity in the commercially important king scallop, *P. maximus*. A range of concentrations over an exposure period of 48 hours were examined. As an emerging aquaculture species in Europe this species is the focus of continuing efforts to optimise culture protocols. However difficulties in the culture of this species have been associated with the transition from the larval to juvenile stages, with protracted metamorphosis, low development synchronicity and variable survival (Nicolas *et al*., 1998; Robert and Nicholas, 2000). Increasing development synchronicity and survival would reduce hatchery production time and costs, and increase yields. Previous studies have demonstrated that epinephrine and L-DOPA have induced significant metamorphosis in *P. maximus*, although induction levels and synchronisation remain relatively low (Chevolot *et al*., 1991; Nicholas *et al*., 1996; Nicholas *et al*., 1998).

The current study, detailed in Chapter 2, increased our knowledge of induced metamorphosis in the scallop *P. maximus*. The chemical KCl can be added to the list of effective agents in this bivalve species, whilst the effectiveness of L-DOPA was reinforced. KCl at 20mM and L-DOPA at 10^{-6}M induced significantly ($P<0.05$) higher rates of larval development, with KCl increasing secondary shell growth by 208% and L-DOPA increasing gill formation by 128% compared to the controls after 1 week. Nevertheless, the benefit of KCl must be off-set against a drop in survival, not seen with L-DOPA. In contrast the first examination of NH_4Cl, acetylcholine chloride and GABA with *P. maximus* has shown that these chemicals are unsuccessful as metamorphic inductive agents, at least over the concentration and exposure range tested. Epinephrine also proved ineffective, in contrast to previous studies (Chevolot *et al*., 1991; Nicholas *et al*., 1996; Nicholas *et al*., 1998) although this is likely a consequence of the hydrochloric acid used to dissolve the epinephrine. The induction of synchronous larval development in this species remains unachieved with maximum metamorphosis after 1 week just 28.9±3.3% for gill formation with optimum L-DOPA treatment and 12.3±2.4% for secondary shell growth with optimum KCl treatment. The results indicate that sensitivity to the tested chemical agents is low.

At present recommendations cannot be made for hatchery application, as a sufficiently effective and economically viable solution to the long development period in *P. maximus* has not yet been found. However, this study contributes to our increasing understanding of induction in this species.

Induction in *Mytilus edulis*

Assessment was made of the chemicals L-DOPA, epinephrine, KCl and NH_4Cl as a means of inducing larval metamorphosis, as well as their impact on survival and growth, in the commercially important blue mussel, *M. edulis*. A wide range of concentrations over exposure periods of 24, 48 and 72 hours were examined. This species is the focus of on-going research aimed at reducing the reliance on natural sources of seed animals and improving the economic viability of future hatchery operations. As *M. edulis* has a low unit value (Brake *et al*., 2001) it is essential for future hatchery operations to maximise production yield whilst minimising production time and cost.

There is limited information on the influence of exogenous chemicals on metamorphosis in *M. edulis*. Previous studies have assessed a handful of chemicals including KCl, L-DOPA, acetylcholine chloride, GABA and 3-isobutyl-1-methylxanthine (IBMX). However these studies were generally short, concluding after 48 hours, were primarily focused on the settlement response of larvae and often relied upon the assessment of small numbers of larvae (≤20) (Cooper, 1982; Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). Therefore these may not have been sufficiently long enough to characterise the full impact, either positive or negative, of using the different chemical agents or been large enough to accurately determine the full effect of the chemical due to the inherent variability seen within, as well as between batches of bivalve larvae (Bonar *et al*., 1990). So far L-DOPA, IBMX and acetylcholine chloride have all been shown to stimulate increased settlement in *M. edulis*, but only L-DOPA has been shown to increase metamorphosis. KCl and GABA have to date proven ineffective as either settlement or metamorphic inducers.

The current study, detailed in Chapter 3, increased our knowledge of induced metamorphosis in the mussel *M. edulis* by adopting an extended assessment period beyond the treatment window, with cultures containing thousands of larvae. Larval metamorphosis was quantified after 72 hours and again after 1 week, as well as the influence on survival and growth over the same period. Larval susceptibility to L-DOPA, epinephrine, KCl and NH_4Cl proved to be limited as none of the assessed chemicals induced a significant lasting improvement in metamorphosis compared to untreated controls. The results for L-DOPA contrast with previous work that found L-DOPA to be an effective

metamorphic inducer in *M. edulis* (Cooper, 1982). This may be a consequence of larval immaturity in the present study or larval variability. High concentrations generally proved to be toxic and led to lower levels of development. Excess K^+ did however induce a slight improvement in larval growth at concentrations of 1.3mM and 13.4mM ($1.3x10^{-3}$ and $1.3x10^{-2}$ respectively) after exposure for 24 and 48 hours. The ability to induce metamorphosis in *M. edulis* using a chemical agent remains elusive. Even though this study did not lead to the identification of an improvement in culture practice for *M. edulis*, it does contribute to our expanding knowledge of metamorphic control in this species. Notably several of the chemicals tested in this study have proven effective at inducing metamorphosis in related Mytilidae, including epinephrine, K^+, NH_4^+ and GABA (García-Lavandeira *et al*., 2005; Yang *et al*., 2008; Yang *et al*., 2013) indicating that the metamorphic receptors and signalling pathways are potentially different in *M. edulis*, and the level of response in this genus may be species-specific. A number of other chemical agents including methoxyphenamine, clonidine, serotonin, as well as organic solvents including ethanol, methanol, ethylene glycol and acetonitrile, have proven effective in related Mytilidae and may prove beneficial in *M. edulis* (Yang *et al*., 2008; Yang *et al*., 2013). Arising from this study it is recommended that future induction studies with *M. edulis*, and any other species, are conducted over sufficient time to observe the full effect of the treatments.

Future work on bivalve induction

Future work should focus on assessing alternative chemicals from the exhaustive list identified as acting as cues in marine invertebrates and which have yet to be tested with either *P. maximus* or *M. edulis*. An alternative chemical may hold the key to induction of metamorphosis in both species. Further assessment should also be directed towards the influence of exposure period, as short exposure of minutes to a few hours has proven effective in other species (Coon *et al*., 1985; Kingzett *et al*., 1990; Beiras and Widdows, 1995). It is also critical that with any new chemical that the influence of concentration and exposure period are evaluated in detail over a sufficient period to assess any latent influence post-treatment.

The lack of effect with L-DOPA in the present study with *M. edulis*, in contrast to the findings of Cooper (1982), suggests that the larvae in this study were not competent to metamorphose. Effectiveness of an inducer is dependent upon the competence of larvae to undergo metamorphosis, however there is often inherent variability in the susceptibility between and within experimental larval groups (Bonar *et al*., 1990; Chevolot *et al*., 1991). Therefore the result in one batch may not

be repeated in the next batch. It would be valuable to quantify the interaction between development stage and chemical inducers in *M. edulis*, and also *P. maximus*. Coon *et al*. (1990) assessed the temporal relationship between the onset of behavioural and morphogenetic competence in *C. gigas* using L-DOPA and epinephrine as inducers. Competence was correlated with, but not dependent upon, larval size and eyespot development. However, interestingly larval response to these chemicals differ temporally, with larvae displaying a settlement response to L-DOPA, before showing a metamorphic response to epinephrine, which in turn preceded the metamorphic response to L-DOPA. They also determined that for larvae past the onset of competence the apparent stimulus threshold to induce metamorphosis progressively decreases. The temporal separation of the various facets of competence suggests that settlement and metamorphosis is a multi-step process with competence developing through different mechanisms (Coon *et al*., 1990).

However, it requires consideration whether chemical agents can or should be used within the cultivation of animals intended for human consumption. Alternative means of inducing metamorphosis should therefore possibly be considered. Several studies have demonstrated that the application of short periods of thermal shock, elevating culture temperatures for up to several hours, can induce increased rates of metamorphosis in marine invertebrate larvae (Kroiher *et al*., 1992; Gaudette *et al*., 2001; Boettcher, 2005). Heat shock treatment has been associated with a shift in the pattern of protein synthesis and the formation of heat-shock proteins (Kroiher *et al*., 1992). Assessment of this approach in bivalves such as *M. edulis* and *P. maximus* could be beneficial and should be considered for future study.

Influence of environmental factors on attachment and detachment of post-larvae

Maximising the retention of valuable seed animals is a priority for bivalve aquaculture. In Europe mussel production is reliant upon collecting wild seed, either by dredging areas of natural settlement or using artificial substrates to act as collectors, and then on-growing to a marketable size (4-7 cm) in 1-3 years. On-growing is usually undertaken either using bottom culture or suspended culture on bouchots, rafts or long-lines (Spencer, 2002; Maguire *et al*., 2007). Bottom culture is based upon the transfer of mussels from natural exposed beds, to managed sheltered plots where density is reduced to improve growth and fattening (Spencer, 2002). Initial stocking density and management after re-laying are critical. Average yields are probably close to 1 ton of marketable mussels for every ton of seed. However, if poorly managed, this may decline due to drifting, storms and

predation (Dare, 1980; Spencer, 2002). With bottom culture there is little stopping seed from dispersing. However, using predator-proof intertidal fenced plots can increase productivity up to 8 tons of mussels for every 1 ton of seed (Dare, 1980). Bouchots consist of rows of vertical poles set in the sea-bed in the intertidal zone close to the low-water mark of spring tides. They are used both for spat collection and on-growing. Spat are collected on poles or horizontal lines of coir rope on metal frames, with seed mussels transferred to tubular nets wound round the growing poles. Even when initially confined, seed are known to migrate from bouchot stockings to cover the surface of the pole itself, and therefore require thinning (Spencer, 2002). Raft culture sees mussels attached to ropes suspended beneath a floating rafts, whilst the long-line systems consist of a series of horizontal ropes buoyed at or close to the surface, with vertical ropes at intervals carrying the mussel crop (Spencer, 2002). For both raft and long-line culture systems collector ropes are suspended in the same manner as culture ropes. Once spat are approximately 10mm in size they are removed and either placed in cotton mesh or synthetic stockings over the culture rope, or as seen in Spain, bandaged to the rope with fragile cotton mesh tape (Spencer, 2002). In some cases long wooden pegs are also inserted along the ropes at intervals to reduce the likelihood of loss from slippage as the mussels grow (Spencer, 2002). Critically the loss of *M. edulis* seed (1.5 to 5.0mm shell length) from rope cultures can be extremely high, with 50% in the first 2-4 months, though at high stocking densities losses can exceed 98% within 12 months (Dare and Davies, 1975). The production systems employed are heavily reliant upon the mussels remaining attached and not dispersing. Research to identify factors influential in the retention of *M. edulis* is essential to the development of effective management strategies.

The culture of scallops has traditionally utilised spat collectors, typically mesh bags filled with plastic and nylon material, suspended during expected spatfall periods (Brand *et al*., 1980; Paul *et al*., 1981; Spencer, 2002). Spat approximately 5mm in size are then transferred to vertically suspended pearl nets in open water, before transfer to lantern nets at approximately 30mm until they reach a marketable size (Millican, 1997; Spencer, 2002). Alternatively they can be sown to the sea bed once 30mm in size, although plates of steel or aluminium fencing, or aluminium cages are potentially required to prevent unacceptable levels of loss from predation (Bergh and Strand, 2001; Spencer, 2002). They can also be hung from ropes in shallow, sheltered areas from a hole drilled in an ear once 40-60mm in size (Spencer, 2002). With hatchery production now feasible the cultivation of *P. maximus* typically involves transferring hatchery settled spat, between 1-5mm in size, from indoor nurseries, were they are held in down-welling systems, to outdoor off-shore or on-shore nursery systems for on-growing to 15-30mm in size (Magnesen and Christophersen, 2007; Andersen *et al*.,

2011). Spat undergo transfers from hatchery to nursery, from one equipment type to another or from one farm to another, and limiting transfer stress and duration has been shown to improve survival, particularly of small seed (2mm shell height) (Andersen *et al*., 2011). Off-shore nurseries utilise a number of methods, including suspended pearl and lantern nets (Millican, 1997), as well as suspended stacks of trays and cages, or mesh baskets within frames laid directly on the sea floor (Andersen *et al*., 2011). Seed as small as 2mm are transferred to nursery systems (Bergh and Strand, 2001; Andersen *et al*., 2011). On-shore nursery systems have been developed in Norway. These consist of land-based raceways with spat initially settled on collector bags or screens within the hatchery placed directly in the system or in mesh trays respectively, fed with natural seawater filtered to 100µm at a flow rate of 2cm second^{-1} (Magnesen and Christophersen, 2007). This system, with *P. maximus* growth from 1.5mm up to saleable seed size of >15mm shell height, is seen as an intermediate means of bridging the gap between hatcheries and grow-out in the sea to improve production (Magnesen and Christophersen, 2007). Whilst *P. maximus* seed are cultivated in an enclosed environment that reduces the risk of dispersal, it is still critical to provide optimum conditions for growth and survival, and maximising attachment and understanding the factors affecting it are essential.

Environmental factors play a key role in the control of byssus attachment and detachment of many bivalve species. Identifying and understanding the impact of influential factors is essential to optimise the management and culture of commercially important species, such as *M. edulis* and *P. maximus.* Furthermore many bivalve species display motile behaviour facilitating secondary dispersal post-metamorphosis, which has been linked to unfavourable conditions or a shift in habitat requirement (Sigurdsson *et al*., 1976; Sörlin, 1988; Beukema and de Vlas, 1989; Roper *et al*., 1995; Baker and Mann, 1997; Lundquist *et al*., 2004). This behaviour represents an important tool in the selection of a suitable habitat, however for aquaculture it represents a risk to the effective management of a valuable and finite resource.

Attachment, detachment, dispersal and mortality in *Mytilus edulis*

M. edulis is known to possess highly mobile life stages able to relocate to new habitats. However the factors affecting seed retention and dispersal are not fully understood. The current study, detailed in Chapter 4, assessed the impact of factors including substrate type, attachment period, feeding level, water agitation, and seed density on attachment, retention and mortality of seed mussels

between 0.97 and 3.85mm in shell length. The ability to assess the quality of *M. edulis* seed and predict future performance based upon their pedal crawling activity was also investigated.

Substrate type and attachment period played a pivotal role in determining the level of seed attachment, although food availability did not. Previous work has shown that substrate properties (Pearce and Bourget, 1996; Devakie and Ali, 2002), as well as attachment period (Gagné *et al*., 2012) all influence bivalve attachment. A number of experimental substrates were examined to reflect surfaces of varying rugosities on which to assess seed retention. Several of which were natural fibre materials which were assessed with a view to providing novel materials for deployment on benthic on-growing plots or suspended structures. Seed showed a clear preference for smooth glass and natural fibre wool felt, with attachment rates of up to 89.2% and 95.2% after 6 hours respectively. Attachment to other natural fibre materials (soy, hemp and cotton), plus a slate material, was lower. Whilst glass is unsuitable for large scale application in a hatchery or in open water, the wool felt offers a potentially attractive mechanism that is both an abundant and renewable material, on which seed could be settled in a hatchery system before being laid on benthic on-growing plots or suspended from floating structures wrapped round bouchots or ropes.

Food availability, water agitation, density and substrate all play a critical role in seed retention, although not mortality. Byssal production and quality can be compromised by the availability of energetic resources (Carrington, 2002; Moeser and Carrington, 2006; Babarro *et al*., 2008) with the ability of nutritionally compromised seed to remain attached being reduced (Carton *et al*., 2007). The current study demonstrated the opposite, with fed seed showing increased detachment and dispersal compared to seed receiving limited or no food. Fed seed were more likely to undertake active dispersal, with ≥75% of detachment associated with dispersal to alternative attachment sites. Seed on restricted feeding regimes, although less likely to detach, were also more likely to remain in situ, since <40% of detached seed relocate, indicating seed may lack the energy reserves for active dispersal. Hydrodynamic pressures are known to influence byssus attachment in *M. edulis*, thereby influencing mussel retention with byssus production and strength increased by increasing water agitation, turbulence or flow velocity, and reduced in static water environments (Glaus, 1968; Price, 1982; Dolmer and Svane, 1994; Lachance *et al*., 2008). This was the case in the current study, with water agitation significantly increasing retention ($P<0.05$), and reducing the influence of feeding. Water agitation decreased dispersal behaviour to alternative attachment sites by 62 to 100% and total detachment by 55 to 100%. Water movement dramatically increased the likelihood of retention. Bivalve density is also an influential factor on recruitment and dispersal patterns

(Commito *et al*., 1995; Turner *et al*., 1997; Hunt and Mullineaux, 2002). As found in other species, including the bay scallop *Argopecten irradians concentricus* (Powers and Petersen, 2000) and the soft shelled clam *Mya arenaria* (Hunt and Mullineaux, 2002) the detachment and dispersal of *M. edulis* is proportional to seed density, with total detachment rates of between 0.067 to 0.158 seed per attached seed cm^{-2} and active dispersal rates of 0.016 to 0.059 seed per attached seed cm^{-2}. However, the rate of detachment and active dispersal is significantly influenced by substrate type, with the rate of loss higher from the smooth glass than from the fibrous wool material. Notably little detachment or dispersal is recorded from the wool substrate at a density of up to approximately 50 seed cm^{-2}. From an aquaculture perspective substrate type has a significant impact upon carrying capacity with some substrates offering higher retention of more seed.

The implication based upon this study, is that recommended parameters for maximising the chance of *M. edulis* seed attachment and retention, is to use a wool substrate, stocked at approximately 50 seed cm^{-2}, with undisturbed attachment for 6 hours, and with attached seed subjected to some form of water agitation. Feeding a suitable dietary quantity is obviously necessary, and the increase in dispersal associated with feeding should be prevented by the water agitation. These parameters provide a detailed base upon which future investigation can build, however they should also be considered for testing in hatchery operations.

The ability to predict future performance of bivalve seed by determining seed quality and fitness represents a useful tool for bivalve aquaculture. In the current study pedal crawling could not be used to predict either attachment or subsequent retention, however a link between seed activity and seed mortality was identified. Seed displaying increased mobility exhibited higher survival, with 80 to 100% lower mortality than sedentary seed. This indicates that seed activity could be used as a simple predictor for seed performance, with activity related to level of fitness.

Attachment, detachment and dispersal in *Pecten maximus*

In pectinids rapid attachment, and high and stable attachment rates have been associated with high growth and survival (Paul, 1980a,b; Christophersen and Strand, 2003). The current study, detailed in Chapter 5, assessed the impact of factors including substrate type, substrate pre-conditioning, attachment period, feeding ration, agitation and illumination on attachment of juvenile scallops between 1.9 and 7.2mm in shell length. The impact of substrate type, substrate pre-conditioning

and feeding ration on detachment of juvenile seed was also examined in a benthic water flume over increasing velocities from 0 to 12.6cm second^{-1}.

Attachment of bivalves is affected by substrate properties and condition (Pearce and Bourget, 1996; Devakie and Ali, 2002). This study established that scallop juveniles have a preference for the textured slate surface, with mean attachment rates up to 75.6±14.4% after 24 hours over other test substrates. However attachment could be variable. As has previously been found (Gagné *et al*., 2012), the length of attachment period also proved critical, with periods longer than 1 hour essential to allow juveniles sufficient time to attach. Attachment could be boosted by pre-conditioning substrates in flow-through tanks of unfiltered seawater for 1 to 2 weeks. However, agitation (static vs agitated conditions), illumination (24 hours illumination vs 24 hours darkness) and feeding regime (starvation, 0.025g, 0.05g and 0.15g organic weight of microalgae g^{-1} spat (live weight) week^{-1}) had no impact on attachment.

In terms of detachment, water velocity, substrate type, substrate condition and feeding ration are all key factors affecting juvenile scallop retention. In each experiment detachment increased with increasing water velocity, as previously found for smaller *P. maximus* juveniles (Gagné *et al*., 2012). Previous studies have demonstrated that the influence of substrate properties can be modified by changing water velocities (Roper *et al*., 1995; Lundquist *et al*., 2004). In this study the influence of substrate type on retention was variable, although over the course of the study slate ensured the greatest chance of retention, with attachment up to 100% at 12.6±0.2 cm second^{-1}. Interestingly pre-conditioning substrates for too long (i.e. 2 weeks) compromises juvenile retention under increasing water velocities. This may be a consequence of biofilm shearing or erosion as a consequence of the hydrodynamic forces in the flume (Trulear and Characklis, 1982). Excess biofilm material may not provide a stable attachment structure, therefore hatcheries should take care that biofilm growth on attachment structures is monitored and controlled. As previously stated bivalve attachment and retention is also linked to the availability of energetic resources, with lower attachment and retention in nutritionally compromised animals (Carton *et al*., 2007; Babarro *et a*l., 2008). This was also the case with *P. maximus*, with detachment highest by animals starved for 6 days prior to attachment. Feeding juveniles a ration of 0.025g microalgae.g^{-1} juveniles.week^{-1} was enough to maximise retention. Tailoring feeding levels is essential to optimise production costs.

The implications of this study, is that recommended parameters for maximising juvenile *P. maximus* attachment and retention in water velocities up to 12.6±0.2cm second^{-1}, include utilising a slate type

substrate, pre-conditioned for 1 week, with juveniles having been fed a diet of at least 0.025g microalgae.g^{-1} juveniles.$week^{-1}$, and allowed to attach for 24 hours. These parameters provide a detailed base upon which future investigation can build, and they can also be considered for testing in hatchery operations.

Future work on attachment and detachment of bivalve post-larvae

There is a long list of environmental and culture variables that could be examined in both *M. edulis* and *P. maximus.* However, there are some specific areas of interest arising from investigations conducted with the two species in this study.

In *M. edulis* key areas for further work include hydrodynamic forces and the application of large scale trials. The influence of wave action, turbulence and flow velocity on byssus production and strength has been examined in *M. edulis* (Glaus, 1968; Price, 1982; Dolmer and Svane, 1994; Lachance *et al*., 2008), however there remains many variables that could be examined under flowing water regimes. Influential factors should not be considered in isolation, since the effects of one factor can modify and be modified by another (Paul, 1980a), with water velocity known to modify bivalve dispersal behaviour (Lundquist *et al*., 2004). Following the success of the work with *P. maximus* within a flume in this study, it could be rewarding to apply similar protocols to *M. edulis*. Interesting factors to assess include substrate type, substrate pre-conditioning, and feeding ration, among others. Unfortunately the flume used for *P. maximus* did not exist at the time of the work on *M. edulis*. Preliminary work in a 10metre benthic flume indicated that between 90 and 100% of attached mussel seed on wool substrates could resist detachment for 96 hours at velocities up to 28cm $second^{-1}$. This work must be treated with caution as it was conducted in a system without thermal regulation in which water temperatures exceeded 26±1˚C and would therefore require confirming. However, it is likely that a system with a maximum water velocity significantly greater than 28cm $second^{-1}$ would be required to test variables with *M. edulis* since mussels on benthic plots along the Menai Strait (North Wales, UK) can be exposed to velocities in the region of 50cm $second^{-1}$ adjacent to Beaumauris and 130cm $second^{-1}$ by Caernarfon, during spring tides (CAMS, 2014).

The influence of dietary ration in *M. edulis* should also be given further consideration. The influence of alternative feeding regimes pre- and post-attachment on *M. edulis* should be examined further, and specifically within a flume environment. In this case the questions are: will feeding increase

active dispersal? Will water flow prevent dispersal or will it facilitate dispersal of nutritionally compromised seed?

A priority for future studies should also be to examine the wool felt material, and similar substrates, in larger scale applications within the hatchery, but particularly in open water environments. This material offers an attractive mechanism which is abundant and renewable.

In *P. maximus* key areas for further work include the examination of alternative substrates, diet and effect of higher water velocities. In the current study attachment of juvenile *P. maximus* to test substrates could be highly variable, and high attachment rates cannot be guaranteed, even to the optimum material of slate. It is therefore important to examine alternative substrate materials, especially ones which could be used within the industry. Slate tiles could easily be placed within settlement and nursery systems particularly in land-based systems, although the industry typically employs plastics, nylon and polyethylene materials (Brand *et al*., 1980; Millican, 1997; Magnesen and Christophersen, 2007). It should also be considered for future investigation that the length of attachment period may have a greater effect, and longer attachment periods may reduce attachment variability. Therefore a wider range of attachment periods should also be investigated in order to optimise handling protocols. The impact of feeding rations on juvenile *P. maximus* retention is another topic for further investigation, with the potential to define the relationship between energy requirements for retention and other biological processes, and the impact of feeding level on the partitioning of resources.

Following the success of the flume with *P. maximus,* future work should continue to utilise this approach. It is a highly effective means of assessing the influence of additional environmental and culture variables on bivalve attachment and retention, as well as the influence of water velocity itself. However, it would be beneficial to be able to examine retention of *P. maximus* juveniles, and other species, over a wider range of velocities, specifically higher velocities in order to fully examine the limits of attachment, particularly over extended time periods. This is particularly important, since optimising other variables such as substrate and attachment period may lead to improved attachment and retention alone.

Diet clearly plays an important role in attachment and retention of bivalves, as shown in the current study. Therefore, arising from this study and the work by Gagné *et al*. (2012) it would be interesting to examine the influence of both quantity and quality of diet on *P. maximus* attachment and

retention further. Diet constituents and ration play a major role in determining *P. maximus* growth (Laing and Psimopoulous, 1998; Andersen *et al*., 2013) with diet affecting fatty acid composition (Delaunay *et al*., 1993). In *M. edulis* the combination of microalgae has proven important both for survival but also for settlement, with the ratio of fatty acids playing a significant role (Pettersen *et al*., 2010). Interestingly the ratio of n-3 to n-6 long chained polyunsaturated fatty acids, influenced settlement and was a key factor in determining larval performance (Pettersen *et al*., 2010). The combination of microalgae in bivalve diets is therefore critical. Quantifying the influence of proportional inclusion of different microalgae's and optimising the quantity necessary for optimum attachment and retention may provide further practical knowledge for optimising production capabilities of *P. maximus*, as well as other species.

Further examination of the influence of illumination on *P. maximus* behaviour is also advisable. Illumination plays a significant role in the recruitment and dispersal of marine invertebrates and the impact of light on behaviour has been demonstrated in other bivalve species (Bayne, 1964; Kobak, 2001). It would not only be interesting to further assess the influence of illumination on attachment, but also on subsequent detachment and dispersal. It would be valuable to conduct a detailed assessment of the influence of light intensity, stimulus direction, as well as photoperiod length in *P. maximus*.

Conclusion

In conclusion this study provides clear insights into the biology and behaviour of the commercially important bivalve species *P. maximus* and *M. edulis*, whilst it also forms the basis for future endeavours with them.

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