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Developing hatchery culture techniques for the winged pearl oyster, *Pteria penguin* (Röding, 1798)

Thesis submitted by
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for the degree of Doctor of Philosophy in Aquaculture of the School of Marine and Tropical Biology
James Cook University.
September 2011
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Statement of contribution of others

Project supervisor Professor Paul Southgate contributed to this research by providing consultation on experimental design and by making editorial suggestions during the drafting process of this thesis and all resulting scientific publications.

The embryos and larvae used to complete research outlined in Chapters 5, 6 and 7 of this thesis were cultured at the Aquaculture Facility of the Ministry of Agriculture, Food, Forests and Fisheries at Sopu in the Kingdom of Tonga, managed at the time by Poasi Ngaluafæ.

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Matthew Wassnig          Date
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This PhD thesis is dedicated to my mother, Anne, who instilled in me a fascination for nature and the drive to always continue learning.
Abstract

Pearl culture has traditionally relied on collecting juvenile pearl oysters (family: Pteriidae) from the wild and growing them to an appropriate size to be used in pearl production; a method that has become increasingly less viable due to a corresponding depletion in wild populations. Hatchery propagation of juvenile pearl oysters is now a necessity in regions where collection from the wild can no longer sustain commercial pearl production. The cultured pearl industry in the Indo-Pacific includes production of half-pearls or ‘mabè’ from *Pteria penguin* (Röding 1798). Pearl production from *P. penguin* has become progressively more reliant on hatchery culture of oysters; however, efficient production is constrained by a lack of knowledge regarding optimal culture techniques. This study aimed to develop hatchery culture techniques that could be implemented by industry to improve the prospects for pearl production from *P. penguin* and decrease the fishing pressure currently placed on wild populations.

The natural spawning season for *P. penguin* typically spans only a few months of each year, providing a short window for hatchery production of juveniles. A lack of knowledge regarding the diet and conditions required to optimise energy uptake by adult *P. penguin* has impeded the development of brood-stock conditioning programs that could be used to encourage gametogenesis outside of the natural spawning season. This study examined the pattern of suspension feeding by *P. penguin* in response to variations in microalgae diet, food concentration and water temperature. Brood-stock were placed in temperature controlled flow-though chambers that supplied individual oysters with a constant concentration of suspended microalgae. Feeding behaviour was quantified as the rate at which water was cleared of algae cells (clearance rate; CR) and the fraction of organic carbon absorbed during digestion (absorption efficiency; AE). The results showed that CR was...
greater when feeding on the two flagellate species *Isochrysis* sp. Tahitian (T-Iso) and *Pavlova* sp. (mean = 32 L h$^{-1}$ oyster$^{-1}$) when compared to the diatom *Chaetoceros muellieri* (27 L h$^{-1}$ oyster$^{-1}$). At temperatures of 24-28°C *P. penguin* maintained a stable CR with increasing food availability up to the maximum concentration tested (50 x 10$^3$ cells mL$^{-1}$). Mean AE was highest for T-Iso (61%) and not influenced by food concentration. Decreased CR and AE in response to a rapid reduction in water temperature during summer, reflected the 35% lower CR and 47% lower AE observed during the colder austral winter, suggesting temperature contributes to differences in suspension feeding between seasons.

The high feeding capacity of *P. penguin* raised the issue that in order to undertake brood-stock conditioning, hatcheries would require the facilities to culture large volumes of live microalgae. Pearl farms that use *P. penguin* are typically located in regional areas where the technical capacity for mass algae culture is not available. An experiment was conducted to assess the viability of using commercially available concentrated microalgae and a unique flow-through aquarium system to condition brood-stock prior to the natural spawning season. Fifteen *P. penguin* of a similar size were distributed between 5 identical 30 L ‘flow-through aquaria’. A mixed diet of concentrated microalgae from the Instant Algae® range was supplied to brood-stock for a period of 40 days with periodic increases (10 day intervals) in food concentration and water temperature up to a maximum of 40 x 10 cells mL$^{-1}$ and 28°C. Histological examination of gonad tissue was conducted at the conclusion of the study so that the reproductive condition of each oyster could be categorized using the five stages for pearl oyster gonad development, ranging from inactive to ripe. The same process was conducted for 15 similar sized *P. penguin* that were held in ocean culture for the same time period. The reproductive state of conditioned animals suggested that male *P. penguin* produced spermatozoa at a rate exceeding that observed in a wild environment over the same time period. The production of mature oocytes in experimental females was less reliable,
attributable to the period for conditioning being too short for the production of energetically expensive oocytes.

In order to develop techniques for hatchery culture of juvenile *P. penguin*, it was first necessary to understand the processes of embryogenesis and larval development for this species. Following standard methods used in the hatchery culture of other pearl oyster species, *P. penguin* eggs were spawned, fertilized and incubated until they hatched into shelled veliger larvae. Larvae were then fed a diet of live microalgae until developing a foot and being deemed competent to settle. Embryos and larvae were sampled periodically during hatchery culture to be examined under scanning electron microscope (SEM). The resulting high resolution images were then used to map the approximate timing of developmental stages. These stages included the first cleavage (1 h post-fertilisation; hpf), morula (2.5 hpf), blastula (4.5 hpf), gastrula (5.5 hpf), trochophore (7 hpf), D-stage (20 - 22 hpf), prodissoconch II (3 - 6 days post-hatching into D-stage; dph), umbone (10 - 12 dph) and pediveliger (22 dph). Comparison with patterns of embryogenesis and larval development in other oviparous oyster species revealed a similar sequence of key events, with differences occurring in the timing of developmental stages, shell structure and shell shape.

Embryo incubation is a period of pearl oyster culture that is typically characterised by excessive mortality. This study addressed the issue of embryo mortality by examining the effects of egg stocking density and the application of antibiotics during incubation. A factorial experimental design combined three egg densities (10, 50 and 100 mL⁻¹) and three antibiotic treatments (Control - no antibiotic; 5 mg mL⁻¹ streptomycin-sulfate; 5 mg mL⁻¹ tetracycline:erythromycin 2.5:2.5 mg mL⁻¹). Antibiotics were added to the culture medium as a single dose and fertilised eggs were incubated for a period of 24 h. Tetracycline:erythromycin (1:1) improved mean survival (23%), but yielded an average of
only 9% more veliger larvae than control aquaria due to interference with development. The antibiotic streptomycin-sulfate improved mean survival by 16% when compared to control aquaria, without significantly compromising development. A high egg density of 100 mL\(^{-1}\) did not significantly reduce survival, but resulted in a 5% reduction in normal development to D-stage. It is recommended that eggs be stocked at a density \(\leq 50\) mL\(^{-1}\) and mortality be minimised by treating the culture medium with the antibiotic streptomycin-sulfate.

Hatchery culture in regional areas is often impossible because farms cannot afford the facilities required to produce the live microalgae used as a food source for larvae. Concentrated algal paste supplied by Instant Algae® has been successfully trialled as an alternative food source during hatchery culture of *P. penguin*, but the feeding regime that promotes optimal larval growth and development is yet to be determined. Experiment 1 assessed the combined effects of stocking density and feed ration on the survival and growth of *P. penguin* larvae during D-stage (1-8 days post-fertilisation). Experiment 2 examined the effects of the same treatments on the survival and growth of larvae during umbo-stage (8 - 17 days post-fertilisation). Both experiments used a factorial design combining 3 egg stocking densities (Experiment 1: 2, 6 and 10 larvae mL\(^{-1}\); Experiment 2: 1, 3 and 5 larvae mL\(^{-1}\)) and 3 levels of feed ration (Experiment 1: 5, 10 and 15 \(\times 10^3\) cells mL\(^{-1}\); Experiment 2: 10, 15 and 20 \(\times 10^3\) cells mL\(^{-1}\)). Survival during D-stage was significantly enhanced (by 105%) in aquaria stocked at <10 larvae mL\(^{-1}\), whereby a density of 6 mL\(^{-1}\) maximised larval production per volume of culture medium. An intermediate feed ration of 10 \(\times 10^3\) cells mL\(^{-1}\) maximised both survival and growth during D-stage. Increasing the initial stocking density of umbo-stage larvae from 1 to 3 mL\(^{-1}\) resulted in a significant reduction of both survival (360%) and growth (16%). Growth of umbo-stage larvae stocked at 1 mL\(^{-1}\) increased significantly (7%) when feed ration remained below 20 \(\times 10^3\) cells mL\(^{-1}\).
Optimising the rate of larval settlement during pearl oyster hatchery cultivation is critical to maximising the production of juvenile spat for commercial use and is reliant on providing suitable stimuli. This study used two experiments to investigate the effects of (1) treating the culture medium with alternate concentrations of three chemical compounds (Serotonin; GABA; KCl) both in the presence/absence of a bio-film and (2) exposure to five substrate types (red nylon mesh with 5 mm and <1 mm pore sizes; black fibreglass mesh with 3mm and 1mm pore sizes; transparent smooth plastic) both in the presence/absence of a chemical cue (KCl), on recruitment of *P. penguin* pediveliger larvae. After 48 h, settlement was 65% greater in aquaria containing a substrate covered by a naturally formed bio-film than in control aquaria. After 72 h, settlement of larvae in aquaria treated with serotonin (10⁻³ M) or KCl (20 mM) was significantly greater than in control aquaria by 75% and 84%, respectively, while exposure to GABA had no effect. Settlement in response to 20 mM KCl was enhanced by the presence of a red nylon mesh substrate with 5 mm pore size.

The findings of this PhD project provide practical knowledge regarding techniques for efficient hatchery culture of *P. penguin*. The specific aims of this study place emphasis on facilitating hatchery propagation in regional communities within the Indo-Pacific. This research will aid in increasing pearl production from hatchery bred *P. penguin* and therefore alleviate much of the pressure currently being placed on overexploited wild populations.
List of publications originating from this thesis


Wassnig, M., Southgate, P.C. (to be submitted). The effects of microalgae diet, food ration, temperature change and season on the feeding behaviour of *Pteria penguin*.

Wassnig, M., Southgate, P.C. (to be submitted). The effects of stocking density and feed ration on the survival and growth of winged pearl oyster (*Pteria penguin*) larvae fed a diet of concentrated algal paste.
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Chapter 1

General introduction

1.1. Pearl oyster taxonomy

The currently accepted taxonomic classification of pearl oysters is as follows (Wada and Tëmkin, 2008):

Phylum: Mollusca

Class: Bivalvia Linnaeus, 1758

Subclass: Pterimorpha Beurlen, 1944

Order: Pterioida Newell, 1965

Suborder: Pteriina Newell, 1965

Superfamily: Pterioidea Gray, 1847

Family: Pteriidae Gray, 1847

Genus: Electroma Stoliczka, 1891

Genus: Pterelectroma Iredale, 1939

Genus: Pinctada Röding, 1798

Genus: Pteria Scopoli, 1777

Genera utilized for commercial pearl production

Most species from the family Pteriidae live in tropical and subtropical latitudes in the Indo-Pacific region (Wada and Tëmkin, 2008). There are in the order of 50 species in total, the majority of which belong to genera Pinctada and Pteria (Mikkelsen et al., 2004). Approximately 14 Pinctada and 20 Pteria species are found in littoral and sub littoral zones.
of continental shelf regions in the Indo-Pacific, where their habitat ranges from rocky reefs to sandy bottom substrates (Wada and Têmkin, 2008).

1.2. Pearl oyster biology

1.2.1. Morphology

The following brief description of the soft tissue anatomy in pearl oysters is taken from more detailed accounts by Velayudhan and Gandi (1987), Fougerouse-Tsing and Herbaut (1994) and Fougerouse et al. (2008). Fig. 1.1 shows the generalized positioning of organs and other soft body tissues within the shell of a pearl oyster. The anterior region of the soft tissue consists of a foot, byssus and mouth, surrounded by labial palps. In between the labial palps lie large pigmented eulamellibranch gills (ctenidia) containing frontal, lateral and latrofrontal cirri that are specialised for capturing suspended particles. A prominent adductor muscle is located in a posterior-ventral position within the shell. A dorsally located visceral mass encompasses much of the coiled intestine, digestive gland and gonad, while the central region of the tissue contains the heart and excretory system. The mantle consists of two lobes that line the inside of the two shell valves, enclosing all other soft tissue and organs. The mantle is responsible for the secretion of shell material.

Pearl oysters typically possess a straight shell hinge with 1-2 small tooth-like thickenings, a cavity below the anterior angle where byssal threads exit the oyster and a scaly surface on the outer shell (Bueno et al., 1991; Strack, 2006). The shell grows concentrically from the umbo, consists of approximately 95% calcium carbonate in the form of calcite and aragonite and is characterised by the following three layers (Fougerouse et al, 2008):

1. periostracum - an outer layer composed primarily of proteins;
2. ostracum - a medial prismatic layer composed of calcite crystals in an organic matrix;
3. hypostracum - an inner nacreous layer (mother-of-pearl) composed of aragonite crystals in an organic matrix.

Fig. 1.1: Diagram illustrating the generalized soft tissue anatomy of a pearl oyster.  
(Source: Fougerouse-Tsing and Herbaut, 1994)

1.2.2. Reproduction

Pearl oysters are protandrous hermaphrodites; that is, over time the sex of an individual changes from mature male to female, thus the sex ratio within a population approaches 1:1 with increasing age and size up to a threshold above which females outnumber males (Rose et al., 1990; Hernandez-Olalde, 2003; Milione et al., 2011). Changes in sex ratio can also vary in relation to environmental conditions over time (Saucedo and Monteforte, 1997), for
instance increased food availability and temperature during summer causing an increase in the percentage of females within a population (Milione and Southgate, 2012a). Repeated rhythmical sex reversal has been observed in several species and is thought to be primarily attributed to variations in energy reserves within the tissue, whereby higher energy levels favour the female condition (Saucedo and Southgate, 2008). The reproductive cycle of both sexes can be described using a categorical system consisting of the following five distinct stages (Tranter, 1953):

1. inactive - gametes yet to develop
2. developing - follicles partially filled with gametes
3. ripe - mature follicles full of gametes
4. spawning - partially emptied follicles
5. spent - emptied follicles undergoing regression

Sperm and eggs are simultaneously released into the water column where fertilisation occurs upon contact. The period of year during which pearl oysters spawn is influenced by environmental factors such as water temperature and food availability (Gervis and Sims, 1992). Therefore, the regularity and duration of the spawning period for a particular species can vary with latitude, ranging from year-round to seasonally discrete (Gervis and Sims, 1992; Saucedo and Southgate, 2008).

1.2.3. Life history

The following brief description of embryonic and larval development in pearl oysters is based on the findings of Wada (1953), Alagarswami et al. (1982), Alagarswami et al. (1983), Rose and Baker (1994), Araya-Nuñez et al. (1995) and Doroudi and Southgate (2003). The pattern of embryogenesis is consistent with other bivalves, entailing morula, blastula, gastrula and trochophore stages, before becoming free-swimming ‘D-stage’ veligers with a calcified shell.
The transition from fertilised egg to veliger larvae that are capable of feeding on exogenous food sources typically occurs within 24 h. New larval shell (prodissococonch II) is gradually added to the pre-existing shell formed during embryonic development (prodissococonch I) and the hinge structure changes from being straight to umbonal by 8-12 days post-fertilisation (‘umbo-stage’; Fig. 1.2). Pediveliger larvae develop a prominent ‘eye-spot’ and functional foot that allows them to search the available substrate (i.e. reef or other sessile organisms) for a suitable position to attach using byssal threads. The period between hatching and settlement can vary between species and populations, and according to environmental parameters such as water temperature and food availability, but is generally completed within 20-28 days post-fertilisation. It is thought that far less than 1% of fertilised eggs survive to settlement (Southgate, 2008).
Soon after settling, larvae metamorphose into juvenile ‘spat’ (Fig. 1.2) when the velum is reabsorbed and gills and a mantle are formed (Rose and Baker, 1994; Saucedo and Southgate, 2008). Pearl oysters feed primarily on picoplankton and nanoplankton by capturing the suspended organisms using specialized cirri within their eulamellibranch gills (Numaguchi, 1994; Pouvreau, 2000; Yukihiro et al., 2006). The quality and quantity of food, in conjunction with water temperature, determine a pearl oyster’s metabolic rate and scope for
growth (Yukihiro et al., 1998b; 2000; Lucas, 2008). The age at which pearl oysters reach sexual maturity is species-specific, ranging from 5-6 months in smaller species (e.g. *Pinctada fucata*) to 1-2 years in larger species (e.g. *Pinctada maxima*) (Tranter 1958; Rose et al., 1990; Milione and Southgate, 2011). Somatic growth continues but slows once the oyster reaches sexual maturity (Saucedo and Southgate, 2008). Smaller pearl oyster species have a lifespan of approximately 4 years, while larger species live for approximately 7-8 years and up to a maximum of 15 years (Gervis and Sims, 1992; Taylor and Strack, 2008).

1.3. Hatchery culture of pearl oysters

1.3.1. Rationale

Pearl culture of all species has traditionally relied on collecting spat from the wild and growing them to an appropriate size for pearl production (Southgate and Beer, 1997). This method has become less viable over time due to wild populations suffering prolonged exploitation (Southgate, 1995). Hatchery propagation has become increasingly important as a source of oysters for the pearl industry and is now a necessity in regions where the collection of wild spat no longer provides adequate numbers of oysters to sustain pearl production on a commercial scale (Southgate, 2008).

Hatchery propagation not only ensures a consistent supply of oysters, it also allows for the selection of commercially desirable genetic traits such as increased growth rate, disease tolerance and improved pearl quality (Evans et al., 1995, Kvingedal et al., 2010). Family lines can be established using breeding pairs of oysters with superior genetic traits, resulting in greater production of high quality pearls (Knauer et al., 1995). There is recent evidence to suggest that gametes produced from genetically superior oysters can be cryopreserved to be used at a later date (Acosta-Salmón et al., 2004), in particular the use of
preserved sperm to fertilise eggs during future spawning events (Lyons et al., 2005; Acosta-Salmón et al., 2007).

1.3.2. General methodology

1.3.2.1. Spawning and fertilisation

Pearl oyster brood-stock are typically kept in ocean culture suspended from a surface line in panel nets at a depth of 2-6 metres, allowing them to undergo reproductive maturation according to the natural spawning season for that geographical area (Gervis and Sims, 1992; Southgate, 2008). Brood-stock are visually inspected to determine their reproductive condition and ripe individuals of both sexes are then transported to the hatchery for artificial spawning induction (Rose, 1990; Southgate, 2008).

Thermal shock is the most effective way to induce spawning of pearl oysters, although other techniques such as water changing, chemical induction, exposition in the air and excessive feeding have also been used with some success (Araya-Nuñez et al., 1991; Rose and Baker, 1994; Southgate and Beer, 1997; Liang et al., 2001; Southgate, 2008). Thermal shock is achieved by alternating exposure of brood-stock to ambient and elevated (+5-6°C) water temperatures until spawning commences (Gervis and Sims, 1992; Southgate, 2008).

Once they have begun releasing gametes, individual oysters are removed from the communal spawning vessel and placed into separate aquaria to complete spawning (Southgate and Beer, 1997; Southgate et al., 1998a). Sperm and eggs from individual brood-stock can then be examined microscopically so that only motile sperm and negatively buoyant eggs are used for fertilisation (Rose, 1990). The gametes from both sexes are combined in two separate aquaria, one for sperm and another for eggs. Small volumes of sperm suspension are added slowly to the egg mixture until there are several sperm available
to each egg (Southgate, 2008). Fertilised eggs can be identified by the presence of the first polar body following initial meiotic division (Alagarswami et al., 1983; Rose and Baker, 1994; Doroudi and Southgate, 2003).

1.3.2.2. Embryo incubation

Fertilized eggs are rinsed onto a mesh sieve to remove superfluous spermatozoa and placed into large incubation tanks (500-2000 L) containing gently aerated 1µm filtered seawater (FSW) (Araya-Nuñez et al., 1991; Rose and Baker, 1994; Doroudi and Southgate, 2003). Egg density has been shown to have a significant effect on the survival of embryos and is not recommended to exceed 30-50 eggs mL\(^{-1}\) (Southgate et al., 1998a; Rose, 1990). A broad spectrum antibiotic such as streptomycin-sulfate is often added to incubation tanks in an effort to reduce the proliferation of harmful bacteria (Southgate and Beer, 1997; Beer, 1999). Fertilised eggs are allowed 24 h at ambient water temperature (usually 26-30°C) to complete embryogenesis and develop into free swimming veligers before being transferred to larval culture tanks (Southgate, 2008).

1.3.2.3. Larval culture

Similar to methods for embryo incubation, larvae are placed into large rearing tanks (500-2000 L) containing gently aerated FSW held at an ambient water temperature (26-30°C) (Araya-Nuñez et al., 1991; Rose and Baker, 1994; Doroudi and Southgate, 2003). The recommended stocking density during larval culture of pearl oysters is between 1-5 larvae mL\(^{-1}\) (Alagarswami et al., 1989; Rose, 1990; Southgate and Beer, 1997; Martinez-Fernández et al., 2003; Saucedo et al., 2007).

Larvae are fed a mixed microalgae diet typically consisting of two or more golden-brown flagellates (Prymnesiophyte), with the addition of at least one diatom (Bacillariophyte)
as they reach the umbo-stage and approach metamorphosis (Southgate, 2008). Feed ration is measured as cells per millilitre of culture medium and varies with stocking density (Doroudi and Southgate, 2000). Generally feed ration starts at $1-5 \times 10^3$ cells mL$^{-1}$ on day 1 and escalates periodically, reaching $25-50 \times 10^3$ cells mL$^{-1}$ at completion of the hatchery run (Rose, 1990; Chellum et al., 1991; Southgate and Beer, 1997; Martinez-Fernández et al., 2003). Bacteria levels rise with the decomposition of uneaten food and accumulation of excretory products, therefore it is important to regularly (every 2-3 days) empty larval rearing tanks and clean them before refilling with FSW (Alagarswami et al., 1989; Rose, 1990; Southgate and Beer, 1997; Martinez-Fernández et al., 2003).

1.3.2.4. Settlement

Once larvae have developed a foot and eye spot they are ready to settle and metamorphose into the juvenile form (see Section 1.2.3.). Competent pediveligers within a certain size range (usually 220-250 µm antero-posterior) are collected on a mesh sieve and transferred from larval culture to settlement tanks (Rose, 1990; Southgate, 2008). ‘Spat collectors’ made from artificial materials such as nylon mesh or plastic slats are suspended within settlement tanks to provide a substrate for settlement (Taylor et al., 1998; Southgate and Beer, 2007). Settlement in pearl oysters is asynchronous and may occur over several days (Southgate, 2008). Larvae are thought to cease feeding activity whilst attaching and metamorphosing (Yu et al., 2010). A flow-through system can be implemented to facilitate water exchange once settlement has occurred (Southgate and Beer, 1997; Martinez-Fernández and Southgate, 2007). The provision of external chemical cues has been shown to encourage settlement of pearl oyster larvae and can be achieved either naturally, by allowing spat collectors to form an epifloral bio-film on their surfaces prior to deployment (Zhao et al., 2003; Su et al., 2007;
Yu et al., 2008), or artificially, by dissolving neuroactive chemical compounds into the culture medium (Doroudi and Southgate, 2002; Zhao et al., 2003; Yu et al., 2010).

1.4. Description of *Pteria penguin* (Röding, 1798)

1.4.1. Morphology

The soft tissue anatomy of *Pteria penguin* follows the generic body plan for pearl oysters (Fig. 1.3) with the exception of some characteristics that are unique to *Pteria* spp. In contrast to other genera, *Pteria* spp. possess an oval shaped-adductor muscle, and mantle lobes that are not fused to the visceral mass (Wada and Tëmkin, 2008). Another morphologically distinct feature is the rectum, which passes through the heart and terminates with a subtriangular-shaped anal funnel (Wada and Tëmkin, 2008).

The most obvious distinctions between *Pteria* spp. and other pearl oysters relate to shell morphology. *Pteria* spp. have a shell that is much wider than it is high, a deeper right valve, and a hinge consisting of a sub-umbonal tooth in the left valve and ridge in the right valve, each with an opposing socket (Bueno et al., 1991; Gervis and Sims, 1992; Wada and Tëmkin, 2008) (Fig. 1.3). The shell of *P. penguin* is distinctly oblique in its outline and has a pronounced hinge that that resembles a wing, giving rise to the common name ‘winged pearl oyster’ (Gervis and Sims, 1992; Wada and Tëmkin, 2008) (Fig. 1.3). The exterior surface of the shell and inner prismatic margin are brown or black in colour and the nacre is typically iridescent silver with a pink or gold tint (Strack, 2006; Wada and Tëmkin, 2008). *P. penguin* is the largest species in the genus, with a maximum shell size (antero-posterior) exceeding 230 mm and capable of reaching 300 mm (Strack, 2006; Southgate, 2008). The maximum hinge length is 250 mm (Oliver, 1992).
1.4.2. Habitat and distribution

*P. penguin* tend to aggregate on rocky reefs in channels 5-35 m deep with high velocity currents (Ito, 1999). This species is known to settle preferentially on arborescent sessile organisms, in particular whip corals and gorgonians (Besley *et al.*, 1998; Beer and Southgate, 2000).

*P. penguin* has a wide distribution spanning from the Red Sea and east coast of Africa, throughout the tropical eastern Indo-Pacific, encompassing southern Japan, northern Australia, Southeast Asia, southern China and areas of both Micronesia and Melanesia (Strack, 2006; Wada and Têmkin, 2008) (Fig. 1.4).
1.4.3. Commercial exploitation

The relatively narrow mantle cavity of *Pteria* spp. presents difficulties for round pearl production (Mao *et al.*, 2004; Yu *et al.*, 2004); however, species from the genus are commonly used to produce valuable blister pearls (Southgate *et al.*, 2008). This type of pearl is analogous to the nacreous blisters that form naturally on the internal surface of the shell as the result of nacre covering shell protuberances (Taylor and Strack, 2008). Commercially produced half-spherical blister pearls are referred to as ‘mabé’, derived from the Japanese name for winged pearl oysters, Mabé-gai (Strack, 2006). The process for mabé production involves gluing up to five plastic nuclei to the inner nacreous shell layer, then allowing sufficient time for nacre to cover the nuclei to the desired thickness (6-12 months), before finally cutting the resulting pearls from the shell (Taylor and Strack, 2008). The methods are
relatively simple when compared to those for round pearl production and on this basis have facilitated economic pearl production in developing countries and coastal communities (Ruiz-Rubio et al., 2006; Teitelbaum and Ngaluafé, 2008).

A large shell size and unique nacre colouration have resulted in *P. penguin* being the most widely used species for mabé production (Southgate et al., 2008). Commercial culture of mabé from *P. penguin* began in the 1950’s on the Ryukyu Islands in Japan, a location that remains the stronghold of mabé production today, producing roughly 200,000 pearls per annum (Hisada and Fukuhara, 1999; Southgate et al., 2008). Similar to Japanese production, the mabé industry in China consists of a few large companies (Yu et al., 2004). Thailand has a mixture of large-scale and smaller family run mabé pearl farms (Bussawarit, 1995). A Japanese pearl company introduced *P. penguin* to the Kingdom of Tonga in 1975 with a view to establishing an industry within the Pacific; however, the Japanese abandoned the project not long after and the local industry was reduced to a few small locally owned farms (Malimali, 1995; Finau, 2005). The Tongan industry remains modest, despite a large area available to pearl farming that if developed to its full potential could yield an estimated 750,000 pearls per annum (Finau, 2005). *P. penguin* is also cultured on a small scale in Australia where the industry is dominated by round pearl production from *Pinctada* spp. (Southgate et al., 2008). Methods for round pearl production performed in Mexico using the closely related species *Pteria sterna* (Nava et al., 2000; Kiefert et al., 2004) have been recently been adopted for *P. penguin*. Successful round pearl production from *P. penguin* in southern China is likely to result in a greater contribution of this species to global pearl production in the future (Yu et al., 2004; Southgate et al., 2008).

The adductor muscle of *P. penguin* is highly prized in Japan where it is used in the sushi trade (Gervis and Sims, 1992). Other countries have been known to export the meat to Japan in a dried form called “kaibashira” (Gervis and Sima, 1992). The shell of *P. penguin* is
used for the traditional manufacture of buttons, inlay and jewellery in Asia and the Pacific (Philipson, 1989).

1.5. Hatchery culture of *Pteria penguin*

1.5.1. Current status

The near exhaustion of *P. penguin* populations in Japan and China has meant that commercial pearl production from this species is now dependent on hatchery propagation (Liang et al., 2001; Yu et al., 2004; Southgate et al., 2008). Spat fall is also particularly low in areas such as The Kingdom of Tonga, where *P. penguin* is an exotic species and does not recruit reliably in the wild (Teitelbaum and Ngaluafa, 2008). A joint venture between Australia’s James Cook University and the Tongan Ministry of Agriculture, Forests and Fisheries has recently been undertaken to commence hatchery culture of this species in an effort to provide local farmers with the oysters required to expand pearl production (Teitelbaum and Ngaluafa, 2008).

1.5.2. Limitations

There is a paucity of information regarding hatchery culture of *P. penguin*, which is thought to be a consequence of several factors including the modest scale of the industry, a reliance on traditional methodology and a reluctance of privately owned farms to release sensitive information that may benefit their competitors. The aim of this section is to outline the particular challenges faced during hatchery culture of *P. penguin* and thus highlight where research is required to improve production techniques.
1.5.2.1. Narrow spawning season

The natural spawning season for *P. penguin* typically spans a short period of each year (November - March) (Milione and Southgate, 2012a), unlike other tropical pearl oyster species such as *Pinctada maxima* and *Pinctada margaritifera* which experience more extended spawning seasons (Tranter, 1958; Rose, 1990; Acosta-Salmón and Southgate, 2005). Annual hatchery production of juveniles is therefore limited by the short period during which brood-stock possess ripe gonads. Aquarium culture methods have been used to successfully stimulate the development of ripe oocytes and active spermatozoa outside of the natural spawning in the pearl oyster *Pinctada mazatlanica* (Saucedo *et al*., 2001), but these techniques are yet to be developed for *P. penguin*. A major impediment to designing an effective land-based brood-stock conditioning program for *P. penguin* is a lack of knowledge regarding the diet and conditions required to optimise ingestion and digestion. Furthermore, suspension feeding is optimised by maintaining a constant favourable food level (Winter, 1978), requiring large volumes of marine microalgae which few hatcheries have the facilities and technical capacity to produce (Southgate, 2008).

1.5.2.2. Embryo mortality

Embryo incubation is typically characterised by high rates of mortality (Southgate, 2008) and is a part of the life-cycle of pearl oysters for which our knowledge of optimal culture conditions is lacking. Eggs must be stocked at a high density to ensure an adequate number of larvae for the next phase of production; however, this is likely to cause mortality by resulting in mechanical interference between eggs (Blaxter, 1956), greater bacterial proliferation from the introduction of decaying matter on the surface of eggs (Blaxter, 1956; Gruffydd and Beaumont, 1970) and the transfer of bacterial infections from parental gonad tissue (Riquelme *et al*., 1994; Jorquera *et al*., 2001). The effect of egg density on survival of *P.


*penguin* embryos and the efficacy of antibiotics in reducing bacteria induced mortality are currently unknown.

### 1.5.2.3. Limited technical capacity for larval rearing

Hatchery culture of *P. penguin* in coastal communities is difficult due to the limited funding and technical capacity available for the culture of live microalgae as a food source for larvae (Southgate *et al.*, 2006; Teitelbaum and Ngaluafe, 2008). Concentrated algal paste has been trialled as an alternative food source to live microalgae during *P. penguin* hatchery culture in the Kingdom of Tonga (Teitelbaum and Ngaluafe, 2008); however, the feeding regime that promotes optimal larval growth and development is yet to be determined. Concentrated algae cells have negative buoyancy and are no longer motile, therefore behave differently to live algae when introduced to larval rearing tanks (Heasman *et al.*, 2000). Furthermore, the effects of feed ration during larval culture vary according to density (Doroudi and Southgate, 2000; Liu *et al.*, 2010), thus both factors need to be considered in efforts to provide larvae with sufficient nutrition, without causing reduced water quality as a result of excessive metabolic waste and the decomposition of unconsumed food (Loosanoff and Davis, 1963; Doroudi *et al.*, 1999b).

### 1.5.2.4. Suboptimal recruitment

Optimising the rate of larval settlement during pearl oyster hatchery cultivation is critical to maximising the production of juvenile spat for commercial use and is reliant on providing suitable stimuli (Doroudi and Southgate, 2002; Zhao *et al.*, 2003; Saucedo *et al.*, 2005). Providing suitable cues for settlement is also required to synchronise settlement, which may otherwise prolong production by occurring over a number of days (Southgate, 2008). Molluscan species are known to exhibit a high degree of specificity in relation to settlement
cues (Hadfield, 1984), thus traditional methods for promoting settlement of pearl oysters may not be relevant to *P. penguin*. The ability of *P. penguin* larvae to respond to specific water-borne and surface-bound settlement cues in regards to detection and alteration of behaviour (Grassle *et al.*, 1992; Tamburri *et al.*, 1992) are at this stage unknown.

1.6. Major objectives of this study

Despite the economic value of pearls produced from *P. penguin* and the call for hatchery grown spat, there is very little published research regarding appropriate methods for hatchery production of this species. This study aimed to address the issues currently limiting efficient hatchery culture of *P. penguin*. The following is a list of the main objectives for this research with reference to the corresponding thesis chapter.

1. Identify conditions that optimise ingestion and absorption efficiency of microalgae by *P. penguin* brood-stock (Chapter 2).

2. Trial the use of a specialised aquarium system and a concentrated food source to maintain and condition *P. penguin* brood-stock (Chapter 3).

3. Describe embryonic and larval development of *P. penguin* (Chapter 4).

4. Examine the effects of egg stocking density and antibiotics on the survival and development of *P. penguin* embryos (Chapter 5).

5. Examine the effects of stocking density and feed ration on the survival and growth of *P. penguin* larvae fed a diet of concentrated algae (Chapter 6).

6. Examine the effects of various chemical and physical cues on the behaviour and settlement of *P. penguin* larvae (Chapter 7).
1.7. Study sites

1.7.1. James Cook University: Marine and Aquaculture Research Facilities Unit (MARFU)

James Cook University’s main campus is situated in Townsville, North Queensland, Australia. Adjacent to the campus is the University’s Marine and Aquaculture Research Facilities Unit (MARFU). This facility provides students with laboratory space to conduct scientific experiments relating to the fields of marine biology and aquaculture. Filtered seawater is stored at the facility so that it is continuously available. The complex provides a laboratory for the culture of marine microalgae, various outdoor tank systems, temperature controlled wet laboratories with seawater supply, equipment for filtering/treating seawater and a large range of aquaria of various designs and volumes.

1.7.2. Kingdom of Tonga Ministry of Agriculture, Food, Forests and Fisheries (MAFFF): Aquaculture Facility

James Cook University has collaborated with the Australian Centre for International Agricultural Research (ACIAR) and the Tongan Ministry of Agriculture, Food, Forests and Fisheries (MAFFF) to undertake the project “Winged Oyster Pearl Industry Development in Tonga (FIS/2006/172)”. A component of the research for this project was conducted at the shellfish hatchery located at the MAFFF Aquaculture Facility. This small scale facility is used to undertake mass culture of pearl oyster larvae. Larvae are cultured under commercial hatchery conditions within 2000 L tanks containing filtered seawater pumped from a tropical reef offshore. A purpose built laboratory is used for the analysis of biological samples. In the absence of facilities for the production of live microalgae, preserved algae is commonly used as food source during larval production.
The following data chapters were written as stand-alone articles for publication in scientific journals; therefore, the individual introduction sections for each chapter may contain some repetition of information presented within the General Introduction and prior data chapters. Full names for technical terms and species are used when first mentioned within each chapter.
Chapter 2

Suspension feeding by *Pteria penguin*

2.1. Introduction

Bivalve grazing can affect benthic ecosystems by changing phytoplankton community composition and limiting nutrient availability (Prins *et al*., 1998; Levinton *et al*., 2001; Jiang and Gibbs, 2005), as well as influencing rates of bio-deposition (Haven and Morales-Alamo, 1966; Hatcher *et al*., 1994; Nunes *et al*., 2003). Knowledge regarding suspension feeding of commercial bivalves can be used to ensure that intensive farming in near-shore environments remains sustainable without significantly altering the supply of nutrients or habitat characteristics of local ecosystems (Heral, 1993; Pouvreau *et al*., 1999; Nunes *et al*., 2003). Furthermore, studies conducted during the natural breeding season provide insight to the energy requirements of brood-stock undergoing reproductive maturation, thus are a valuable source of information during the design of brood-stock conditioning programs (Yukihiro *et al*., 1998a).

Bivalves are known to maximise energy uptake by altering their feeding behaviour in response to the composition and abundance of food. Two popular methods for quantifying feeding activity in bivalves are clearance rate: the rate at which ambient water is cleared of particulate matter; and absorption efficiency: the amount of organic carbon retained during the process of digestion (e.g. Navarro and Winter, 1982; Stenton-Dozey and Brown, 1992; Gardner, 2002). In addition to altering pumping rate, some bivalves maximise feeding efficiency through active retention of nutritional particles and rejection of excess or unsuitable particles as pseudofaeces (Jorgensen, 1990; Ward *et al*., 1998; Urrutia *et al*., 2001). Absorption efficiency of retained particles is often then optimized by controlling the
level of intracellular digestion and gut retention time (Brillant and MacDonald, 2000; 2002). Increased water temperature plays an important role in feeding efficiency by enhancing ingestion and metabolic rate during periods when energy is required for the production of gametes (Muranaka and Lannon, 1984; van Erkom Schurink and Griffiths, 1992; Saucedo et al., 2001); this occurs up to an optimal temperature beyond which digestion is restricted by the denaturation of catalytic enzymes (Shumway, 1982).

Pearl oysters have the highest recorded feeding rates among bivalve molluscs (Numaguchi, 1994; Yukihira et al., 1998a; Pouvreau et al., 2000) and can maintain efficient digestion at high food concentrations (Yukihira et al., 1998b). Their superior feeding capacity has been attributed to high pumping rates and a relatively large gill area (Pouvreau et al., 2000; Lucas, 2008). Energy absorption by pearl oysters has been shown to increase with food availability up to a certain threshold, where their capacity for ingestion and digestion is surpassed and energy is wasted producing pseudofaeces (Yukihira et al., 1998b). The pattern of this process and conditions for optimum growth differ between pearl oyster species and the diet to which they are exposed (Yukihira et al., 1998b). Feeding efficiency of pearl oysters has been shown to increase with water temperature up to a species-specific thermal optimum (Numaguchi, 1994; Yukihira et al., 2000).

Previous research examining the feeding behaviour of pearl oysters has focused on species from the genus Pinctada (e.g. Sato et al., 1964; Numaguchi, 1994; Yukihira et al., 1998a; Pouvreau et al., 1999; Loret et al., 2000), resulting in a paucity of information for all other species, including Pteria penguin. This study examined the effects of microalgae diet, food concentration and water temperature on the clearance rate and absorption efficiency of phytoplankton by P. penguin. The primary aim was to acquire the knowledge of feeding capacity required to design a brood-stock conditioning program. It was hypothesized that
clearance rate and absorption efficiency would vary according to experimental conditions and that optimum levels would exist within the levels tested.

2.2. Materials and Methods

2.2.1. Experimental animals

*P. penguin* brood-stock used in hatchery propagation by James Cook University are held in ocean culture conditions within pearl oyster nets suspended from a long-line at Orpheus Island, north Queensland, Australia (18°36’24 S, 146°29’10 E). Two months prior to participating in feeding trials experimental oysters were transported to similar ocean culture conditions at the Australian Institute of Marine Science (AIMS), north Queensland, Australia (19° 15’ S, 147° 05’ E), so as to be accessible to the experimental laboratories located at the Marine and Aquaculture Research Facilities Unit (MARFU) in Townsville, Queensland. Oysters were habituated to laboratory conditions for a period of 24 h prior to feeding trials, during which time they were held in flowing unfiltered seawater and allowed to feed naturally. Shell height (SH), the longest distance from umbo to the edge of the outer shell, was used as the standard measure of oyster size (Wada and Tëmkin, 2008).

2.2.2. Microalgae culture

All unicellular microalgae used in feeding trials (*see Sections 2.2.6 - 2.2.8*) were cultured using techniques described by Minaur (1969). Pure culture strains were obtained from the Australian National Algae Culture Collection (ANACC) (CSIRO Laboratories, Hobart, Tasmania, Australia) and maintained in 250 mL Erlenmeyer flasks illuminated by fluorescent lamps for a 12 h photoperiod. Due to the large volume of algae required, high density cultures were transferred for mass culture in 60 L sterile plastic bags filled with UV treated 1 µm filtered seawater (FSW), held upright within cylindrical metal cages. Cultures were supplied
with an f/2 nutrient medium (AlgaBoost, AusAqua Pty Ltd., Wallaroo, South Australia, Australia) and harvested during the logarithmic phase of growth.

2.2.3. Aquarium system

An aquarium system consisting of 10 identical 8 L flow-through chambers (Fig. 2.1) (after Widdows, 1985; Yukihira et al., 1998a) was used to measure clearance rate (CR): the volume of water that each oyster cleared of algal cells per unit of time. Experimental chambers contained a single oyster sitting on a gridded false floor that allowed faeces to fall away from the animal and empty chambers served as controls. Incoming FSW containing a consistent concentration of suspended microalgae (see Sections 2.2.6 - 2.2.8) was pumped through each chamber at a flow rate of 30 ± 2 L h⁻¹, set according to previous studies conducted on other similar sized pearl oyster species (Yukihira et al., 1998a; Yukihira et al., 1998b). FSW entered each chamber at a point near to the bottom and moved upwards past the oyster before flowing out at the top. FSW was heavily aerated prior to entering the chambers to ensure oxygen levels remained above 5.0 mg L⁻¹ and 75% saturation and microalgae remained suspended. Oxygen concentration entering chambers was continuously monitored using a YSI 55 digital dissolved oxygen meter (YSI Australia, Morningside, Queensland, Australia).
2.2.4. Measuring clearance rate

Clearance rate (CR) (L h\(^{-1}\)) was measured for each oyster individually at 1 h intervals after it had commenced feeding normally and took place over a total period of 4 h (Yukihira et al., 1998a). Normal feeding behaviour of oysters was typically observed within 30 minutes of being placed into chambers, signified by a slight gap between shell valves (5 – 15 mm) and a subtle water current from the posterior end of the oyster (Lucas, 2008). Algae concentration was quantified using a FlowCAM® (Fluid Imaging Technologies, Yarmouth, Maine, U.S.A.); an integrated system for rapidly analysing particles in a fluid by combining the capabilities of flow cytometry, microscopy and laser imaging. CR was calculated using the
flow rate of water entering each experimental chamber (F) (L h⁻¹), estimates of the concentration of algae surrounding each oyster (C0) (cells mL⁻¹) and the concentration of algae at the inflow (C1) and outflow (C2) of each chamber (after Hildreth and Crisp, 1976):

\[
CR \ (L \ h^{-1} \ oyster^{-1}) = F \ (C1 - C2)/C0
\]

2.2.5. Measuring absorption efficiency

The faeces remaining in each chamber after 4h feeding trials were collected and placed on pre-rinsed/pre-combusted GF/C filter papers. Faecal samples were then dried, weighed to the nearest 0.0001 g and ashed at 450°C for 5 h before being weighed again (Yukihira et al., 1998a). Samples of the microalgae used as a food source were captured by filtering 2 L of the algae suspension onto pre-rinsed/pre-combusted GF/C filter papers. The microalgae samples were then dried, weighed and ashed using the same methods described above for faecal samples. The percentage of organic material absorbed during digestion, referred to as absorption efficiency (AE), was determined by comparing the fraction of dry faeces (f) lost on ashing with the fraction of dry algae (a) lost on ashing (after Conover 1966):

\[
AE \ (%) = 100 \times (a - f)/(1 - f)a
\]

2.2.6. Effect of microalgae diet

This experiment was conducted during the month of November to coincide with the period of year when *P. penguin* in north-eastern Australia are reproductively active (Milione and Southgate, 2012a). Seventy two oysters (APM: 179 – 240 mm) were divided into three groups of 24 so that there was no significant difference (one-way ANOVA; α = 0.05) in the mean SH of each group. Clearance rate (CR) and absorption efficiency (AE) were measured for each group of 24 oysters while exposed to a particular species of microalgae; (1) *Isochrysis* sp. Tahitian (T-Iso) (Haptophyceae) (CSIRO strain code: CS-177), (2) *Pavlova* sp.
(Haptophyceae) (CS-50) and (3) Chaetoceros muelleri (Bacillariophyceae) (CS-176). Eight oysters were placed in separate aquarium chambers and two chambers were left empty to act as controls, thus 3 feeding trials were required to test all 24 oysters exposed to a particular microalgae. An ambient water temperature of 26-28°C was used throughout the experiment. Preliminary research showed that adult *P. penguin* feed without producing pseudofeces at algae cell densities up to 55 - 60 x 10^3 cells mL^-1. For the purpose of this experiment, microalgae were supplied at a standard concentration of 40 ± 2 x 10^3 cells mL^-1 to ensure faecal samples did not include undigested material in the form of pseudofeces. One-way ANOVA and *post-hoc* Tukey tests were used to determine if there were significant differences in mean CR and AE between microalgae diets.

### 2.2.7. Effect of food concentration at various water temperatures

This experiment was conducted during the months of November and December the following year. Seventy-two oysters (SH = 185 - 243 mm) were divided into four groups of 18 so that there was no significant difference in the mean SH for each group. Nine oysters were placed in separate aquarium chambers and a single chambers was left empty to act as a control, thus 2 feeding trials were required to test all 18 oysters exposed to a particular microalgae concentration. Microalgae species *Isochrysis* sp. Tahitian (T-Iso) was used as a food source based on the results for the previous experiment on microalgae diet (*see Section 2.3.1*). CR and AE were measured for each group of oysters while exposed to one of four food concentrations (20, 30, 40 and 50 x 10^3 cells mL^-1) at an ambient water temperature of 28°C. The process described for testing the effects of food concentration above were repeated at water temperatures of 20 and 24°C. The temperatures used were within the annual range previously recorded for north-eastern Queensland (*Yukihira et al.*, 2000). Water temperature was gradually adjusted from an ambient 28°C at a rate of 2°C per 6 h to the required level.
during the standard 24 h laboratory acclimation period that preceded all feeding trials. The same methods of analysis described in Section 2.2.6 were used to examine feeding behaviour at the various water temperatures.

2.2.8. Effect of season

Feeding behaviour was examined again during July of the following year, which is the period of year when water temperature is lowest and *P. penguin* are in a “resting” state, therefore no longer reproductively active (Milione *et al.*, 2011). Using the methods previously described in Section 2.2.7., CR and AE were measured while exposed to two concentrations of T-Iso (20 x 10^3 and 40 x 10^3 cells mL^{-1}) at an ambient water temperature of 20°C. Oysters were allocated to experimental groups to ensure no significant difference in mean SH compared to the oysters used to assess feeding behaviour under the same conditions during summer. Data from both seasons were compared using one-way ANOVA to identify significant differences in CR and AE.

2.3. Results

2.3.1. Effect of microalgae diet

The monitoring of control chambers indicated that rigorous aeration within the systems header tank and a fast flow rate ensured microalgae did not fall out of suspension at a detectable rate, thus food concentration remained stable within control chambers in the absence of oysters. There was no significant difference in the clearance rate (L h^{-1} oyster^{-1}) of *P. penguin* when fed the flagellates *Isochrysis* sp. Tahitian (T-Iso) and *Pavlova* sp., however, CR was significantly greater (F = 3.422, df = 2, p = 0.038) when fed flagellates compared to the diatom *Chaetoceros muelleri* (Table 2.1). A significant difference in mean absorption efficiency (%) existed between all three microalgae species (F = 16.528, df = 2, p < 0.001)
whereby the highest values were recorded for T-Iso, followed by Pavlova sp. and finally Chaetoceros muelleri (Table 2.1).

Table 2.1: Clearance rate (CR) and absorption efficiency (AE) of Pteria penguin when feeding on three species of microalgae.

<table>
<thead>
<tr>
<th>Algae species</th>
<th>CR (±SD) (L h(^{-1}) oyster(^{-1}))</th>
<th>AE (±SD) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-Iso</td>
<td>31.8 ± 8.5(^{a})</td>
<td>61.6 ± 14.1(^{a})</td>
</tr>
<tr>
<td>Pavlova sp.</td>
<td>32.2 ± 6.9(^{a})</td>
<td>43.6 ± 18.8(^{b})</td>
</tr>
<tr>
<td>C. muelleri</td>
<td>27.4 ± 4.9(^{b})</td>
<td>28.0 ± 10.8(^{c})</td>
</tr>
</tbody>
</table>

* Microalgae supplied at a standard concentration of 40 x 10\(^3\) cells mL\(^{-1}\).
** Superscript letters indicate Tukey groupings.

2.3.2. Effects of food concentration at various water temperatures

An increase in the concentration of algae had a significant effect on CR at 20°C (F = 18.583, df = 3, p < 0.001) and 24°C (F = 34.229, df = 3, p < 0.001), but not at the ambient summer temperature of 28°C. At the lowest tested water temperature of 20°C, CR increased with food concentration up to a cell density of 40 x 10\(^3\) cells mL\(^{-1}\) before decreasing at 50 x 10\(^3\) cells mL\(^{-1}\) (Fig. 2.2). CR improved with increasing water temperature at each food concentration, except for at 40 x 10\(^3\) cells mL\(^{-1}\) (Fig. 2.2).
Fig. 2.2: Mean (± SE) clearance rate (L h⁻¹ oyster⁻¹) of *Pteria penguin* when fed *Isochrysis* sp. Tahitian (T-Iso) over a range of cell concentrations (cells mL⁻¹), at water temperatures of 20°C (solid line), 24°C (broken line) and 28°C (dashed line).

At water temperatures >24°C, greater food availability resulted in a positive relationship between the mean number of algae cells ingested and food concentration (Fig. 2.3). Ingestion rate dropped markedly when exceeding a food concentration of 40 x 10³ cells mL⁻¹ at 20°C due to the drop in clearance rate (see Fig 2.2). Mean AE did not change significantly with increasing algae concentration up to the maximum level tested. Overall for all food concentrations, mean AE was greater at 28°C when compared to both the two lower water temperature treatments (Fig. 2.4). No production of pseudofaeces was observed during feeding trials.
Fig. 2.3: Mean (± SE) number microalgae cells ($10^6$ h$^{-1}$ oyster$^{-1}$) ingested by *Pteria penguin* when fed *Isochrysis* sp. Tahitian (T-Iso) over a range of cell concentrations (cells mL$^{-1}$), at water temperatures of 20°C (solid line), 24°C (broken line) and 28°C (dashed line).
3.2.3. Effect of season

During winter (July; ambient water temperature 20°C), CR and AE of *P. penguin* did not change significantly when food concentration was doubled from 20 x 10^3 cells mL^-1 to 40 x 10^3 cells mL^-1. This stable feeding behaviour was in keeping with the trend observed at an ambient water temperature during summer (28°C) (Fig 2.2). When pooling the data for both food concentrations at ambient water temperatures, *P. penguin* demonstrated a significantly lower mean CR (F = 482.883, df = 1, p < 0.001) and AE (F = 38.096, df = 1, p < 0.001) of T-Iso during winter (CR = 11.8 ± 4.6; AE = 32.6 ± 10.3) compared to summer (CR = 33.4 ± 8.4; AE = 69.3 ± 13.3), with a decrease in CR and AE of 35% and 47%, respectively.

![Fig. 2.4](image)

**Fig. 2.4:** Mean (± SE) absorption efficiency (%) of *Pteria penguin* when fed *Isochrysis* sp. Tahitian (T-Iso) at a range of cell densities (20, 30, 40 & 50 x 10^3 cells mL^-1) at three different water temperatures (°C).
2.4. Discussion

Pearl oysters from the genus *Pinctada* are considered to have very high clearance rates (CR), exceeding those recorded for 26 other bivalve species, including clams, mussels, scallops and giant clams (Yukihiro *et al.*, 1998a). The data generated during this study allows the filtering capacity of pearl oysters from two genera to be compared. The size of *P. penguin* used in this study and another study that used *Pinctada margaritifera* (SH = 121 – 180 mm) and *Pinctada maxima* (SH = 132 – 237 mm) (Yukihiro *et al.*, 1998b), all fall within the bounds expected for mature adults (Wada and Temkin, 2008). When fed *Isochrysis* sp. Tahitian (T-Iso) at a density of $20 \times 10^3$ cells mL$^{-1}$ and ambient temperature of 28°C, the CR’s for *P. margaritifera* and *P. maxima* (Yukihiro *et al.*, 1998b) were approximately 32 and 29 L h$^{-1}$ oyster$^{-1}$, respectively, similar to the 33 L h$^{-1}$ oyster$^{-1}$ recorded during this study for *P. penguin* using similar methods. However, when food concentration was doubled to a density of $40 \times 10^3$ cells mL$^{-1}$, the CR of *P. penguin* remained relatively stable, whereas CR for the two *Pinctada* species was reported to decline by an average of 59% (Yukihiro *et al.*, 1998b).

Bivalves have been known to stabilize filtration rate at food concentrations approaching ingestion saturation, therefore minimizing the energy wasted in rejecting food material as pseudofeces (Foster-Smith, 1975). The ability of *P. penguin* to ingest high concentrations of algae without producing pseudofeces may in part explain the similar CR observed at each food concentration up to the maximum tested, at both summer and winter ambient water temperatures. Further research is required to determine if CR would remain stable at food concentrations beyond $50 \times 10^3$ cells mL$^{-1}$. When feeding at 20°C, the CR of *P. penguin* dropped with an increase in food concentration above $40 \times 10^3$ cells mL$^{-1}$, suggesting that the ability to maintain CR at high food concentrations is temperature dependent. *P. penguin* have been shown to grow more rapidly in turbid near-shore environments with high concentrations of particulate matter when compared to habitats further offshore (Milione and
Southgate, 2012b). Being able to maintain filtration rate in turbid conditions may account for the wide ecological range inhabited by *P. penguin* when compared to *Pinctada* spp., which prefer the oligotrophic waters of offshore rocky reefs (Wada and Tëmkin, 2008). The high filtering capacity of *P. penguin* may also explain why this species is found predominantly in areas of high current (Ito, 1999) where food replenishment occurs rapidly. In habitats where the availability of particulate organic matter is low, such as the tropical lagoons often used for pearl culture (Pouvreau *et al*., 1999), it is conceivable that intensive farming of *P. penguin* could impact local seston composition.

The dimensions of cells for each microalgae species used in this study (diameter 6-9 µm) fall within the bounds retained by bivalves with over 90% efficiency (Mohlenberg and Riisgard, 1978; Riisgard, 1988). Prior studies have shown that compared to other bivalves, pearl oysters demonstrate little capability for pre-ingestive sorting of particles to maximize the organic fraction of ingested material (Hawkins *et al*., 1998; Yukihiro *et al*., 1999); therefore, a slight discrepancy in CR when feeding on different microalgae is likely attributed to differences in the ingestibility of individual cells. While pre-ingestive selection in pearl oysters is limited, there is evidence for post-ingestive selection of ingested material for enhanced digestion by *P. margaritifera* (Loret *et al*., 2000; Lucas, 2008). Likewise, clam and scallop species are known to select certain ingested material based on physical dimensions and chemical cues for longer periods of gut retention and further intracellular digestion (Decho and Luoma, 1991; Brillant and MacDonald, 2000; 2002). Of the microalgae species offered to *P. penguin* in this study, significantly greater absorption efficiency (AE) was recorded for *Isochrysis* sp. Tahitian (T-Iso), supporting previous observations of a high AE of T-Iso by *Pinctada* spp. (Yukihiro *et al*., 1998b). Further research is required to discern if the relatively greater AE of T-Iso observed in this study is based purely on digestibility or
provides evidence for selection of this high energy food source (Whyte, 1987) for enhanced digestion.

There is some conjecture about whether or not bivalves are capable of compensating for annual changes in water temperature by retaining a constant level of feeding activity throughout the year (Widdows and Johnson, 1988; Kittner and Riisgard, 2005). This study supports previous findings that CR of phytoplankton by pearl oysters is negatively affected by a reduction in water temperature (Numaguchi, 1994; Yukihiro et al., 2000). As has previously been shown for *Pinctada fucata* (Numaguchi, 1994), *P. margaritifera* and *P. maxima* (Yukihiro et al., 2000), *P. penguin* demonstrate reduced ingestion during winter, attributable to a reduction in respiration and thus metabolic rate (Kobayashi and Tobata, 1949; Lucas, 2008). AE of the algae T-Iso by *P. penguin* was significantly reduced in winter when compared to summer when examined at ambient water temperatures, indicating a greater sensitivity of digestion to environmental change than that seen in *P. margaritifera* and *P. maxima* for which season had little effect on AE of T-Iso (Yukihiro et al., 2000). The difference in CR and AE of the microalgae T-Iso by *P. penguin* between summer and winter resembles the pattern seen when rapidly reducing water temperature during summer, suggesting that a reduction in water temperature serves either as a cue or dictates reduced feeding activity in winter.

The results of this study indicate that *P. penguin* alter their filtration and digestion efficiency in response to changes in diet, food availability and water temperature. As is the case for all filter feeders, *P. penguin* alter phytoplankton composition and rates of biodeposition within their local habitat, thus population sizes in areas of pearl farming must be managed to prevent adverse affects to local ecosystem dynamics. This study provides insight into the conditions required to optimise feeding efficiency and energy uptake during the period of year that *P. penguin* are reproductively active.
Chapter 3
Using concentrated microalgae to maintain and condition

*Pteria penguin*

3.1. Introduction

Hatchery production of *Pteria penguin* is reliant on the controlled spawning of mature brood-stock during the natural spawning season; this typically spans only a few months of each year (Milione and Southgate, 2012a). Hatchery production outside of the natural spawning season has been successfully achieved for species of scallop (Turner and Hanks, 1960; Sastry, 1963; Villalaz, 1994; Monsalvo-Spencer *et al.*, 1997) and clam (Loosanoff and Davis, 1950; Helm and Bourne, 2004; Ojea *et al.*, 2008) via exposure to adequate water temperatures and appropriate feeding regimes of live microalgae. Hayashi and Seko (1986) were the first to observe maturation of a pearl oyster species (*Pinctada fucata*) in response to cultured microalgae, although the mono-specific diet used did not yield fully mature brood-stock. Saucedo *et al.* (2001) used aquarium culture methods to successfully stimulate the development of ripe oocytes and active spermatozoa in the pearl oyster *Pinctada mazatlanica*, however once again, gonad development did not reach spawning condition. This may have been due to discontinuous pulse feeding regimes being implemented rather than a continuous food supply.

Pearl oysters typically have a far greater filtering capacity than other commercial bivalve species (Yukihiro *et al.*, 1998a; *see Chapter 2*). Suspension feeding of bivalves is optimised by maintaining a constant supply of food (Winter, 1978), therefore systems for brood-stock conditioning require an ongoing supply of large volumes of marine microalgae which few pearl oyster hatcheries have the facilities and technical capacity to produce.
The advent of commercially available concentrated microalgae products has meant that land-based culture of bivalves can be undertaken in areas where the infrastructure for live microalgae production is not available.

The results of Chapter 2 of this study suggest that providing *P. penguin* brood-stock with a balanced diet containing a high proportion of *Isochrysis* sp. (T-Iso) at a cell concentration of 40-50 x 10^{3} cells mL^{-1} and ambient summer water temperature (~28°C), will replicate conditions during the natural spawning season and facilitate a high rate of energy absorption and on that basis increase the probability of gametogenesis. This study aimed to provide preliminary data regarding the potential of using concentrated microalgae to maintain and condition *Pteria penguin* brood-stock in a unique flow-through aquarium system. It was hypothesised that providing a transition from ambient to optimal feeding conditions during the late austral winter, when brood-stock are in a “resting” state and therefore not reproductively active (Milione and Southgate, 2012a), would stimulate gametogenesis prior to the natural spawning season.

### 3.2. Materials and Methods

#### 3.2.1. Aquarium system

The aquarium system used in this study consisted of 5 identical 30 L ‘flow-through aquaria’ capable of holding several oysters each. The system was segmented into separate aquaria so that in the event an experimental oyster died, the decaying matter would not negatively impact all oysters in the system. Similar to the aquarium design used in Chapter 2 (Fig. 2.1), filtered seawater (FSW) (to 1 µm) was pumped from a temperature controlled 1200 L tank to a heavily aerated header tank designed to distribute the water evenly into flow-through aquaria at a rate of 60 ± 2 L h^{-1} aquarium^{-1}, providing a 100% water exchange per aquarium every 30 min. FSW entered each flow-through aquarium at a point near to the bottom and
moved upwards past the oyster before flowing out at the top. Gentle aeration within individual aquaria was used to ensure that oxygen levels remained above 5.0 mg L\(^{-1}\) and 75% saturation and algae cells remained in suspension. Oxygen concentration was monitored daily using a YSI 55 digital dissolved oxygen meter (YSI Australia, Morningside, Queensland, Australia). Aquaria were emptied and cleaned once a day to remove faecal matter.

3.2.2. Study design

This study was undertaken at James Cook University’s Marine and Aquaculture Research Facilities Unit (MARFU) in northern Queensland, Australia, and used \(P.\ penguin\) previously held in pearl nets suspended from a long-line in Pioneer Bay, Orpheus Island (18°36′24 S, 146°29′10 E). The study was conducted for a period of 40 days from late August to early October, to assess if aquarium culture techniques could be used to promote gamete production prior to the natural spawning season of November to March (Milione and Southgate, 2012a). Fifteen adult \(P.\ penguin\) (mean shell height (SH) = 204 mm) were divided evenly across the 5 aquaria, thus 3 oysters per aquarium.

A mixed diet of concentrated microalgae from the Instant Algae® range (Instant Algae®, Reed Mariculture Incorporated, Campbell, CA, USA, 95008) was supplied to brood-stock continuously throughout the study period. In terms of the number of cells mL\(^{-1}\), the diet consisted of 50% \(Isochrysis\) sp. (Haptophyceae), 25% \(Pavlova\) sp. (Haptophyceae), 15% \(Thalassiosira\ weissflogii\) sp. (Bacillariophyceae) and 10% \(Tetraselmis\) sp. (Chlorophycophyceae). One mg L\(^{-1}\) dry weight of the mixed diet was equal to approximately 10,000 cells mL\(^{-1}\).

Brood-stock were continuously provided with suspended concentrated algae, which at the beginning of the study period was supplied at a concentration of 10 x 10\(^3\) cells mL\(^{-1}\), approximately twice the average phytoplankton concentration commonly encountered by
pearl oysters in their natural habitat on the Great Barrier Reef (Yukihiro et al. 2008a). Food concentration was increased by $10 \times 10^3$ cells mL$^{-1}$ at 10 day intervals beginning on day 11. Water temperature was maintained at an ambient 23.5°C for the initial 10 days and increased by 1.5°C at ten day intervals beginning on day 11. This regime was based on the results of Chapter 2, which showed an increase in energy absorption by *P. penguin* with food concentration and water temperature up to $40 \times 10^3$ cells mL$^{-1}$ and 28°C, respectively.

3.2.3. Monitoring feeding behaviour

The rate at which ambient water is cleared of algae cells was measured for each aquarium every 3 days beginning on day 4 and the data used to calculate clearance rate (CR) (L h$^{-1}$) (*see Section 2.2.4*). CR values for aquaria containing multiple brood-stock could not be used to accurately estimate CR on a per oyster basis as was done in Chapter 2, however the data could be converted to a simple scale of 1-100 to convey the overall pattern of feeding behaviour over time, termed here as relative CR. Data was converted to relative CR by having the upper most clearance rate measurement equal 100 and scaling all other measurements accordingly.

The faeces remaining in each aquarium were collected every 3 days to monitor absorption efficiency over time (AE), that is, the proportion of available organic material absorbed during digestion (*see Section 2.2.1*). Energy absorption (EA) (J h$^{-1}$) per aquarium was estimated as the product of the energy content of the food source (J mg$^{-1}$), algal concentration (mg L$^{-1}$), relative clearance rate (L h$^{-1}$) and absorption efficiency (%) (following Widdows et al., 1985). As described above for CR, the data for EA was also converted to a relative scale of 1-100 to convey the overall pattern of feeding behaviour over time, termed here as relative EA.
Proportional (%) data were arcsine square-root transformed prior to statistical analyses. Plots of residual values revealed no relationship with predicted data and all data sets were confirmed to be approximately normally distributed. Linear fixed effects modelling was conducted using tank as a random variable to determine if there were significant changes (α = 0.05) in CR, AE and EA between tanks and over time. Significant differences are reported within the text.

3.2.4. Assessing reproductive condition

Ten *P. penguin* (mean SH = 212 mm) were dissected at the commencement of the study so that their gonad tissue could be preserved within FAAC, a formaldehyde based fixative solution (formaldehyde 4%, acetic acid 5%, calcium chloride 1.3%). The same method was used at the conclusion of the 40 day study to preserve the gonad tissue of the 15 *P. penguin* (mean SH = 204 mm) placed in experimental chambers and also 15 similar sized *P. penguin* (mean SH = 201 mm) that had been feeding on natural seston at Orpheus Island. One-way ANOVA revealed no significant difference in mean SH between the oysters used to gather baseline data and the two study groups (experimental and ocean culture). Histological analysis was performed on samples of gonad tissue from each oyster according to the methods described by Milione and Southgate (2011b). Sections of tissue were removed from the preserved visceral mass, embedded in wax and sliced to >10 µm to provide cross-sections that could be mounted on glass slides and stained using Haematoxylin and Eosin. Observations at 100X magnification were used to categorize each oyster’s condition into one of the five stages of pearl oyster gonad development described by Tranter (1958): (1) inactive (gametes absent), (2) developing (partially filled follicles), (3) ripe (mature filled follicles), (4) spawning (partially emptied follicles) and (5) spent (empty follicles undergoing regression).
3.3. Results

3.2.1. Feeding behaviour

There was no significant effect of tank in any data set precluding to feeding behaviour, indicating that oysters within each of the five tanks demonstrated similar behaviour. Mean CR remained relatively stable throughout the experiment despite increased food concentration with time (Fig. 3.1A). Linear fixed effects modelling and post-hoc Tukey analysis indicated that the only significant difference between data collected at various sampling times occurred between the lowest reading on day 16 and the highest reading on day 31 (F = 30.608, p = 0.022).

Absorption efficiency (AE) decreased significantly with time (F = 2.713, p < 0.001) in an approximate linear fashion according to the equation y = -2.61x + 93.98 (R² = 0.92), dropping from 89% on day 4 to 62% on day 37. Post-hoc Tukey analysis revealed that AE dropped significantly during the last three sampling times when compared to the first 30 days of the experiment. For instance, AE was significantly lower (F = 19.455, p < 0.001) at day 37 when compared to day 28.

Overall EA also changed significantly with time (F = 13.613, p < 0.001). Despite decreased AE, an increase in CR and higher food availability resulted in a significant (F = 124.268, p < 0.001) increase in energy absorption (EA) (J h⁻¹) of over 350% from day 4 to day 37. The largest increase in EA between consecutive sampling times occurred when increasing food concentration from 20 x 10³ cells mL⁻¹ to 30 x 10³ cells mL⁻¹ on day 21 (F = 60.208, p < 0.001) (Fig. 3.1B).
Fig. 3.1: Feeding behaviour of *Pteria penguin* over time when feeding on a mixed diet of concentrated algae. Food concentration and water temperature were increased at ten day intervals beginning on day 11. A) Mean (± SE) relative clearance rate; B) mean (± SE) relative energy absorption.
3.2.2. Reproductive condition

Baseline reproductive condition at the commencement of the experiment was assessed as being in the early stages of development. After a period of 40 days in flow-through aquaria, having experienced relatively rapid increases in food concentration and water temperature, the gonad condition of experimental oysters ranged from being inactive (gametes absent) to ripe (mature follicles) (Table 3.1). Three experimental oysters had inactive gonads, indicating that not all animals obtained enough energy to develop gametes; however, of the remaining oysters, all were either in the later stages of gonad development or ripe. The *P. penguin* sampled from ocean culture at Orpheus Island at the conclusion of the experiment were also classified as either developing or ripe, however in comparison to experimental oysters, fewer had reached the ripe stage (Table 3.1). Example images of histological sections of developing and ripe gonad stages are shown in Fig. 3.2.

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Inactive</th>
<th>Developing</th>
<th>Ripe</th>
<th>Spawning</th>
<th>Spent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>10</td>
<td>0</td>
<td>7 (M), 3 (F)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Experimental</td>
<td>15</td>
<td>3</td>
<td>2 (M), 3 (F)</td>
<td>5 (M), 2 (F)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ocean culture</td>
<td>15</td>
<td>0</td>
<td>6 (M), 5 (F)</td>
<td>3 (M), 1 (F)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 3.1:** Stages in the reproductive cycle of male (M) and female (F) *P. penguin* at the commencement of the study and after 40 days exposure to experimental conditions or ocean culture, as determined via histological examination of gonad samples.
Fig. 3.2: Images of developing and ripe gonad stages for *P. penguin*.
A) Developing male with expanding follicles (Fo) and spermatogonia (Sg) lining the follicle walls; B) ripe male with densely packed spermatozoa; C) developing female with both young oocytes (Yo) and mature oocytes (Mo); D) ripe female with densely packed mature oocytes.
3.4. Discussion

*P. penguin* brood-stock were exposed to increasing levels of water temperature and algae concentration over a period of 40 days within a specially designed set of flow-through aquaria. Feeding behaviour and energy absorption were monitored to determine if brood-stock were able to effectively gain energy from concentrated microalgae. The results of Chapter 2 suggest a positive influence of increased water temperature on ingestion rate by *P. penguin*, a phenomenon that also occurs for some other species of other pearl oyster (Numaguchi, 1994; Yukihiro *et al.*, 2000) as well as other bivalves including species of temperate oyster (Hutchinson and Hawkins, 1992), clam (Sobral and Widdows, 1997) and mussel (Kittner and Riissgard, 2005). For these bivalves, higher water temperatures are assumed to facilitate greater ingestion through the elevation of respiration and thus metabolic rate (Kobayashi and Tobata, 1949; Lucas, 2008).

Oysters did not eject excess feed as pseudofeaces at any point throughout the study, including at the highest food concentration of $40 \times 10^3$ cells mL$^{-1}$. This supports the conclusion made in Chapter 2 that *P. penguin* have a unique ability to maintain ingestion efficiency at high food concentrations when compared to pearl oyster species belonging to the genus *Pinctada*, which typically begin producing pseudofeaces at algae densities of $10 - 30 \times 10^3$ cells mL$^{-1}$ (Yukihiro *et al.*, 1998b). *P. penguin* require a high filtering capacity to maintain feeding efficiency when faced with the heavy concentrations of suspended matter present in near shore environments, as opposed to the clear oligotrophic waters preferred by *Pinctada* spp. (Wada and Tëmkin, 2008). Clearance rate (CR) by *P. penguin* remained relatively stable while being held in flow through aquaria for 40 days, despite increases in food concentration over time. A plateau in feeding behaviour at high food concentrations has been seen previously in other bivalve species and is thought to be a mechanism for avoiding energy wastage associated with the production of pseudofeaces at food concentrations.
surpassing maximum ingestion capacity (Foster-Smith, 1975). Short-term absorption efficiency (AE) of microalgae by *P. penguin* decreased with increasing ingestion rate, most probably due to decreased gut retention time and corresponding enzymatic digestion (Widdows, 1978; Sibly, 1981; Iglesias, 1992). This effect was less evident in the results for feeding trials conducted in Chapter 2, perhaps because the period of exposure to high food concentrations was less prolonged. A stable CR allowed for overall energy absorption (EA) to escalate with greater food availability despite the corresponding decrease in short-term AE.

The reproductive condition of oysters after the 40-day long study period suggested that male *P. penguin* were able to access the energy required to produce spermatozoa at a rate exceeding that observed in a wild environment over the same time period. The production of mature oocytes in females was less reliable. Milione and Southgate (2011b) found that approximately 20% of adult *P. penguin* from the same Orpheus Island population used in this study possess inactive gonads during the month of August, therefore it is possible that the oysters that were inactive at the conclusion of this study actually entered the aquarium system in that condition. It is also possible that gonad condition regressed from the developing stage to being inactive due to inadequate energy to maintain gamete production or an adverse response to the artificial conditions. Ripe gametes are typically achieved earlier in male than female oysters (Loosanoff and Davis 1952), thus the period of optimal energy absorption provided in this study (10 - 20 days) may not have been long enough to allow for the reliable production of energetically expensive oocytes. This notion is supported by the results of Saucedo *et al.* (2001) who found a positive relationship between the proportion of female *Pinctada mazatlanica* producing ripe oocytes and the period of exposure to high densities of microalgae (75 x 10³ cells mL⁻¹), with periods of 45 - 60 days yielding the greatest oocyte production.
This study provides preliminary observations regarding the use of concentrated microalgae to promote gametogenic activity in *P. penguin*, however more research is required to determine the nutrition and time period required to reliably bring female brood-stock to spawning condition. Further research should also be conducted to assess if the techniques used in this study can be used to extend the annual period of hatchery production by maintaining brood-stock in a ripe condition in the months following cessation of the natural spawning season.
Chapter 4

Embryonic and larval development of *Pteria penguin*

4.1. Introduction

Knowledge concerning early-life development of pearl oysters has become increasingly important as more commercial pearl farms choose to propagate juveniles for future pearl production rather than rely on collection of oysters from the wild. The majority of literature documenting early developmental stages in pearl oysters has focused on species belonging to the genus *Pinctada* (Wada, 1942; Minaur, 1969; Alagarswami *et al*., 1982; Alagarswami *et al*., 1989; Rose and Baker, 1994; Doroudi and Southgate, 2003), thus very little information is available regarding embryonic and larval development of *Pteria* spp. Existing literature regarding the genus *Pteria* concentrates on the species *Pteria sterna*, an oyster that differs from *Pteria penguin* in both morphology and geographical range (McAnally-Salas and Valenzuela-Espinoza, 1990; Araya-Nuñez *et al*., 1991; 1995; Southgate *et al*., 2008). An understanding of the processes underpinning embryonic and larval development in *P. penguin*, particularly the timing of developmental stages, is central to the advancement of hatchery techniques for this commercially important species.

In addition to its commercial relevance, knowledge of early-life development of *P. penguin* is required to distinguish morphological characteristics that can be used to accurately identify the species within plankton communities. The ability to identify embryos and larvae of a bivalve species is necessary to investigate patterns of dispersal, settlement and recruitment of wild populations (Gribben and Hay, 2003; Da Costa *et al*., 2008). A chronological description of early developmental stages of a bivalve species can only be achieved by culturing the species within a hatchery environment and making periodic observations.
This study aimed to describe embryonic and larval development in *P. penguin* with the aid of high resolution imagery achieved using scanning electron microscopy (SEM). The detailed depiction of embryogenesis and early shell formation will add to existing knowledge of these processes in bivalve molluscs, in particular to that of tropical bivalves for which there is limited information. Furthermore, the findings of this study will form the basis for future research pertaining to hatchery culture of *P. penguin* and the monitoring of recruitment in wild populations.

### 4.2. Materials and methods

The following is a brief description of the hatchery methods used to culture *P. penguin* larvae during this study, which were based on those previously developed for other pearl oyster species and are summarised in greater detail within the general introduction (see Section 1.4.2).

*P. penguin* brood-stock were collected from Orpheus Island, off the north Queensland coast of Australia (18°36′24 S, 146°29′10 E) and induced to spawn using the standard methods of thermal stimulation. Fertilised eggs were stocked at a density of 50 mL⁻¹ in 3 replicate 500 L incubation tanks filled with UV treated, 1-µm filtered sea water (FSW) with a temperature of 27 ± 1°C. The broad spectrum antibiotic streptomycin-sulphate was added to the incubation tanks at a concentration of 5 mg L⁻¹ to reduce the proliferation of harmful bacteria (Southgate and Beer, 1997).

After 24 h of incubation, D-stage larvae were collected from each tank on a 25 µm nylon sieve mesh and transferred to 500 L larval rearing tanks containing FSW at a density of 3 larvae mL⁻¹. The static culture systems were drained, cleaned and refilled with FSW on day 4 and then every two days until larval settlement occurred on day 22. Each tank received constant gentle aeration, while salinity was kept at 34 - 35 ‰ and pH at 8.15 - 8.19. A water
temperature of 27 ± 1°C was maintained, which is within the range experienced by wild larvae during the peak spawning period in northern Australia (Beer and Southgate, 2000).

D-stage larvae were fed a 1:1 mixture of the golden-brown flagellates *Isochrysis* sp. Tahitian (T-Iso) (CSIRO strain code: CS-177) and *Pavlova* sp. (CS-50) until day 12, when the diatom species *Chaetoceros muelleri* (CS-176) was incorporated into the diet to provide a 1:1:1 ratio of the three species on the basis of cell numbers (Southgate, 2008). The daily feeding rate was 1800 cells mL\(^{-1}\) on day 1 of larval culture, 3000 mL\(^{-1}\) during days 2 - 4 and 5000 mL\(^{-1}\) on day 3, after which ration was increased by approximately 1000 cells mL\(^{-1}\) day\(^{-1}\).

Embryos were sampled every 15 mins during the first 3 h and then every half hour until D-stage larvae developed (see Section 1.2.3. for details on pearl oyster life history). Once reaching D-stage, larvae were sampled daily until settlement occurred. Samples were concentrated in 2.5% gluteraldehyde in seawater with a pH of 8.15 - 8.19. Concentrated embryos and larvae were prepared for examination using scanning electron microscopy (SEM) by washing in a phosphate buffer, rinsing with distilled water and then dehydration through a graduated series of ethanol (Turner and Boyle, 1974). After dehydration, the samples were placed momentarily in chloroform solution before being allowed to dry in a sealed glass Petri dish. The Petri dish contained filter paper soaked in chloroform so the samples could dry within a vaporous environment. Once dry, samples were mounted on aluminium stubs and gold coated. A manual outlining the methods used to prepare samples for SEM is provided as Appendix 4.

Common descriptive terminology for embryogenesis and larval development of bivalve molluscs was used to describe development and shell formation following prior studies in this field (e.g. Wada, 1942; Loosanoff and Davis, 1963; Kniprath, 1979; 1980; Eyster and Morse, 1984; Weiss et al., 2002). Growth during the larval period was monitored
by measuring the shell length (antero-posterior measurement; APM) and height (dorso-ventral measurement; DVM) of 20 individuals every 2 days.

4.3. Results

4.3.1. Embryogenesis

Fig. 4.1A shows a single sperm, with a round nucleus and broad acrosome, resting on the contoured surface of an egg. Newly fertilised eggs had a mean diameter of 45 ± 1.3 µm. Polar bodies became evident within 28 minutes of fertilization, indicating meiotic division (Fig. 4.1B). The first cleavage occurred at approximately 1 h post-fertilisation (hpf), with the second cleavage following rapidly, resulting in 4 blastomeres (Fig. 4.1C). The blastomeres divided unequally creating cells of various sizes, including macromeres and smaller micromeres. The large macromere at the vegetal pole of 8-cell embryos (Fig. 4.1D) was roughly twice the diameter of neighbouring micromeres which measured 10 µm in diameter. This large macromere underwent a series of unequal divisions, eventually producing a daughter cell that divided bilaterally to create the two largest cells present in the 28-cell embryo (Fig. 4.1E). Morula stage occurred within 2.5 hpf and more than 70% of embryos had reached the blastula stage by 4.5 hpf (Fig. 4.1F). Embryos were oval shaped at 5 - 6 hpf (Fig. 4.1G), before beginning to extend along their longitudinal axis to form a ‘cone shape’ with a broad anterior region and narrower posterior region (Fig. 4.1H). A large blastopore could be seen by 7 hpf, indicating that gastrulation had commenced (Fig. 4.1H). Cilia first appeared on the surface of young trochophores by 7 hpf and posterior cilia at the apical plate continued to elongate (15 - 20 µm long) and thicken during the following 2 h to form an apical tuft (Fig. 4.1I). A summary of the timing of developmental stages is provided in Table 4.1.
Fig. 4.1: Embryonic development of *Pteria penguin*. A) Single sperm on egg surface; B) fertilised egg displaying polar body (0.5 h post-fertilisation); C) embryos after first and second divisions (1.25 hpf); D) embryo after fourth division (1.5 hpf); E) morula (2.5 hpf); F) blastula (4.5 hpf); G) to gastrula (6 hpf); H) early trochophore (7 hpf); I) trochophore prior to shell formation (7.5 hpf). Abbreviations: at, apical tuft; b, blastopore; pb, polar body; pl, polar lobe.
Table 4.1: Timing of the stages in embryonic development of five pearl oyster species.

<table>
<thead>
<tr>
<th></th>
<th><em>Pinctada fucata</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Pinctada maxima</em>&lt;sup&gt;b&lt;/sup&gt;</th>
<th><em>Pinctada margaritifera</em>&lt;sup&gt;c&lt;/sup&gt;</th>
<th><em>Pteria sterna</em>&lt;sup&gt;d&lt;/sup&gt;</th>
<th><em>Pteria penguin</em>&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>27-29</td>
<td>27-29</td>
<td>28</td>
<td>24</td>
<td>26-28</td>
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<tr>
<td>Polar body</td>
<td>-</td>
<td>-</td>
<td>24 min</td>
<td>24 min</td>
<td>28 min</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; Division</td>
<td>45</td>
<td>40 min</td>
<td>-</td>
<td>90 min</td>
<td>60 min</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; Division</td>
<td>60 min</td>
<td>60 min</td>
<td>2 h</td>
<td>4 h</td>
<td>75 min</td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; Division</td>
<td>108 min</td>
<td>-</td>
<td>-</td>
<td>4 h</td>
<td>90 min</td>
</tr>
<tr>
<td>Morula</td>
<td>-</td>
<td>3 h</td>
<td>-</td>
<td>4-5 h</td>
<td>2.5 h</td>
</tr>
<tr>
<td>Blastula</td>
<td>6 h</td>
<td>-</td>
<td>-</td>
<td>5-6 h</td>
<td>4.5 h</td>
</tr>
<tr>
<td>To gastrula</td>
<td>6.75 h</td>
<td>5 h</td>
<td>5 h</td>
<td>6-12 h</td>
<td>5-6 h</td>
</tr>
<tr>
<td>Trochophore</td>
<td>11 h</td>
<td>7 h</td>
<td>8-12 h</td>
<td>12-16 h</td>
<td>7-14 h</td>
</tr>
<tr>
<td>D-stage</td>
<td>20-21 h</td>
<td>18-24 h</td>
<td>24 h</td>
<td>21-24 h</td>
<td>18-22 h</td>
</tr>
</tbody>
</table>

*Sources of data: a) Alagarswami et al., 1982; b) Rose and Baker, 1994; c) Doroudi and Southgate, 2003; d) Araya-Nuñez et al., 1995; and e) this study.*
4.3.2. Early shell formation

Soon after the blastopore had begun to enlarge at the anterior end of the trochophore (7 hpf), the formation of a shell gland resulted in another depression at the dorso-posterior end, termed the shell field invagination (sfi). At 7.5 hpf, the sfi was concave in shape and 2-3 times larger than the round blastopore (Fig. 4.2A). Roughly 8 - 9 hpf, the sfi began to extend along the dorsal surface of the posterior end of the trochophore creating a deep narrow crevice (Fig. 4.2B). Organic shell material (pellicle) extended across the aperture of the sfi and began to accumulate at either side of the central crevice, forming the beginnings of what would later develop into the two valves of the larval shell (Fig. 4.2C). The early shell material continued to expand forming a ‘saddle’ shape over the trochophore (11 - 12 hpf), during which time the central hinge depression everted and flattened (Figs. 4.2D, E and F). The central region between the two valves broadened and the shell valves thickened over the following 5 - 8 h, covering an increasing proportion of the trochophore and compressing the animal laterally (Figs. 4.2G and H). The previously ‘cone shaped’ trochophore adopted a ‘heart shape’ between 14 and 16 hpf and before assuming the ‘D shape’ common to newly hatched bivalve larvae. Early organic shell material appeared wrinkled at 17 hpf, suggesting that the process of calcification had not yet begun (Fig. 4.2I). Shell mineralisation commenced once the periostracum spanned the whole epithelial surface, giving rise to D-stage larvae with a calcified shell by 20 - 22 hpf.
Fig. 4.2: Early shell formation in *Pteria penguin*. A) Trochophore displaying initial shell field invagination (8 hpf); B) trochophore with elongated shell field invagination (8.5 hpf); C) early shell material (encircled) on either side of the narrow crevice that will form the hinge (9hf); D) partially everted hinge region (10hpf); E) shell extended over the trochophore surface to form a ‘saddle’ shape (12 hpf); F) developing shell and flat surface of hinge region (13 hpf); G) spread of shell material over the surface of ‘heart’ shaped trochophore (14 hpf); H) lateral compression of trochophore approaching D-stage (15 hpf); I) trochophore completely covered with shell material prior to calcification. Abbreviations: b, blastopore; h, hinge; sfi, shell field invagination.
4.3.3. Larval development

Within 1 h of hatching, D-stage larvae (Fig. 4.3A) were capable of utilizing their velum and associated cilia to actively swim through the water column and achieve a relatively even distribution within the tank volume. Velar retractor muscles attached to the shell at points near to the hinge. Larvae possessed a simple digestive tract, consisting of an oesophagus, stomach, digestive gland and intestine, all of which became more distinct with increasing shell size. Initial growth lines in the larval shell were visible by 3 days post-hatching (dph) and prodssonconch I and II were clearly identifiable during late D-stage (Fig. 4.3B). By 7 dph, D-stage larvae displayed dentition with lateral tooth and socket joints present on the inner surface of both valves, adjacent to the central region of the hinge (Fig. 4.3C). A broken section of a D-stage shell is magnified in Fig. 3D, to show the thin inner and outer prismatic shell layers and thicker homogenous granular layer in between. By 12 dph, more than 80% of larvae had become umbone (Fig. 4.3E), possessing a cardinal tooth and adjacent socket directly below the umbo (Fig. 4.3F). Umbonal larvae developed an asymmetrical shell shape (Fig. 4.3G) as a result of skewed growth towards the posterior end of the deepest valve, which became increasingly more pronounced as the larvae approached settlement (Fig. 4.3H). A red pigmented ‘eye-spot’ could be seen through the partially transparent shell of fast growing individuals at approximately 18 dph. Fig. 4.3I shows the outer shell edge of a pediveliger larvae, where the inner-most shell layer has peeled back during sample preparation to reveal the amorphous calcium carbonate (acc) matrix that makes up the bulk of the shell. Pediveligers were capable of proactively using their foot to crawl on the tank surface, during which time slight gill ciliation became evident. A summary of larval development is provided in Table 4.2.
Fig. 4.3: Larval development of *Pteria penguin*. A) Early D-stage larvae with calcified shell (1 day post-hatching); B) D-stage with prodissoconch I and II (6 dph); C) D-stage hinge of a single valve showing dentition and a section of broken shell magnified in Fig. 3D (7dph); D) section of broken shell showing three shell layers; E) early umbo-stage (8dph); F) umbo hinge (12 dph); G) late umbo-stage (14 dph); H) pediveliger (21 dph); I) edge of pediveliger shell showing the peeling back of the inner prismatic layer to reveal the internal shell matrix. Abbreviations: acc, amorphous calcium carbonate; bs, section of broken shell; ct, central cardinal tooth; g, granular shell layer; ip, inner prismatic shell layer; op, outer prismatic shell layer; s, socket opposing hinge tooth; t, hinge tooth.
Table 4.2: Timing (T) of the stages of larval development of five pearl oyster species and their mean size (S) in µm at each stage

| Source of data: a) Alagarswami et al., 1983; b) Rose and Baker, 1994; c) Doroudi and Southgate, 2003; d) Araya-Nuñez et al., 1995; and e) this study. |

<table>
<thead>
<tr>
<th>Stage</th>
<th>Pinctada fucata(^a)</th>
<th>Pinctada maxima(^b)</th>
<th>Pinctada margaritifera(^c)</th>
<th>Pteria sterna(^d)</th>
<th>Pteria penguin(^e)</th>
</tr>
</thead>
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<tr>
<td>Temperature (°C)</td>
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<td>25-29.5</td>
<td>27-29</td>
<td>23</td>
<td>26-28</td>
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<tr>
<td>Stage</td>
<td>T</td>
<td>S</td>
<td>T</td>
<td>S</td>
<td>T</td>
</tr>
<tr>
<td>D-stage</td>
<td>21 h</td>
<td>67.5</td>
<td>18-24 h</td>
<td>85</td>
<td>24 h</td>
</tr>
<tr>
<td>Early umbone</td>
<td>10 d</td>
<td>-</td>
<td>8 d</td>
<td>110</td>
<td>8 d</td>
</tr>
<tr>
<td>Umbone</td>
<td>12 d</td>
<td>135</td>
<td>10 d</td>
<td>114</td>
<td>12 d</td>
</tr>
<tr>
<td>Eye spot</td>
<td>15 d</td>
<td>210</td>
<td>20 d</td>
<td>205</td>
<td>22 d</td>
</tr>
<tr>
<td>Pediveliger</td>
<td>20 d</td>
<td>230</td>
<td>22-24 d</td>
<td>230</td>
<td>22+ d</td>
</tr>
</tbody>
</table>

Sources of data: a) Alagarswami et al., 1983; b) Rose and Baker, 1994; c) Doroudi and Southgate, 2003; d) Araya-Nuñez et al., 1995; and e) this study.
4.3.4. Larval growth

Twenty-four h after fertilisation, D-stage larvae had a mean shell length (antero-posterior measurement; APM) (± SE) of 83 ± 3.7 µm (Fig. 4.4) and height (dorso-ventral measurement; DVM) of 63 ± 5.1 µm, of which approximately 3.5% was attributed to prodissoconch II. Larvae grew in an approximate linear fashion during D-stage (APM: y = 8.42x + 74.6, R² = 0.97; DVM: 4.80x + 59.9, R² = 0.98) until developing an umbone 12 dph, when mean APM (± SE) and DVM were 129 ± 6.4 µm and 120 ± 4.5µm, respectively (Fig. 4.4). Shell size increased rapidly between days 12 and 20, at a mean growth rate of 16.4 µm APM and 9.1 DVM day⁻¹ (Fig. 4.4). Growth slowed after day 20 and by day 22 more than 70% of surviving larvae had reached the eyed pediveliger stage with a mean APM and DVM of 233 ± 31.3µm and 211 ± 25.1 µm, respectively (Fig. 4.4). Larvae showed a mean increase in shell size of 7.2 µm APM and 7.0 µm DVM day⁻¹ during the 22 day larval period. Growth of *P. penguin* larvae in terms of shell size from initial D-stage to settlement is best modelled by the polynomial equations: APM = 0.39x² – 2.09x + 92.3 (R² = 0.97) and DVM = 0.27x² – 0.14x + 72.0 (R² = 0.96) (Fig. 4.4). The data for growth was heteroscedastic, with size becoming more variable with age (Fig. 4.4).
4.4. Discussion

The clarity of images generated using scanning electron microscopy (SEM) is partly attributed to the novel methods used for drying samples prior to gold coating. The technique of rinsing samples with chloroform and allowing them to dry within a vaporous chloroform environment was effective in eliminating non-evaporable deposits from the surface of samples and avoiding the shrinkage commonly encountered when using critical point or air drying techniques (Bozzola and Russell, 1999). Critical point drying is thought to be the most
effective method for the preservation of fine scale structures (Bozzola and Russell, 1999); however, this study demonstrates the successful use of an alternative technique to dry delicate and complex specimens for examination using electron microscopy.

*P. penguin* eggs are roughly spherical and possess an uneven and highly contoured surface. This study provides the first highly magnified image of a sperm from a pearl oyster species. The sperm nucleus is ovoid in shape with a short broad acrosome situated at the tip. The morphology is similar to that of sperm from the temperate oyster *Crassostrea virginica* (family: Ostreidae), but very different to the arched head and elongated acrosome seen in bivalve families Dreissenidae, Mytilidae and Veneridae (Niijima and Dan, 1965; Moueza *et al.*, 1999; 2006; McAnlis *et al.*, 2010). The ‘spiralian’ pattern of cleavage described for *P. penguin* is typical of that previously observed in other bivalve species, suggesting that the sequence is essential to constructing the bivalve body plan (Henry and Martindale, 1999; Kin *et al.*, 2009).

The timing of respective developmental stages during embryogenesis varies between pearl oyster species; however, each complete the transition from fertilized egg to D-stage larvae within 18 - 24 h of fertilisation. This relatively rapid rate of embryogenesis is also seen in other tropical and sub-tropical oviparous oysters (Southgate and Lee, 1998; Kakoi *et al.*, 2008). In contrast, temperate species generally require 32 - 48 h to reach D-stage (Fujita, 1929; Roughley, 1933; Loosanoff and Davis, 1963; Galstoff, 1964; Dinamani, 1973). This is in keeping with the notion that water temperature is the most important variable in explaining the decrease in developmental rates of marine invertebrate embryos with increasing latitude (Hoegh-Guldberg and Pearse, 1995). While it may be beneficial for oyster hatcheries to maximise water temperature and therefore the rate of development, the threshold for normal development should not be exceeded (Dos Santo and Nascimento, 1985; Doroudi *et al.*, 1999a). It is particularly important to maintain optimal water temperature during the early
cleavage stages when bivalve embryos are most sensitive to mortality caused by temperature shock (Wright et al., 1983).

High resolution imagery achieved using SEM allowed this study to provide a detailed record of early shell formation in a Pteriidae species. The initial stages of shell formation during bivalve embryogenesis are well understood, whereby a shell field invagination (sfi) is formed when ectodermal cells migrate inwards to form the shell gland (Kniprath, 1980; Waller, 1981; Eyster and Morse, 1984). Casse et al. (1998) found that the microvilli-bearing cells that form the pore of the invagination in the scallop *Pectin maximus* (family: Pectinidae) are the cells responsible for pellicle secretion. The sfi can occur at various developmental stages in molluscs (Gros et al., 1997) and there is conjecture relating to whether the shell gland is typically first present before, during or after gastrulation (Waller, 1981). This study shows that in *P. penguin*, the sfi first becomes evident during gastrulation, following the presence of a blastopore. As expected, the sfi appears sooner after fertilisation in *P. penguin* than in bivalve species with a slower rate of embryonic development (Casse et al., 1998).

The concave shape of the initial sfi resembles that observed in the surf clam *Spisula solidissima* (family: Mactridae) (Eyster and Morse, 1984); however, rather than expanding, the sfi in *P. penguin* stretches laterally, creating a deep narrow crevice similar to that more recently documented for the scallop *Pecten maximus* (Casse et al., 1998) and the oyster *Saccostrea kegaki* (Family: Ostreidae) (Kakoi et al., 2008). Observations of shell secretion in *Spisula solidissima* suggested that bivalves develop two opposing shells by expanding their shell field over both sides of the trochophore in a saddle or ribbon shape (Eyster and Morse, 1984). This pattern was again recently observed in bivalve families Lucinidae (Gros et al., 1997), Veneridae (Moueza et al., 1999; 2006) and Ostreidae (Kakoi et al., 2008) and has now been confirmed by this study to occur in at least one member of the family Pteriidae. SEM images show that the early central hinge region in *P. penguin* broadens and then folds
outward to form a flat surface. The inner region of the shell gland in bivalves is thought to evert to form the mantle epithelium (Neff, 1972), followed by the onset of shell mineralisation and calcification during the late trophophore stage (Eyster, 1986; Moueza et al., 2006). This study found that shell calcification in *P. penguin* does not take place until the periostracum spans the entire epithelial surface. The rippled striations on the outer surface of the newly calcified D-stage shell reflect the punctate-stellar pattern common to young bivalve shells (Waller, 1981; Eyster and Morse, 1984; Hayakaze and Tanabe, 1999).

This study is the second to observe a change in shape during the trophophore stage of a pearl oyster species, suggesting it is a phenomenon common to the family Pteriidae. Doroudi and Southgate (2003) comment that *Pinctada margaritifera* trophophores extend along their longitudinal axis, resulting in the anterior region becoming broader than the posterior region. This study describes a similar transformation from ovoid to ‘cone shape’ in *P. penguin*, whereby the blastopore is present at the centre of the broad anterior region and shell formation initiates on the dorso-posterior surface. Early shell material compresses the trophophore laterally, giving rise to a distinctive ‘heart shape’ for the few h preceding D-stage

A larval period of 18 - 24 days is common to many oviparous oyster species, regardless of latitudinal distribution (Loosanoff and Davis, 1963; Dinamani, 1973; Gerdes, 1983; Buroker, 1985; Southgate and Lee, 1998; Kakoi et al., 2008). Oviparous oysters are thought to have evolved over a relatively narrow range of ecological conditions (Buroker, 1985), which may account for the similarity in larval period. The suggested reason for such a long planktonic stage is that it enables gene flow over a wide geographical range (Grassle, 1972), which in turn minimises inbreeding and facilitates population growth. Oviparous oysters typically inhabit patchy habitats such as rocky reef beds. In the absence of an ability to recognise whether their immediate environment is an ideal habitat, the combination of
large spawning events and a long planktonic period increases the chance that a proportion of progeny will settle in an environment that fosters survival and future reproduction (Strathmann, 1974; Jablonski and Lutz, 1983; Buroker, 1985).

The supply of live microalgae during hatchery rearing of pearl oyster species is based on previous research determining optimal diet and feed ration (Southgate, 2008), leaving water temperature as the primary determinant of development time (Alagarswami et al., 1983; Araya-Nuñez et al., 1995; Doroudi et al., 1999a). Water temperatures in excess of 25°C result in an optimal larval period of 18 - 24 days in sub-tropical pearl oyster species (Table 4.2), but a proportion of larvae will fail to maintain the rapid rate of development and effectively become ‘trapped’ in early larval stages until eventually dying (Rose and Baker, 1994). This may contribute to the greater variation in size of *P. penguin* larvae approaching settlement age. Some pearl oyster species, such as *P. sterna*, breed at a time of year when water temperatures are lower and generally demonstrate a comparatively slow rate of development (Table 4.2) (Serrano-Guzman and Salinas-Ordaz, 1993; Araya-Nuñez et al., 1995).

Shell structure in D-stage *P. penguin* larvae is consistent with patterns observed for other pearl oyster species, including daily growth lines and lateral hinge dentition (Rose and Baker, 1994; Doroudi and Southgate, 2003). This study shows a prominent cardinal tooth and socket in umbonal larvae. A similar hinge structure is present in the juvenile and adult *Pteria* morphotype, described by Morton (1995) as a single tooth that interlocks between two smaller teeth on the opposing valve. In addition to this tooth and socket, *P. penguin* are also known to possess a ridge on the right valve, coupled by an opposing indentation on the left valve (Wada and Tëmkin, 2008). *P. penguin* larvae possess the three layers common to molluscan shells (Carriker and Palmer, 1979; Waller, 1981; Weiss et al., 2002); an outer prismatic layer below the periostracum, an inner prismatic layer adjacent to the mantle and a
thick homogenous granular layer in between. Doroudi and Southgate (2003) did not
distinguish between the inner prismatic and granular layers during their study on embryonic
and early larval development of *P. margaritifera*. The lack of a ‘clear-cut boundary’ between
these layers has also been observed in larvae of the temperate oyster species *Crassostrea
gigas* (Weiss *et al.*, 2002). SEM analysis suggests that an inner prismatic layer is relatively
prominent in *P. penguin*, but the outer prismatic layer is almost non-existent in some areas.

*P. penguin* possess a large flat umbo, rather than the typical cone-shape umbo of *P.
sterna* (Dr. Pedro Saucedo, pers. comm.) and *Pinctada* spp. (Rose and Baker, 1994; Doroudi
and Southgate, 2003), and skewed shell growth towards the posterior end of the deepest
valve. This species typically settles in areas of high current (Ito, 1999), where an
asymmetrical shell shape may decrease resistance of the shell to fast flowing water, therefore
reducing susceptibility to detachment. A pronounced hinge is present in the adult morphotype
to aid in supporting the asymmetrical shell shape, but this does not begin to develop until
after larval settlement.

The knowledge generated in this study addresses the current deficiency of information
regarding the processes of embryonic and larval development in tropical bivalves and sheds
particular light on the process of early-shell development. In addition to facilitating
monitoring of wild populations via identification in plankton samples, this research outlines
the processes underpinning early-life development in *P. penguin*, central to the advancement
of research regarding hatchery culture of this commercially important bivalve.
Chapter 5

Minimising mortality of *Pteria penguin* embryos

5.1. Introduction

Maximising hatchery output requires an understanding of the conditions that support development and survival. Pearl oyster eggs incubated in captivity typically require 18-24 h to undergo embryogenesis and pass through the trophophore stage to become free-swimming ‘D-stage’ veligers with a calcified shell (see Table 4.1). This phase is typically characterised by high rates of mortality (Southgate, 2008) and is a part of the life-cycle of pearl oysters for which our knowledge of optimal culture conditions is lacking.

The survival rate of pearl oyster embryos is dependent on endogenous energy reserves passed on by brood-stock (Utting and Millican, 1997); however, the mass mortality often experienced during hatchery incubation is most likely the result of exogenous factors. Pearl oysters have been shown to tolerate a wide range of water temperatures and salinities (Doroudi *et al*., 1999a; O’Connor and Lawler, 2004), suggesting that mass mortality during incubation is primarily caused by poor water quality and the proliferation of harmful bacteria. Hatcheries typically expose incoming seawater to filtration and ultraviolet radiation to eliminate pathogens (Minaur, 1969; Rose and Baker, 1994; Araya-Nuñez *et al*., 1995), leaving the eggs themselves as the main route for bacterial contamination. Stocking incubation tanks with a high density of eggs is important to ensure an adequate number of larvae for the next stage of production, but doing so may compromise survival and development by introducing excessive decaying matter on the surface of eggs (Blaxter, 1956; Gruffydd and Beaumont, 1970) and transferring bacterial infections from parental gonad tissue (Riquelme *et al*., 1994; Jorquera *et al*., 2001). For pearl oysters of the genus *Pinctada*,

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the proportion of fertilised eggs to reach D-stage during egg incubation generally decreases with increasing stocking density (Southgate et al., 1998a).

The assumption that mass mortality of embryos is linked to pathogenic infection has resulted in incubation protocols for pearl oysters often including the application of a broad spectrum antibiotic (Southgate, 2008). In research conducted on the culture of fish eggs, Peck et al. (2004) demonstrated that when compared to other chemical disinfectants, antibiotic solution is the most effective anti-microbial for maximising egg hatch rate. Furthermore, controlling bacterial growth in seawater used for bivalve culture is best achieved with the addition of two or more antibiotics at the same time (Walne, 1958; Fitt et al., 1992; Doroudi, 2001; Stoeckel et al., 2004). The antibiotic(s) most appropriate for promoting survival in a given aquaculture species is dependent on their physiological response to treatment of the culture medium and the nature of the bacteria they are exposed to.

At present, efficient hatchery production of *P. penguin* is constrained by a lack of knowledge regarding optimal conditions for the culture of eggs, larvae and juveniles of this species. This study aimed to assess the impact of both egg density and antibiotics on (a) the survival of newly fertilised eggs and (b) the proportion of surviving embryos that undergo normal development to achieve D-stage within 24 h. It was hypothesised that survival of *P. penguin* embryos would decrease at higher egg densities and increase when treating the culture medium with antibiotics. It was anticipated that the positive effects of antibiotics on survival would become more pronounced with increasing density and that neither factor would have a significant effect on embryonic development.

### 5.2. Materials and Methods

This study was conducted at the Aquaculture Facility of the Ministry of Agriculture and Food, Forests and Fisheries at Sopu in the Kingdom of Tonga (21°07’21”S; 175°13’36”W).
Fifty brood-stock were collected from a long-line located 500 m offshore and cleaned, before being induced to spawn via repeated air exposure (Victor et al. 2001). The method for spawning induction involved placing the oysters in a shallow spawning tank for a period of 1 h, before completely draining the tank and leaving the oysters exposed to direct sunlight for 10 min. The tank was then refilled with UV-treated 1µm filtered sea water (FSW) at a temperature of 28°C and the oysters left undisturbed for 30 min. This process was repeated twice before several males began releasing sperm, prompting the females to release eggs 3-5 minutes later. Once spawning had commenced the procedures for achieving fertilisation followed the standard techniques for pearl oysters, as described in the general introduction (see Section 1.4.2).

Forty minutes was allowed for fertilisation to take place, by which time greater than 80% of eggs displayed the first polar body. Fertilised eggs remained submerged as they were collected onto a 20 µm sieve and rinsed with FSW. Eggs were then stocked into 5 L plastic aquaria with lids. This study used a factorial design combining 3 egg stocking densities (10, 50 and 100 mL\(^{-1}\)) and 3 antibiotic treatments: (1) the absence of any antibiotic (control); (2) 5 mg L\(^{-1}\) of the standard antibiotic applied during pearl oyster incubation (streptomycin-sulfate; Southgate, 2008); and (3) 5 mg L\(^{-1}\) of the antibiotic combination shown to benefit pearl oyster veliger larvae in a prior study (tetracycline:erythromycin, 2.5:2.5 mg L\(^{-1}\); Doroudi, 2001). Antibiotics (≥98% potency), manufactured by Sigma-Aldrich (St Louis, Missouri, 63103, U.S.A), were diluted with FSW to a concentration of 1 g L\(^{-1}\) before being added to incubation aquaria prior to stocking with eggs. A relatively low concentration of antibiotics was used in an effort to avoid the deformities seen in pearl oyster veliger larvae exposed to high antibiotic concentrations (>5 mg L\(^{-1}\); Doroudi, 2001). The treatment combinations were conducted in triplicate resulting in 27 aquaria, maintained at a temperature of 27 ± 1°C and given continuous gentle aeration.
The experiment was terminated after 24 h, which has been shown to be sufficient time for fertilised eggs from *P. penguin* to develop into shelled veligers or ‘D-stage’ larvae (*see Chapter 4*). The contents of each aquarium were collected on a 25 µm nylon sieve mesh and concentrated in separate 50 mL vials containing 4% formaldehyde solution in buffered seawater. Triplicate 1 mL sub-samples were removed from each vial and the number of larvae in each was counted using a high power optical microscope at 20X magnification. Embryos or larvae showing signs of tissue decay were classified as deceased. Embryos that survived 22 h without having formed a calcified shell were assumed to have experienced some degree of slowed or impaired development. Counts were used to estimate: (a) the total proportion of fertilised eggs that had ‘survived’ 24 h of incubation (trochophores + D-stage) and (b) the proportion of surviving embryos that had undergone ‘normal development’ and were therefore D-stage larvae with a calcified shell. Data were square-root arcsine transformed prior to analysis by two-way ANOVA. Significant differences between treatment means were determined using a post-hoc Tukey test (α = 0.05).

**5.3. Results**

5.3.1. Embryo survival

A two-way ANOVA examining the effects of stocking density and antibiotic treatment on the survival (including all stages of development) of *P. penguin* eggs during incubation revealed no significant interaction between the two factors. The addition of antibiotics to incubation tanks significantly influenced survival (F = 11.279, df = 2, p < 0.001), whereby mean percentage survival was approximately 20% greater in aquaria treated with antibiotics and maximised with the application of tetracycline:erythromycin (1:1) (Fig. 5.1A). Egg stocking density did not have an overall significant effect on survival, however, the combination of tetracycline:erythromycin (1:1) was significantly (F = 4.180, df = 2, p = 0.028) more
effective (≈ 13.5%) at enhancing survival at low and intermediate egg densities than at a high egg density (Fig. 5.1B). Furthermore, when egg density was high, the addition of streptomycin-sulfate did not increase survival when compared to larvae in control aquaria without antibiotic.

5.3.2. Embryo development

A two-way ANOVA examining the effects of stocking density and antibiotic treatment on the percentage of surviving *P. penguin* embryos that developed normally to become D-stage veligers, showed no significant interaction between the two factors. Both factors, when considered individually, had an overall significant effect on successful development to D-stage (density: F = 14.418, df = 2, p < 0.001; antibiotic: F = 58.372, df = 2, p < 0.001). The mean proportion of normally developed D-stage larvae was significantly reduced at the highest egg density (Fig. 5.2A), although the difference was small in magnitude (≈ 5%). At each level of egg stocking density, the antibiotic treatment of tetracycline:erythromycin (1:1) caused significant (10 mL⁻¹, F = 19.465, df = 2, p < 0.001; 50 mL⁻¹, F = 26.888, df = 2, p < 0.001; 100 mL⁻¹, F = 18.123, df = 2, p < 0.001) developmental impairment, resulting in a lower proportion of D-stage larvae (≈ 11.5%) and corresponding higher proportion of trochophores (Fig. 5.2B). The impaired development caused by tetracycline:erythromycin (1:1) became more pronounced with increasing egg density.
**Fig. 5.1:** Mean (± SE) percentage survival of *Pteria penguin* eggs after 24 h incubation. A) In control aquaria that received no antibiotic (C), aquaria treated with 5 mg mL⁻¹ streptomycin-sulfate (S) and aquaria treated with 5 mg L⁻¹ tetracycline:erythromycin (1:1) (T+E); B) survival at three egg densities (10 □, 50 ■ and 100 □□ eggs mL⁻¹). Superscript numbers represent Tukey groupings within individual antibiotic treatments.
Fig. 5.2: Mean (± SE) percentage of *Pteria penguin* embryos that developed normally into D-stage larvae after 24 h incubation. A) At three levels of egg stocking density (mL\(^{-1}\)); B) when exposed to three antibiotic treatments (control with no antibiotic □; 5 mg L\(^{-1}\) streptomycin-sulfate ■ and 5 mg L\(^{-1}\) tetracycline:erythromycin (1:1) ). Superscript numbers represent Tukey groupings within individual density treatments.
5.4. Discussion

Hatchery production of pearl oyster species belonging to the genus *Pinctada* has shown that the proportion of eggs that survive and develop normally to become D-stage veligers, when using a typical egg density of 20-50 mL\(^{-1}\), can vary greatly from 6% to 75-80% (Southgate *et al.*, 1998a). A disparity in egg quality, i.e. the amount and condition of endogenous reserves available as an energy source during embryogenesis (Utting and Millican, 1997), can account for differences in the performance of embryos between multiple spawning events from the same species, however frequent instances of mass mortality are attributable to exogenous factors (Southgate *et al.*, 1998a). When a controlled spawning event is achieved within a hatchery, the focus is to minimise mortality of eggs and maximise normal development of the available embryos.

Bivalve larvae are susceptible to mortality caused by pathogenic bacteria, which are known to destroy juveniles via direct invasion or contact (Guillard, 1959). Douillet and Langdon (1993) tested the effects of 21 bacterial strains on larvae of the temperate oyster *Crassostrea gigas* and found most to be detrimental to survival. Bacteria from the genus *Vibrio* have been identified as being particularly dangerous for oyster species (DiSalvo *et al.*, 1978; Jones, 2007) and are known to cause prolific mortality during hatchery production (Sainz-Hernández and Maeda-Martínez, 2005). A number of prior studies on early-life culture of bivalves have reported the benefits associated with implementing broad-spectrum antibiotics to control bacterial contamination and therefore decrease mortality (e.g. Walne, 1958; Fitt *et al.*, 1992; Stoeckel, *et al.*, 2004). The benefits of antibiotic application for increasing survival of shelled larvae have been demonstrated for the pearl oyster *P. margaritifera* (Doroudi 2001), and this study has confirmed such benefits during the incubation of newly fertilised *P. penguin* eggs.
It is common for pearl oyster incubation tanks to be treated with a broad-spectrum antibiotic, such as streptomycin-sulfate (Southgate and Beer, 1997; Southgate, 2008), but until now there has been no formal comparison with survival under control conditions without antibiotic. The results of this study show that the presence of streptomycin-sulfate increased survival of *P. penguin* embryos over an incubation period of 24 h. Doroudi (2001) found tetracycline:erythromycin (1:1) to be beneficial in promoting survival of *P. margaritifera* larvae that had already formed a calcified shell, but prior to this study, this combination of antibiotics was yet to be tested on newly fertilised pearl oyster eggs. Our results indicate that survival of *P. penguin* embryos during the initial 24 h post-fertilisation is enhanced in the presence of tetracycline:erythromycin (1:1), but this treatment did not significantly improve survival when compared to the widely adopted streptomycin-sulfate.

At the highest egg density used in this study of 100 mL\(^{-1}\), streptomycin-sulfate did not support improved survival compared to that in control aquaria. Also, the antibiotic combination of tetracycline:erythromycin (1:1) was significantly less effective at enhancing survival at an egg density of 100 mL\(^{-1}\) when compared to 50 mL\(^{-1}\). Bivalve eggs are capable of carrying decaying matter, in particular superfluous sperm, on their surfaces (Blaxter, 1956; Gruffydd and Beaumont, 1970) and given the contoured exterior of pearl oyster eggs (Doroudi and Southgate, 2003), it is unlikely that simply rinsing with filtered seawater prior to incubation will eliminate all potentially harmful bacteria. Total egg surface area increases proportionally with density, providing greater refuge for dangerous bacteria at higher egg densities. Furthermore, infections transferred from the parental gonad tissue (Riquelme *et al.*, 1994; Jorquera *et al.*, 2001) would be expected to increase with egg density. The low antibiotic dosage of 5 mg mL\(^{-1}\) applied to incubation aquaria in this study may have been insufficient to deal with bacterial loading at a high egg density and on this basis, antibiotics were not as effective in promoting survival compared to at lower densities.
Across each of the egg densities tested, the proportion of embryos with impaired development was significantly greater in aquaria treated with tetracycline:erythromycin (1:1), when compared to control aquaria and aquaria that received the same dosage of streptomycin-sulfate. This suggests interference in early shell development, particularly initial calcification, which occurs in the final transition from the trochophore to veliger stages (Eyster, 1986; Mouëza et al., 2006). Tetracycline is known to interfere with calcification and cause shell deformities in bivalve larvae (Fitt et al., 1992), but this has not been previously demonstrated for embryos. Doroudi (2001) observed some deformity in pearl oyster larvae (P. margaritifera) exposed to tetracycline:erythromycin (1:1) at a concentration of 10 mg mL\(^{-1}\), but not at 5 mg mL\(^{-1}\). The results of this study suggest that the antibiotic combination of tetracycline:erythromycin (1:1) is capable of disrupting early shell formation prior to D-stage, even when applied at a dosage that is safe for shelled larvae. Doroudi (2001) found that increasing the concentration of neomycin-sulfate:streptomycin-sulfate (1:1) from 5 to 10 mg mL\(^{-1}\) did not cause deformity in shelled larvae, but was not recommended because it failed to improve survival.

Rose (1990) recommended that Pinctada maxima eggs be incubated at a maximum density of 30 mL\(^{-1}\), however, Southgate et al. (1998) observed high rates of survival to D-stage of both P. margaritifera and P. maxima at densities ≥100 mL\(^{-1}\). The results of this study show no significant reduction in the survival of P. penguin embryos when doubling stocking density from 50 to 100 mL\(^{-1}\). The impaired development observed at the highest density may be attributed to elevated bacterial levels or mechanical interference between eggs (Blaxter, 1956), but was small enough in magnitude to have little effect on the number of normally developed D-stage larvae. On this basis, a high stocking density is recommended if the tank space available for incubation is limiting adequate production of D-stage larvae for the next phase of production.
In summary, the antibiotic mixture tetracycline:erythromycin (1:1) is effective in enhancing survival of *P. penguin* embryos during a 24 h period of incubation, but hinders development during the transition from trochophore to D-stage. In contrast, streptomycin-sulfate supports greater survival without significantly compromising development. Additional research is required to determine if a longer period of incubation would allow *P. penguin* embryos with impaired development to eventually reach D-stage. If this were the case, we would expect tetracycline:erythromycin (1:1) to support a superior hatch rate of D-stage larvae. Incubating *P. penguin* eggs at a density ≥100 mL⁻¹ can impede normal development and hinder the efficacy of antibiotics when applied at a low dosage. However, increasing stocking density from 50 to 100 mL⁻¹ did not significantly reduce mean survival and therefore resulted in greater production of D-stage larvae. Further research is warranted to determine if higher concentrations of streptomycin-sulfate than those tested in this study can be used to enhance survival when utilising a high egg density.

A draw-back of using antibiotics is the potential threat to the natural environment if not disposed of correctly and the possibility for resistant bacteria to develop. Safety protocols should be followed closely and dosages should not exceed those required for a given density. Whilst antibiotics are still used during the initial 24 h of pearl oyster embryo incubation, their use is not recommended for the longer periods of larval rearing that commence once D-stage is reached, when much larger volumes of culture medium are required (Southgate, 2008). Probiotics have been used successfully to promote growth and survival during larval culture of shellfish, although their efficacy is dependent on the provision of favourable conditions and their specific interaction with the microbial community already present in the culture medium (Vine *et al.*, 2006; Watson-Kesarcodi *et al.*, 2008). Further research is required to identify probiotics that successfully promote survival and development during early-life culture of pearl oysters.
Chapter 6

Enhancing the survival and growth of *Pteria penguin* larvae when fed a diet of concentrated algae

6.1. Introduction

Maximising pearl oyster hatchery output requires an understanding of the conditions that optimise survival and development of larvae. It is thought that newly hatched larvae exhaust their maternally-derived endogenous energy reserves within a few days, becoming reliant on cultured microalgae as a source of energy for immune function, growth and metamorphosis (Gervis and Sims, 1992; Strugnell and Southgate, 2003; Southgate, 2008). If not cultured correctly, live microalgal cells held at high density provide a haven for bacterial infection that can then be transferred to larvae (Heasman *et al*., 2000). Techniques for the mass culture of bacteria free live algae are both labour and resource demanding (Coutteau and Sorgeloos, 1992; Southgate, 2008), thus not viable for many small scale pearl farming operations and in developing countries, which lack the required resources and technical capacity.

Hatcheries for the production of edible oyster species were amongst the first to use concentrated algal paste as a cost effective, low risk alternative food source (Coutteau and Sorgeloos, 1993). Oyster species such as *Crassostrea gigas* and *Saccostrea glomerata* were shown to survive and grow equally well when fed a diet of concentrated algal paste rather than live algae (Heasman *et al*., 2000; Ponis *et al*., 2008). Algal pastes are formed by condensing cells from mass microalgal culture, creating a ‘long-life’ product that can be reconstituted to a lower cell density (Robert and Trintignac, 1997). Research has identified the methods for concentration that optimise the palatability and nutritional value of algal paste for bivalve larvae (Nell and O’Connor, 1991; Heasman *et al*., 2000; Brown and Robert, 2002; Ponis *et al*., 2008). Teitelbaum and Ngaluaf (2008) highlighted the potential of algal
paste in pearl oyster hatchery propagation when they reported its experimental use in successful larval production of *Pteria penguin*.

Prior research has identified golden-brown flagellates as the most appropriate microalgae for maintaining survival and maximising growth of pearl oyster larvae (Minaur, 1969; Rose, 1990; Chellum *et al*., 1991; Martinez-Fernández *et al*., 2004; Martinez-Fernández *et al*., 2006). Algal pastes formulated from this ideal food source are commercially available; however, concentrated algae has negative buoyancy and is no longer motile, therefore behaves differently to live algae when introduced to larval rearing tanks (Heasman *et al*., 2000). On this basis, feeding regimes when using concentrated algal paste are different to those traditionally used for live microalgae, even if the nutritional content of the food source is similar. Feed ration must allow sufficient access to the nutrition required for development, without causing reduced water quality as a result of excessive metabolic waste and the decomposition of unconsumed food (Loosanoff and Davis, 1963; Doroudi *et al*., 1999b). The effects of feed ration vary according to larval density thus both factors need to be considered when devising an appropriate feeding strategy (Doroudi and Southgate, 2000; Liu *et al*., 2010). An understanding of the specific effects of larval density and feed ration when using concentrated algal pastes in hatchery culture is required to ensure efficient larval production and minimise food wastage.

This study aimed to investigate the effects of stocking density and feed ration on the survival and growth of *P. penguin* larvae when using concentrated algal paste as a food source. Two separate experiments aimed to examine the effects of both factors on D-stage and umbo-stage larvae. It was hypothesised that the effect of feed ration on both survival and growth of *P. penguin* would vary with density and that increasing density would have a general negative impact. Maximum larval production per unit of culture medium was expected to occur in aquaria with intermediate levels of both stocking density and feed ration.
6.2. Materials and methods

6.2.1. Larval production

This study was conducted at the Aquaculture Facility of the Ministry of Agriculture and Food, Forests and Fisheries (MAFFF) at Sopu in the Kingdom of Tonga (21º07’21”S; 175º13’36”W). Methods for brood-stock spawning induction and egg fertilisation were the same as those described in Chapter 5 (see Section 5.2). Embryonic development was monitored by examining samples under a high power optical microscope at 200X magnification and incubation was conducted for a period of 22 h, at which time D-stage larvae possessed a calcified shell (see Chapter 3). D-stage larvae from each of 3 egg incubation tanks were gently mixed within a 20 L aquarium. The mean antero-posterior shell measurement (APM) of 50 randomly selected D-stage larvae was 81.0 ± 3.8 µm. The required number of D-stage larvae were distributed amongst the aquaria used for Experiment 1, while the remaining were placed into typical pearl oyster larval rearing conditions (see Section 1.4.2.3) for the 8 days required to reach umbo-stage before being used in Experiment 2. The APM of 50 randomly selected umbo-stage larvae was 105.9 ± 10.7 µm immediately prior to Experiment 2.

6.2.2. Concentrated algae

Concentrated algae paste from the Instant Algae® range (Instant Algae®, Reed Mariculture Incorporated, Campbell, CA, USA, 95008) was used as a food source in both experiments. Three separate Instant Algae products were used; mono-cultured Isochrysis sp. (Haptophyceae), mono-cultured Pavlova sp. (Haptophyceae) and the mixed algae product called ‘Shellfish Diet’ which consists of the previous two species plus Thalassiosira weissflogii (Bacillariophyceae) and Tetraselmis sp. (Chlorophycophyceae). In terms of the number of cells mL⁻¹, the diet for Experiment 1 (D-stage) constituted a 1:1 ratio of Isochrysis
sp. and *Pavlova* sp. The diet for Experiment 2 (umbo-stage) consisted of a 1:1:1 ratio (on the basis of cell number) of *Isochrysis* sp., *Pavlova* sp. and Shellfish Diet, resulting in roughly 43% *Isochrysis* sp., 40% *Pavlova* sp., 10% *Thalassiosira weissflogii* and 7% *Tetraselmis* sp.. Concentrated algae concentration was estimated by diluting the algal paste to 1000 mL and conducting manual cell counts (mL$^{-1}$) using a haemocytometer viewed at 100X magnification. Instant Algae® stocks were kept refrigerated (manufacturers instructions) and light shaking was implemented daily as an effective method to disassociate clumped cells (Aji, 2011).

6.2.3. Experiments

Experiment 1 assessed the combined effects of stocking density and feed ration on the survival and growth of *P. penguin* larvae during D-stage (1-8 days post-fertilisation). Experiment 2 examined the effects of the same treatments on the survival and growth of larvae during umbo-stage (8 - 17 days post-fertilisation). Both experiments used a factorial design combining 3 stocking densities (Experiment 1: 2, 6 and 10 larvae mL$^{-1}$; Experiment 2: 1, 3 and 5 larvae mL$^{-1}$) and 3 levels of feed ration (Experiment 1: 5, 10 and 15 x 10$^3$ cells mL$^{-1}$; Experiment 2: 10, 15 and 20 x 10$^3$ cells mL$^{-1}$), resulting in 9 treatment combinations, each conducted in triplicate within 27 x 5 L plastic aquaria.

Aquaria were filled with UV-treated 1 µm filtered sea water (FSW) and maintained using the same husbandry techniques as those described for routine larval rearing of pearl oyster species (*see Section 1.4.2.3*). Water temperature was held at 28°C by having all 5 L aquaria partly submerged within a temperature controlled water bath. Each aquarium possessed a removable lid and was lightly aerated through a glass tube inserted from the top. The instant algae diet was diluted to 100 mL and placed into each aquarium once a day. To simulate typical husbandry techniques, each aquarium underwent a complete water exchange.
every two days. To complete this process individual aquaria were removed from the water bath and the contents washed through a 25 µm sieve. Larvae caught on the sieve surface were transferred to a clean sterile aquarium pre-filled with FSW and returned to the temperature controlled water bath. Larvae remained submerged in FSW throughout the transfer process.

At the end of each experiment, the contents of individual aquaria were collected on a 25 µm nylon sieve mesh and concentrated in separate 50 mL vials containing 4% formaldehyde solution in buffered seawater. Survival and growth were estimated by removing triplicate 1 mL sub-samples from each 50 mL vial and examining the contents under a high power optical microscope at 200X magnification to: (a) count the number of surviving larvae; and (b) measure the shell length (APM) of 20 individuals. By multiplying estimates of initial density by mean survival it was possible to estimate larval production per unit of culture medium for each aquarium. Transparent larvae with no signs of internal structure were classified as deceased. The normality of raw data was assessed using probability plots of residual values and proportional data were square-root arcsine transformed prior to analysis. Significant differences between treatment means were determined by two-way ANOVA and post-hoc Tukey tests (α = 0.05).

6.3. Results

6.3.1. Experiment 1: D-stage

A two-way ANOVA examining the effects of stocking density and feed ration on the percentage survival of D-stage P. penguin revealed no significant interaction between the two factors. Overall mean survival improved by 105% (F = 61.456, df = 2, p < 0.001) when decreasing stocking density from 10 to 6 larvae mL⁻¹, but a further decrease in density did not significantly alter survival (Fig. 6.1A). Multiplying initial density by survival revealed that utilizing a low density of 2 larvae mL⁻¹ yielded a significant (F = 43.097, df = 2, p < 0.001)
reduction of 260% in the production of larvae per volume of culture medium (Fig. 6.1B). At stocking densities greater than 2 larvae mL$^{-1}$, mean survival increased significantly ($F = 3.180$, df = 2, $p = 0.029$) by an average of 40% when using an intermediate feed ration of 10 x 10 cells mL$^{-1}$ (Fig. 6.1C), rather than a low (5 x 10 cells mL$^{-1}$) or high feed ration (15 x 10 cells mL$^{-1}$).

A two-way ANOVA revealed a significant interaction ($F = 9.07$, df = 4, $p < 0.001$) between the effects of stocking density and feed ration on the shell length of D-stage *P. penguin*, indicating that the effect of feed ration differed according to stocking density. When considered independently, density had an overall significant effect on shell length ($F = 25.22$, df = 2, $p < 0.001$), but the effect was small in magnitude, whereby shell length increased 4% when reducing stocking density from 10 to 6 larvae mL$^{-1}$ and a further 3% when reducing stocking density to 2 larvae mL$^{-1}$ (Fig. 6.2A). Feed ration had the largest effect on larval size at a low stocking density of 2 larvae mL$^{-1}$, whereby growth was reduced significantly ($F = 10.147$, df = 2, $p = 0.002$) by an average of 7% when applying a high feed ration of 15 x 10 cells mL$^{-1}$ (Fig. 6.2B). Conversely, at the highest density of 10 larvae mL$^{-1}$, a feed ration of 15 x 10 cells mL$^{-1}$ resulted in a significant increase ($F = 4.307$, df = 2, $p = 0.015$) in shell length of 2.3% when compared to lower feed rations (Fig. 6.2B).
Fig. 6.1: A) Mean (± SE) survival (%) of *Pteria penguin* larvae during D-stage (days 1-8) at three levels of stocking density (mL$^{-1}$); B) mean number of larvae produced per volume of culture medium (mL$^{-1}$); C) mean survival (%) at three levels of feed ration (5 □, 10 ■ and 15 □□ algae cells mL$^{-1}$). Superscript numbers represent Tukey groupings within individual density treatments.
Fig. 6.2: A) Mean (± SE) antero posterior shell measurement (APM) (µm) of *Pteria penguin* larvae post D-stage (days 1-8) at three levels of stocking density (mL⁻¹); B) mean APM at three levels of feed ration (5 □, 10 ■ and 15 △ algae cells mL⁻¹). Superscript numbers represent Tukey groupings within individual density treatments.
6.3.2. Experiment 2: Umbo-stage

A significant (F = 4.479, df = 4, p = 0.003) interaction between the two factors of stocking density and feed ration on the percentage survival of umbo-stage *P. penguin* indicated that feed ration effected survival differently according to stocking density. Density had an overall significant effect on survival (F = 212.110, df = 2, p < 0.001), whereby survival was improved by 700% when decreasing stocking density from 5 to 3 larvae mL\(^{-1}\) and 360% when decreasing stocking density from 3 to 1 mL\(^{-1}\) (Fig. 6.3A). Multiplying initial density by survival showed that increasing density from 1 to 3 larvae mL\(^{-1}\) did not significantly affect production of larvae per volume of culture medium (Fig. 6.3B). At stocking densities greater than 1 mL\(^{-1}\), mean survival improved significantly (F = 6.750, df = 2, p = 0.003) by an average of 310% when increasing feed ration above the lowest treatment of 10 x 10 cells mL\(^{-1}\) (Fig. 6.3C).

There was no significant interaction between the effects of stocking density and feed ration on the shell length of umbo-stage *P. penguin*. Density had an overall significant effect on growth (F = 206.835, df = 2, p < 0.001), whereby mean size of larvae was elevated by 12% when decreasing density from 5 to 3 larvae mL\(^{-1}\) and a further 16% when decreasing density from 3 to 1 mL\(^{-1}\) (Fig. 6.4A). Feed ration only had a significant effect on larval size when stocking density was low, whereby a high feed ration of 20 x 10\(^3\) cells mL\(^{-1}\) caused a 7% decrease in shell length when compared to lower feed rations (F = 6.576, df = 2, p = 0.002) (Fig. 6.4B).
Fig. 6.3: A) Mean (± SE) survival (%) of *Pteria penguin* larvae during umbo-stage (days 8-17) at three levels of stocking density (mL$^{-1}$); B) mean number of larvae produced per volume of culture medium (mL$^{-1}$); C) mean survival (%) at three levels of feed ration (5 □, 10 ■ and 15 △ algae cells mL$^{-1}$). Superscript numbers represent Tukey groupings within individual density treatments.
Fig. 6.4: A) Mean (± SE) antero-posterior measurement (APM) (µm) of Pteria penguin larvae post umbo-stage (days 8-17) at three levels of stocking density (mL⁻¹); B) mean (± SE) APM at three levels of feed ration (5 □, 10 ■ and 15 ☐ algae cells mL⁻¹). Superscript numbers represent Tukey groupings within individual density treatments.
6.4. Discussion

Mortality during larval culture of pearl oyster species is the most important factor limiting hatchery production (Southgate et al., 2008). Rose and Baker (1994) produced a total of approximately 40,000 settled spat from three separate spawning events of *Pinctada maxima* brood-stock, representing less than 4% of the initial larval population. Likewise, final survival rates during hatchery production of *Pinctada mazatlanica* are also known to be low, recorded previously at 3.1% (Martinez-Fernández et al., 2003) and 2.7% (Saucedo et al., 2005). The proportion of *Pinctada margaritifera* larvae to metamorphose as spat in a study by Alagarswami et al. (1989) was 6.3%, slightly higher than the mean survival to settlement age of 5% achieved during hatchery production of the same species by Southgate and Beer (1997). Doroudi and Southgate (2000) were unsuccessful in improving the survival rate of *P. margaritifera* larvae during efforts to determine optimal levels of density and feed ration. In contrast, Beer (1999) recorded a much higher survival rate of 16% when culturing *P. penguin* larvae at a low density of 1.18 mL$^{-1}$, comparable to the 14% survival observed in low density treatments during this study. These results suggest that the survival rate of *P. penguin* larvae during hatchery culture is relatively high when compared to pearl oyster species belonging to genus *Pinctada*, although the vast majority of larvae still fail to survive to settlement.

What separates this study from others reporting on the survival of pearl oyster larvae during hatchery culture is that concentrated algal paste was used as a food source rather than live algae. This is not the first time that an alternative food source has been trialled for larval culture of a pearl oyster species. The combination of the high cost and the high risk of bacterial contamination associated with live algae production has prompted research investigating alternative food sources for larval culture of *P. margaritifera*. There is evidence that substituting 25-50% of a live algae diet with concentrated dried algae (*Tetraselmis* sp.) is possible without significantly reducing survival or growth of D-stage *P. margaritifera*.
(Southgate et al., 1998b; Doroudi et al., 2002). The use of yeast-based products in conjunction with live algae has been shown to boost the growth rate of umbo-stage larvae (Southgate et al., 1998b). These commercially available ‘off the shelf’ products are most effective when used as partial substitutions to a live diet, therefore the need for live algae remains. This study shows larvae of a pearl oyster species exhibiting high rates of survival when raised on a diet consisting solely of concentrated algae. This finding is particularly important for regions where the culture of live algae as a food source for pearl oyster larvae is cost or technically prohibitive. Formal trials comparing survival of larvae from the same spawning event fed on either concentrated or live algae are yet to be conducted.

The initial stocking density of larvae within rearing tanks was found to be more influential than feed ration on the survival of P. penguin larvae. However, increasing stocking density from 2 to 6 larvae mL\(^{-1}\) did not significantly affect mean survival of D-stage larvae, supporting previous observations for the survival of P. margaritifera larvae at densities of 1 to 5 mL\(^{-1}\) (Doroudi and Southgate, 2000). A further increase in larval density to 10 mL\(^{-1}\) caused a reduction in survival of D-stage P. penguin to almost 50% of that recorded at lower densities. Moreover, survival of umbo-stage larvae dropped from 35% to less than 10% when larval density was raised above 1 mL\(^{-1}\). Increases in density have been correlated with elevated bacterial loading during larval culture of pearl oyster Pinctada fucata (Subhashi et al. 2007). Direct association between larvae and bacteria allows the pathogenic bacteria to destroy juvenile bivalves via cell invasion (Guillard, 1959). Subhashi et al. (2007) found six bacteria isolates within P. fucata rearing tanks that caused a high mortality rate, five of which were identified as Vibrio spp., which are known to be particularly dangerous for oyster species (DiSalvo et al., 1978; Jones, 2007) and are capable of causing prolific mortality during hatchery production (Sainz-Hernández and Maeda-Martínez, 2005).
Mean growth of *P. penguin* larvae was reduced with increasing stocking density resulting in significantly smaller mean shell size (shell length, antero-posterior measurement) during both D-stage and umbo-stages of development. Larval size in tanks with a high stocking density remained relatively low even when feed ration was increased, supporting the notion that factors other than access to nutrition limit growth at high density (Liu *et al*., 2006). Two factors known to influence the growth of bivalve larvae held at high density are competition for space and the degradation of water quality. Elevated density triggers a corresponding increase in the collision rate between free-swimming veligers, causing an immediate loss of energy due to retraction of the velum and therefore cessation of feeding as well as longer term energy loss associated with shell repair (Cragg, 1980; Sprung, 1984a; Liu *et al*., 2006). Higher larval density may also result in the rapid accumulation of toxic metabolites that can be detrimental to both immune function and growth rate (Sprung, 1984a; Yan *et al*., 2006; Raghavan and Gopinathan, 2008).

With environmental variables such as temperature, salinity and oxygen concentration controlled for when culturing bivalve larvae, it is often assumed that survival rate at a given density is largely a function of food availability (Helm and Millican, 1977). The results of this study and previous research conducted on *P. margaritifera* larvae (Doroudi and Southgate, 2000) suggest that feed ration has minimal effect on the survival of D-stage pearl oyster larvae. This could be due to young larvae still being partly reliant on endogenous energy reserves provided within the egg, with the initial content of protein, lipid, triglyceride, and fatty acid reserves within the oocyte yolk being the primary determinant of larval viability rather than exogenous food sources (Bayne, 1983; Gallager and Mann, 1986; Fraser, 1989; Strugnell and Southgate, 2003). In fact, the ingestion of exogenous food sources by bivalve larvae is often limited by a narrow oesophagus (Gallager, 1988; Rico-villa *et al*., 2009).
Doroudi and Southgate (2000) found that the survival of umbo-stage *P. margaritifera* larvae decreased with increasing feed ration of live algae within the range of 2 - 30 x 10^3 cells mL^-1, regardless of stocking density. This trend was thought to be caused by the decomposition of uneaten food resulting in elevated levels of dangerous bacteria. In contrast, the results of this study show that at stocking densities greater than 1 larvae mL^-1, increasing feed ration of concentrated algae from 10 to 20 x 10^3 cells mL^-1 maximised mean survival of umbo-stage *P. penguin* larvae. The superior ability of umbo-stage *P. penguin* larvae to thrive at higher feed rations is likely to facilitate recruitment across a wide ecological range encompassing near shore turbid environments, whereas *Pinctada* spp. are restricted to the oligotrophic waters of offshore rocky reefs (Wada and Têmkin, 2008). An investigation of the effects of feed ration on survival when fed a diet of live microalgae is required to determine if the pattern of feeding behaviour is consistent with that observed during this study when using a diet of concentrated algal paste.

Doroudi *et al.* (1999) demonstrated that the ration of live algae required to maximise survival of *P. margaritifera* larvae (10 x 10^3 cells mL^-1) had to be doubled in order to also maximise growth. Maximum growth of D-stage and umbo-stage *P. margaritifera* larvae occurs at live algae rations of ~8 x 10^3 cells mL^-1 and 25 x 10^3 cells mL^-1, respectively, regardless of changes in larval density (1 - 5 mL^-1). Unlike for *P. margaritifera* larvae, the effects of feed ration on growth of *P. penguin* larvae appear density dependent. Feed ration had the most marked effect on growth of both D-stage and umbo-stage larvae when stocked at a low density (D-stage, 2 mL^-1; umbo-stage, 1 mL^-1), whereby a high feed ration (D-stage, 15 x 10^3 cells mL^-1; umbo-stage, 20 x 10^3 cells mL^-1) caused a significant reduction in shell length. This might be attributed to growth being negatively impacted by the decomposition of unconsumed algae resulting in an accumulation of toxic metabolites and heightened proliferation of bacteria (Loosanoff and Davis, 1963; Sprung, 1984a; 1984b; Liu *et al.*, 2006;
Raghavan and Gopinathan, 2008). Strathman et al. (1993) proposed that food availability not only influences the growth rate of bivalve larvae but also the site of growth, whereby development of feeding structures is maximised when food is scarce in order to enhance ingestion rate, causing a corresponding decrease in shell growth. The contribution of this phenomenon to slow growth of *P. penguin* larvae in conditions of high larval density and low feed ration is an area for future research.

The results of this study provide evidence for successful culture of pearl oysters using concentrated algal paste as a food source rather than live algae. It is recommended that *P. penguin* hatcheries implement a density of 6 mL\(^{-1}\) during D-stage development to maximise larval production per volume of culture medium whilst incurring only a slight reduction in growth. A feed ration of 10 x 10\(^3\) cells mL\(^{-1}\) of concentrated algae optimised mean survival and growth of D-stage larvae when stocked at 6 mL\(^{-1}\). Increasing density of umbo-stage larvae from 1 mL\(^{-1}\) to 3 mL\(^{-1}\) resulted in a marked reduction of both survival and growth, and is therefore not recommended for efficient hatchery production. At a low larval density of 1 mL\(^{-1}\) for umbo-stage larvae, feed ration of concentrated algae should be maintained at 10 x 10\(^3\) cells mL\(^{-1}\) to minimise food costs and avoid decreased larval growth at higher feed rations (≥15 x 10\(^3\) cells mL\(^{-1}\)).
Chapter 7
Enhancing settlement of *Pteria penguin* larvae

7.1. Introduction

Patterns of molluscan species distribution and abundance are influenced to a large extent by factors that promote larval recruitment to particular habitats (Pawlick and Hadfield, 1990). Larval settlement onto the substrate is mediated by stimuli that control subsequent metamorphosis into the juvenile form (Morse, 1985; Pawlick and Hadfield, 1990). There are various environmental cues known to induce settlement, such as those associated with conspecifics (Hadfield, 1978; Burke, 1986; Su *et al*., 2007), the presence of a suitable substrate (Gosling, 2003; Yang *et al*., 2007; Neo *et al*., 2009) and microbial bio-films on the substrate surface (Weiner *et al*., 1989; Tamburri *et al*., 1992; Wieczorek and Todd, 1998). Attachment is often preceded by a sequence of larval behaviours (Gosling, 2003), including pro-actively swimming towards the substrate (Tamburri *et al*., 1992; Wieczorek and Todd, 1998) and the use of a sensory foot to crawl over the substrate surface (Pascual and Zampatti, 1995; Saucedo and Southgate, 2008). Optimising successful settlement during hatchery cultivation of bivalves is critical to maximising production of juveniles for commercial use and is reliant on providing suitable stimuli.

The presence of a microbial bio-film on the surface of substrata has been found to promote settlement in a number of commercial bivalve taxa including scallops (Parsons *et al*., 1993; Leyton and Riquelme, 2008), mussels (Bao *et al*., 2007), oysters (Walch *et al*., 1987; Tamburri *et al*., 1992; Anderson, 1996) and pearl oysters (Zhao *et al*., 2003; Su *et al*., 2007; Yu *et al*., 2010). The response of larvae to the presence of a bio-film depends on whether the cues are water-borne or surface-bound signals (Wieczorek and Todd, 1998) and the ability of larvae to detect and actively swim towards the origin of those cues (Grassle *et al*., 1992;
Tamburri *et al*., 1992; Wieczorek and Todd, 1998). Molluscs show a high degree of substratum chemical specificity (Hadfield, 1984), explaining why microbial community structure, as opposed to microbial abundance, is thought to be the primary determinant of biofilm efficacy at inducing settlement of some bivalve species (Yu *et al*., 2010).

Neuroactive compounds involved with the initiation and control of metamorphosis can be used to induce larval settlement of molluscs (Burke, 1983; Morse, 1985). Larvae rely on exogenous sources for compounds such as γ-aminobutyric acid (GABA), commonly found within natural substrata (Hadfield, 1984; Morse, 1985). Settlement behaviour of some bivalve species can be encouraged by adding dissolved concentrations of GABA to aquaria (Doroudi and Southgate, 2002; Garcia-Lavandeira *et al*., 2005; Mesias-Gansbiller *et al*., 2008), but this method does not work for all species (Beiras and Widdows, 1995b; Grant, 2009; O'Connor *et al*., 2009). The neurotransmitter 5-hydroxytryptamine (serotonin) has been found to increase in concentration within the tissues of bivalve larvae just prior to metamorphosis (Cann-Moisson, 2002) and exposing larvae to external concentrations of this chemical is known to encourage settlement and metamorphosis of some species (Beiras and Widdows, 1995b; Zhao *et al*., 2003; Urrutia *et al*., 2004; Grant, 2009). The depolarization of externally accessible excitable cells via exposure to a simple ion, such as KCl, is sometimes enough to promote settlement of bivalve larvae, providing a cost effective and convenient option for chemical induction (Ke *et al*., 1998; Martinez *et al*., 1999; Yu *et al*., 2008).

Bivalve recruitment is also dependent on the presence of a suitable substrate for larvae to attach to. Substrates made from a variety of materials can be submerged into larval rearing tanks to provide an environment that encourages settlement and increases the surface area available for attachment (Southgate, 2008). Previous research has shown that bivalve larvae show preferences for certain materials based on surface area (Ambrose, 1992), colour (Saucedo *et al*., 2005; Su *et al*., 2007) and texture (Harvey *et al*., 1997; Taylor *et al*., 1998;
Marsden and Lansky, 2000). The physical cues for promoting settlement appear to be species-specific; therefore research is required to determine the individual preferences of commercially important bivalves. Optimising recruitment to substrates is particularly important in the hatchery production of pearl oysters because of their tendency to attach to the sides and bottom of settlement tanks, rather than to collectors that facilitate the transfer of larvae to ocean-based nursery culture (Su et al., 2007; Southgate, 2008).

Previous research investigating settlement cues for pearl oyster larvae has focused on species from the genus *Pinctada* (e.g. Taylor et al., 1998; Doroudi and Southgate, 2002; Zhao et al., 2003; Saucedo et al., 2005; Su et al., 2007; Yu et al., 2008, 2010), thus there is a paucity of information for all other species. This study aimed to improve hatchery production of *Pteria penguin* by investigating the viability of methods used to promote larval recruitment in other hatchery reared bivalves. The effects of chemical compounds, the presence of a biofilm and substrate type on the behaviour, settlement and mortality of *P. penguin* pediveliger larvae were examined. The combined use of bio-films and manufactured chemicals to induce settlement in a pearl oyster species was trialled for the first time. It was hypothesised that each of the chemical compounds would have a similar effect on *P. penguin*, with slight differences in efficacy. It was also predicted that *P. penguin* would preferentially settle onto substrates coated in a bio-film.

### 7.2. Materials and methods

#### 2.2.1. Larval production

This study was conducted at the Aquaculture Facility of the Ministry of Agriculture, Food, Forests and Fisheries (MAFFF) at Sopu in the Kingdom of Tonga (21°07′31″S; 175°13′36″W). Fifty *P. penguin* brood-stock were collected from a long-line located 500 m offshore and cleaned before being induced to spawn via thermal stimulation. The hatchery
methods used to culture *P. penguin* larvae during this study are based on the standard methods summarised within the general introduction (*Section 1.4.2*). The same diet of concentrated microalgae paste described as a food source in Chapter 6 was used during the production of larvae for this study (*see Section 6.2.2*). Stocking density was decreased and feed ration increased as larvae aged, based on the results of Chapter 6 (*Section 6.3*). Larval development was monitored by periodically examining specimens under a high power optical microscope at 200X magnification until the presence of an eye-spot and functional foot signified that larvae were ready to settle on day 22 (*see Section 4.3.3*).

7.2.2. Bioassay techniques

Petri dishes (50 mm diameter x 9 mm deep) used as experimental aquaria contained 10 mL of 0.45 µm filtered test solution (FSW + chemical treatment) (*see Section 7.2.3*) and a single piece of horizontally oriented 25 x 25 mm flat substrate (following Zhao *et al.*, 2003). Eyed pediveliger larvae were collected from larval culture tanks on a nylon sieve with a diagonal pore size of 240 µm and kept submerged until rinsed into a shallow beaker at a approximate density of 1 larvae mL\(^{-1}\). Ten pediveliger larvae were transferred individually into each Petri dish aquarium to achieve a density of 1 larvae mL\(^{-1}\), consistent with the low densities implemented by commercial pearl oyster hatcheries during the later stages of hatchery production (Southgate, 2008). The transparent aquaria were then placed on a black background in a temperature-controlled laboratory maintained at 28 ± 1°C and given a 12:12 h (Light:Dark) photoperiod. Pearl oyster larvae typically cease feeding activity while undergoing settlement (Saucedo and Southgate, 2008; Yu *et al.*, 2010), therefore no food was supplied during experiments.
7.2.3. Experiments

Experiment 1 monitored larval behaviour, settlement and mortality when exposed to 3 chemical compounds (>98% purity) manufactured by Sigma-Aldrich (St Louis, Missouri, 63103, U.S.A), each tested at two concentrations. The neuroactive compounds 5-hydroxytryptamine hydrochloride (serotonin) and γ-aminobutyric acid (GABA) were applied at concentrations of $10^{-4}$ and $10^{-3}$ molar (M), while potassium chloride (KCl) was applied at concentrations of 20 and 50 millimolar (mM). Filtered seawater (FSW) with no chemical additives was used as a negative control. Chemical concentrations were tested both in the presence and absence of a natural bio-film, resulting in 12 treatments, each conducted in triplicate. Aquaria received a single clear plastic microscope cover slip (25 x 25 mm) that was either sterile or given a natural bio-film. Cover-slips were chosen as a substrate to enable comparison with studies on other pearl oyster species (Zhao et al., 2003; Yu et al., 2010) and because of their standard surface area for bio-film conditioning. Natural bio-films were formed on the surface of cover slips by holding them in non-filtered running seawater at a flow rate of 100 L h$^{-1}$ for a period of 48 h prior to the experiment.

Experiment 2 monitored larval behaviour, settlement and mortality when exposed to equal sized pieces (25 x 25 mm) of 5 different substrate materials: (1) red nylon mesh with 5 mm pore size; (2) red nylon mesh with <1 mm pore size; (3) black nylon coated fibreglass screen with 3 mm pore size; (4) black nylon coated fibreglass screen with 1 mm pore size; and (5) clear plastic microscope cover slips. All substrates were sterilised and washed with FSW prior to the experiment. The effect of substrate was tested both in the presence and absence of a chemical cue (20 mM KCl), resulting in 10 treatments, each conducted in triplicate.

Larval behaviour was recorded after 24, 48 and 72 h by examining aquaria using a dissecting microscope and classifying the status of each individual larva according to four
categories: (1) ‘swimming’ through the water column; (2) ‘sitting’ stationary; (3) ‘crawling’ on a surface using their foot and (4) ‘settled’ onto a surface (either substrate or dish) using byssal thread attachment. Fig. 7.1 shows larvae observed under microscope undertaking each of these behaviours. Larvae with decaying internal structures and the absence of beating cilia were recorded as having undergone ‘mortality’. Due to repeated sampling of the same aquaria, mortality was cumulative over time. There was no evidence of larval detachment once settlement had occurred.

The normality of raw data was assessed using probability plots of residual values and proportional data were square-root arcsine transformed prior to statistical analysis. Interactions between factors and significant effects of individual treatments on larval behaviour, settlement and mortality were assessed using ANOVA and post-hoc Tukey tests ($\alpha = 0.05$).
7.3. Results

7.3.1. Experiment 1: The effects of chemical inducers and a bio-film

There was no significant interaction between chemical treatment of the culture medium and the presence/absence of a bio-film on larval behaviour, settlement or mortality. Due to complete mortality in aquaria treated with 50 mM KCl within 48 h, this treatment was removed from the analysis for the 48 h and 72 h sampling periods. The presence of a bio-film
did not significantly influence the proportion of larvae swimming, sitting or crawling, but did significantly increase settlement over the course of the experiment ($F = 6.013$, df = 1, $p = 0.021$). The largest effect of a bio-film on settlement was observed after 48 h, whereby settlement increased from 14.5% in control aquaria to 24% in aquaria containing a plastic substrate covered in a bio-film. The presence of a bio-film did not influence mortality.

Treatment of the culture medium with chemical compounds had an overall significant effect on larval behaviour, settlement and mortality at each sampling time, with the exception of settlement at 24 h (Table 7.1). Swimming behaviour was significantly greater in control aquaria than in aquaria treated with a chemical compound, with the exception of aquaria treated with $10^{-4}$ M serotonin after 24 h (Table 7.1; Fig. 7.2). After 48 h, a large proportion of larvae in the control aquaria displayed crawling behaviour, however those exposed to 20 mM KCl had the highest rate of crawling behaviour at all three sampling times (Table 7.1; Fig. 7.2). Mean settlement was significantly greater in aquaria treated with $10^{-3}$ M serotonin when compared to control aquaria at both 48 h (125% greater) and 72 h (75% greater) and was also significantly greater in aquaria treated with 20 mM KCl at 72 h (84% greater) (Table 7.1; Fig. 7.2). High concentrations of both KCl and GABA were considered toxic after all larvae exposed to 50 mM KCl died within 48 h, and exposure to $10^{-3}$ M GABA resulted in significantly greater mortality than that observed in control aquaria at all three sampling times (mean of 114% greater) (Table 7.1; Fig. 7.2).
Table 7.1: ANOVA results for the effect of chemical treatment on larval behaviour, settlement and mortality at each sampling time. P-values are listed for significant differences between individual treatments and the control of no chemical.
<table>
<thead>
<tr>
<th>Behaviour</th>
<th>F-Stat</th>
<th>df</th>
<th>P-value</th>
<th>Serotonin $10^{-4}$ M</th>
<th>Serotonin $10^{-3}$ M</th>
<th>GABA $10^{-4}$ M</th>
<th>GABA $10^{-3}$ M</th>
<th>KCl 20 mM</th>
<th>KCl 50 mM</th>
</tr>
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<tr>
<td><strong>24 h</strong></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Swimming</td>
<td>10.167</td>
<td>6</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Sitting</td>
<td>4.908</td>
<td>6</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.048*</td>
</tr>
<tr>
<td>Crawling</td>
<td>25.151</td>
<td>6</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001**</td>
<td>-</td>
<td>&lt;0.001**</td>
<td>-</td>
</tr>
<tr>
<td>Settled</td>
<td>2.022</td>
<td>6</td>
<td>0.089</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mortality</td>
<td>22.324</td>
<td>6</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.011**</td>
<td>-</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td><strong>48 h</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Swimming</td>
<td>21.667</td>
<td>5</td>
<td>&lt;0.001</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
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<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>NA</td>
</tr>
<tr>
<td>Sitting</td>
<td>7.126</td>
<td>5</td>
<td>&lt;0.001</td>
<td>0.036**</td>
<td>-</td>
<td>0.001**</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>Crawling</td>
<td>22.716</td>
<td>5</td>
<td>&lt;0.001</td>
<td>0.035*</td>
<td>0.018*</td>
<td>0.018*</td>
<td>0.002*</td>
<td>0.001**</td>
<td>NA</td>
</tr>
<tr>
<td>Settled</td>
<td>3.000</td>
<td>5</td>
<td>0.026</td>
<td>-</td>
<td>0.044**</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Mortality</td>
<td>11.174</td>
<td>5</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001**</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td><strong>72 h</strong></td>
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<td></td>
</tr>
<tr>
<td>Swimming</td>
<td>13.952</td>
<td>5</td>
<td>&lt;0.001</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>NA</td>
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<tr>
<td>Sitting</td>
<td>2.683</td>
<td>5</td>
<td>0.040</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>Crawling</td>
<td>13.288</td>
<td>5</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001**</td>
<td>NA</td>
</tr>
<tr>
<td>Settled</td>
<td>12.370</td>
<td>5</td>
<td>&lt;0.001</td>
<td>-</td>
<td>0.048**</td>
<td>-</td>
<td>-</td>
<td>0.029**</td>
<td>NA</td>
</tr>
<tr>
<td>Mortality</td>
<td>12.947</td>
<td>5</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.001**</td>
<td>-</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Treatment mean significantly lower than the control mean ($P < 0.05$).

**Treatment mean significantly higher than the control mean ($P < 0.05$).
Fig. 7.2. Mean (± SE) percentage of larvae within chemical treatments that displayed each behaviour, were settled, or had undergone mortality at the three sampling times of 24 h, 48 h and 72 h. The treatments were filtered seawater (FSW) control; $10^{-4}$M serotonin (-4M S); $10^{-3}$M serotonin (-3M S); $10^{-4}$M GABA (-4M G); $10^{-3}$M GABA (-3M G); 20mM KCl (20 mM K); 50 mM KCl (50mM K). The treatment 50 mM KCl was removed from the analysis after 24 h due to complete mortality. *Treatment mean significantly lower than the control mean (P <0.05). **Treatment mean significantly higher than the control mean (P <0.05).
3.2. Experiment 2: Effects of substrate type and a chemical inducer

There was no significant interaction between the effects of substrate type and the presence/absence of a chemical treatment (20 mM KCl) on larval behaviour, settlement or mortality. Substrate type did not significantly influence larval behaviour (swimming/sitting/crawling) or mortality at any sampling time; however, settlement in aquaria containing a piece of red nylon mesh with 5 mm between filaments (mean of 15% settled) was significantly higher (F=3.285, df=1, p=0.027) during the first 24 h than in aquaria containing the other four types of substrate (mean of 5% settled). The presence of KCl caused an increase in settlement sooner than observed in Experiment 1, with a significant increase in mean settlement occurring at 48 h (123% greater than control) (F = 8.960, df=1, p=0.006). The highest degree of settlement for a single treatment at the completion of Experiment 2 (72 h) was 35% and occurred in aquaria that contained a piece of red nylon mesh with 5 mm between filaments and were treated with 20 mM KCl.

7.4. Discussion

Substrates exposed to unfiltered seawater quickly develop a microbial film on their surface. These are generally composed primarily of bacteria, microalgae and detritus (Keough and Raimondi, 1995; Bao et al., 2007; Yu et al., 2010). This study recorded the behaviour of pearl oyster pediveliger larvae both in the presence and absence of a bio-film. The presence of a bio-film did not significantly affect the proportion of P. penguin larvae swimming, sitting still or crawling, over a 72 h period. These results are in contrast to those reported for oyster larvae of the genus Crassostrea, which have been shown to swim towards the substrate and initiate crawling behaviour in response to bio-films (Fitt et al., 1990; Tamburri et al., 1992), a reaction brought about by water-soluble peptides originating from the bio-film itself (Zimmer-Faust and Tamburri, 1994). The presence of manufactured chemical inducers in
Experiment 1 was expected to interfere with the effect of water-bourne bio-film associated cues, however, the presence of a bio-film also failed to influence larval behaviour in control aquaria with no chemical treatment. Given that there was no threat of water flow impeding the detection of water-soluble compounds (Turner et al., 1994), it is concluded that *P. penguin* larvae did not alter their behaviour (swimming/sitting/crawling) in response to water-borne cues associated with the presence of a bio-film.

The presence of a bio-film did not appear to influence the behaviour of *P. penguin* larvae other than to significantly increase mean settlement. Some of the settled larvae (~25%) were found attached to the surface of the Petri dish rather than to the bio-filmed substrate. When observing a similar response of pearl oyster (*Pinctada maxima*) larvae, Zhao et al. (2003) concluded that water-borne cues associated with a bio-film can promote attachment to other suitable surfaces in close proximity. Su et al. (2007) found that when given a choice, *Pinctada fucata* larvae preferred to settle on bio-filmed substrates, but some degree of settlement also occurred on non-conditioned surfaces. Very few *P. fucata* larvae settled in response to bio-film conditioned seawater, suggesting that surface-borne cues must also be present in order to encourage settlement (Yu et al., 2010). Settlement of *P. fucata* was not positively influenced by bio-film abundance over time, indicating that microbial community structure and extracellular products, rather than abundance, play an important role in inducing settlement (Yu et al., 2010). The application of natural bio-films within the hatchery is challenging because of the difficulty in controlling their structure and the risk of inappropriate conditioning prolonging settlement (Rose and Baker, 1994; Wieczorek and Todd, 1998). More research is required to determine if water-borne compounds that encourage settlement of *P. penguin* can be isolated for controlled use in the hatchery; this has been reported previously for *Crassostrea* sp. (Zimmer-Faust and Tamburri, 1994). Interestingly, the efficacy of bio-films at inducing settlement was not significantly impacted
by the presence of manufactured chemical cues, suggesting that cues isolated from bio-films could be used in conjunction with other chemical inducers.

External concentrations of serotonin (5-hydroxytryptamine) have been shown to induce settlement and/or metamorphosis in several bivalve genera including *Ruditapes* (Urrutia *et al.*, 2004), *Crassostrea* (Beiras and Widdows, 1995b; Grant, 2009) and *Pinctada* (Zhao *et al.*, 2003; Yu *et al.*, 2008), but the mechanisms involved remain poorly understood. Of seven neuroactive compounds tested in past studies, serotonin was found to be the most effective at inducing settlement (10\(^{-4}\) - 10\(^{-3}\) M) of the pearl oysters *P. maxima* (Zhao *et al.*, 2003) and *P. fucata* (Yu *et al.*, 2008), yielding up to 90% (after 72 h) and 50% (after 48 h) larval settlement, respectively. However, settlement of *P. fucata* dropped to below 20% by 72 h despite only a marginal increase in mortality, indicating that some larvae had detached from the substrate (Yu *et al.*, 2008). During this study 10\(^{-3}\) M serotonin first began to influence behaviour after 24 h, as shown by a reduction in the proportion of swimming larvae when compared to control aquaria. This result supports the notion that high concentrations of serotonin act to reduce ciliated velar activity and therefore swimming behaviour of bivalve larvae (Beiras and Widdows, 1995a; Grant, 2009). Seventy two hours of exposure to 10\(^{-3}\) M serotonin yielded a mean of 40% settlement, with no larval detachment observed.

The reduction in swimming behaviour of *P. penguin* larvae when exposed to \(\gamma\)-aminobutyric acid (GABA) (10\(^{-4}\) M) was similar to that caused by serotonin. This is also thought to be linked to reduced velar activity, effectively dropping larvae out of suspension and onto the substrate (Pawlik, 1990). The lack of *P. penguin* settlement in response to GABA was unexpected given the positive effect of 10\(^{-4}\) M GABA on larval settlement of pearl oysters from the genus *Pinctada* (Doroudi and Southgate, 2002; Zhao *et al.*, 2003; Yu *et al.*, 2008). *P. penguin* now joins the list of other oyster species for which GABA is ineffective at inducing settlement, including those from genera *Crassostrea* (Beiras and
Widdows, 1995b; Grant, 2009) and Ostrea (O’Connor et al., 2009). Exposure to $10^{-3}$ M GABA resulted in significantly greater mortality of P. penguin at each sampling time up to 72 h, supporting previous observations of a high toxicity of GABA to pearl oyster larvae (Doroudi and Southgate, 2002; Yu et al., 2008).

Introducing excess potassium ions ($K^+$) into the culture medium activates the neural pathways that control settlement and metamorphosis in molluscs by depolarizing externally accessible excitable cells (Morse, 1985; Yool et al., 1986). This technique has been shown to induce settlement in bivalve genera Agropecten (Martinez et al., 1999; Zhang et al., 2003), Perna (Ke et al., 1998), Chlamys (Ke et al., 2000), and Pinctada (Zhao et al., 2003; Yu et al., 2008). Treating aquaria with KCl encouraged both crawling behaviour and settlement of P. penguin in this study. A concentration of 20 mM led to significantly greater settlement within 72 h during Experiment 1 and 48 h during Experiment 2, achieving settlement rates on par with those induced by exposure to serotonin (35-40%). The progression from swimming or sitting behaviour to crawling on the substrate surface is a behavioural sequence that often culminates in larval settlement (Gosling, 2003; Saucedo and Southgate, 2008), thus it is probable that the high proportion of larvae still crawling at the end of the experiments would have eventually settled if given enough time. Exposure of P. penguin larvae to 50 mM KCl resulted in 100% mortality within 48 h, indicating that larvae of this species have greater toxic sensitivity to KCl than those of P. fucata (Yu et al., 2008) and P. maxima (Zhao et al., 2003), both of which continued to show increased mean settlement up to and including concentrations of 50 mM. This research suggests that when applied at a concentration of 20 mM, KCl provides P. penguin hatcheries with a chemical means to induce settlement that is easily accessible, safe and inexpensive.

The results of Experiment 2 show that mean settlement in response to KCl was maximised in the presence of a red nylon mesh with 5 mm pore size. The positive effect of
this substrate on inducing settlement was most pronounced during the initial 24 h of the study. Doroudi and Southgate (2002) found that larval settlement of *P. margaritifera* in response to a chemical cue (GABA) was also enhanced by the presence of a nylon substrate. Despite having a reduced surface area when compared to the other tested substrates that were either solid or had smaller pore sizes, red nylon mesh (5 mm) encouraged significantly greater settlement. Vinyl coated black fibreglass screen has similarities in design to the red nylon mesh and has previously been identified as a preferred substrate for *Pinctada mazatlanica* (Saucedo *et al*., 2005), but did not encourage settlement of *P. penguin*. The success of red nylon mesh (5 mm) in promoting settlement could be attributed to factors known to elevate settlement in other pearl oysters including a flat surface (Rose and Baker, 1994), deep red colour (Su *et al*., 2007) and cryptic structure with shaded areas (Alagarswami *et al*., 1983). It is also possible that the artificial substrate bears a resemblance to an ideal habitat found in nature. For instance, the bright colour resembles some gorgonian species known to be a preferred natural substrate for *P. penguin* (Besley *et al*., 1998; Beer and Southgate, 2000) and the empty space between filaments would allow for rapid growth (Southgate *et al*., 2008) and facilitate access to water flow (Ito, 1999).

In summary, cues for settlement of bivalve larvae within a hatchery environment are species-specific. Settlement of *P. penguin* was greater in aquaria containing a substrate covered by a naturally formed bio-film than in sterile aquaria. Treatment of the culture medium with the neuroactive compounds of 5-hydroxytryptamine (serotonin) and γ-aminobutyric acid (GABA) and the chemical potassium chloride (KCl) were all effective at decreasing swimming behaviour of larvae, the first step in the sequence of behaviours known to culminate in settlement. KCl was also effective at increasing the proportion of larvae to crawl over the surface of the substrate in search of a position to settle. Treating the culture medium with either serotonin ($10^{-3}$ M) or KCl (20 mM) resulted in significantly greater larval
settlement, whereas the presence of GABA did not. KCl was equally as effective as serotonin at promoting settlement over 72 h, but comes with the added advantages of being safe for human handling and relatively inexpensive. Given that the efficacy of manufactured chemical inducers was not improved by the presence of a bio-film, the risk of inappropriate substrate conditioning prolonging settlement can be avoided. The combined use of water-bourne cues isolated from bio-films with other chemicals such as serotonin and KCl remains an area for future research. Mean settlement in response to KCl was maximised when a suitable substrate was provided in the form of red nylon mesh with 5 mm pore size. This substrate type led to significantly greater settlement within the first 24 h when compared to four other options, attributable to physical cues resembling those present in an ideal natural habitat.
Chapter 8
General discussion

8.1. Introduction

This study has been successful in addressing some of the major factors currently limiting hatchery production of the winged pearl oyster, *Pteria penguin*. A series of relevant recommendations based on the results of this study will provide industry members with recommendations for efficient production of juvenile oysters for future use in pearl production. Furthermore, this research adds to existing knowledge regarding the physiology and life-cycle of this species and pearl oysters (family: Pteriidae) in general. As is the case for all research projects, this study is not without limitations and should form the foundation for future research aimed at further enhancing culture techniques for this commercially important bivalve.

8.2. Major findings of this study

8.2.1. Feeding physiology

- The rate at which *P. penguin* cleared ambient water of suspended microalgae (clearance rate, CR) was greater when feeding on the two flagellate species *Isochrysis* sp. Tahitian (T-Iso) and *Pavlova* sp. (mean = 32 L h\(^{-1}\) oyster\(^{-1}\)) when compared to the diatom *Chaetoceros muelleri* (27 L h\(^{-1}\) oyster\(^{-1}\)).

- *P. penguin* is the first Pteriidae species to display a stable CR with greater food availability up to an algae concentration of 50 x 10\(^3\) cells mL\(^{-1}\).
• The proportion of organic carbon absorbed by *P. penguin* during digestion (absorption efficiency, AE) was greatest when feeding on T-Iso (61%) and was not influenced by food concentration.

• CR and AE decreased in response to a reduction in water temperature below 28°C during summer, reflecting the 35% lower CR and 47% lower AE observed during the colder austral winter. This suggests that temperature contributes to differences in suspension feeding between seasons.

• The high feeding capacity of *P. penguin* makes brood-stock conditioning a difficult undertaking for hatcheries with limited technical capacity for mass live microalgae culture.

### 8.2.2. Brood-stock conditioning

• Brood-stock were shown to feed efficiently on concentrated microalgae (Instant Algae®).

• Providing brood-stock with a continuous supply of concentrated microalgae, and increasing both food concentration and water temperature over time, is a technique that can be used to promote gametogenesis in *P. penguin* prior to the natural spawning season.

• When using a flow-through aquarium system, male *P. penguin* were able to access the energy required to produce spermatozoa at a rate exceeding that observed in ocean culture techniques over a time period of 40 days; however, the production of mature oocytes in females was less reliable, attributable to the period for conditioning being too short for the production of energetically expensive oocytes.
8.2.3. Embryonic and larval development

- Rinsing samples with chloroform and allowing them to dry within a vaporous chloroform environment provides an alternative technique for the preparation of delicate specimens prior to scanning electron microscopy (SEM).
- This study provides a detailed photograph of a sperm from an oyster belonging to the family Pteridae, which possesses an ovoid shaped nucleus that is different to the arched head and elongated acrosome seen in several other bivalve families.
- The sequence of events during embryogenesis and larval development of *P. penguin* is similar to that previously recorded for *Pinctada* species, with slight differences in the timing of respective developmental stages.
- This study describes early shell formation in a Pteridae species. In brief, a shell-field invagination (sfi) first becomes evident during gastrulation and stretches laterally, creating a deep narrow crevice. The early central hinge region broadens and folds outward to form a flat surface. Shell calcification does not take place until the periostracum spans the entire epithelial surface.
- The larval hinge structure shows a prominent cardinal tooth and socket. The outer prismatic shell layer is almost non-existent in some areas. *P. penguin* larvae possess a large flat umbo, rather than the typical cone-shape umbo of *Pinctada* species, and demonstrate skewed shell growth towards the posterior end of the deepest valve.

8.2.4. Embryo incubation

- Treating incubation tanks with antibiotics is an effective way to reduce excessive mortality.
- Despite a 23% increase in mean survival during incubation, aquaria treated with tetracycline:erythromycin (1:1) yielded an average of only 9% more veliger larvae.
than control aquaria due to interference with development during the transition of trochophore to shelled larvae (D-stage).

- The application of the antibiotic streptomycin-sulfate improved mean survival by 16% when compared to control aquaria, without significantly compromising development.
- Increasing initial stocking density from 50 to 100 eggs mL\(^{-1}\) did not significantly reduce survival, but did result in a 5% reduction in normal development to D-stage.
- Utilising a high stocking density of 100 mL\(^{-1}\) provides the greatest number of D-stage larvae; however, if the supply of eggs is limited, it is recommended that eggs be stocked at a density \(\leq 50\) mL\(^{-1}\) and mortality be minimised by treating the culture medium with the antibiotic streptomycin-sulfate.

8.2.5. Larval culture

- Hatchery culture in regional areas is often impossible because farms cannot afford the facilities required to produce the live microalgae used as a food source for larvae. This study shows acceptable rates of larval survival and growth when fed a diet consisting solely of concentrated microalgae (Instant Algae®).
- Survival during D-stage was significantly enhanced (by 105%) in aquaria stocked at \(<10\) larvae mL\(^{-1}\); a density of 6 mL\(^{-1}\) maximised larval production per volume of culture medium.
- An intermediate feed ration of 10 x 10\(^3\) cells mL\(^{-1}\) maximised both survival and growth during D-stage.
- Increasing the stocking density of umbo-stage larvae from 1 to 3 mL\(^{-1}\) resulted in a significant reduction of both survival (360%) and growth (16%).
- Growth of umbo-stage larvae stocked at 1 mL\(^{-1}\) increased significantly (7%) when feed ration was maintained below 20 x 10\(^3\) cells mL\(^{-1}\).
8.2.6. Larval settlement

- Treatment of the culture medium with manufactured chemical inducers decreased swimming behaviour of pediveliger larvae. KCl was effective at increasing the proportion of larvae to crawl over the surface of the substrate.
- Approximately 24 h after developing a foot, nearly three times more larvae settled onto a red nylon mesh substrate with 5 mm pore size when compared to the same material with a smaller pore size, black fibreglass screen and smooth plastic substrates.
- After 48 h, settlement of *P. penguin* was 65% greater in aquaria containing a substrate covered by a naturally formed bio-film than in control aquaria with no bio-film.
- After 72 h, settlement of larvae in aquaria treated with serotonin (10\(^{-3}\) M) or KCl (20 mM) was significantly greater than in control aquaria with no chemical by 75% and 84%, respectively, while exposure to \(\gamma\)-aminobutyric acid (GABA) had no effect.
- The positive effect of a bio-film on settlement did not change significantly according to chemical treatment of the culture medium, suggesting that the techniques can be used in conjunction.

8.3. Implications of major findings

The major findings regarding the effects of diet, feed ration and water temperature on the feeding behaviour of adult *P. penguin* can be utilized in several capacities. Studies on the feeding behaviour of commercial bivalve molluscs are required to understand their role in ecosystem dynamics (Hawkins *et al.*, 1998). Information regarding commercially cultured species can be used to ensure that large scale farming remains sustainable without significantly altering the local supply of nutrients or habitat characteristics (Heral, 1993; Pouvreau *et al.*, 1999; Nunes *et al.*, 2003). The high feeding capacity of *P. penguin* indicates
the species potential to alter phytoplankton composition and rates of bio-deposition, thus population sizes in areas of pearl farming must be managed to prevent adverse affects to local ecosystem dynamics. The information generated in this study will aid in determining carrying capacities for *P. penguin* culture in near-shore environments.

The results of this study indicate that *P. penguin* brood-stock alter their rate of filtration and digestion efficiency in response to changes in diet, food availability and water temperature. This research provides insight into the conditions required to optimise feeding efficiency and energy uptake during the narrow natural spawning season (Milione and Southgate, 2012a). These findings were implemented when trialling techniques to condition brood-stock outside of the spawning season, using a diet of concentrated algae as a food source. Monitoring of the feeding behaviour of *P. penguin* during the 40-day long conditioning program indicated that brood-stock were capable of effectively ingesting and digesting the concentrated algae. Furthermore, the positive effect on the gonad condition in some experimental oysters highlights the potential of these techniques for brood-stock conditioning aimed at extending the annual breeding period available to *P. penguin* hatcheries.

Hatchery culture of *P. penguin* is undertaken in areas with a low natural spat fall in order to provide an adequate supply of oysters to permit large-scale commercial half-pearl production (Teitelbaum and Ngaluaf, 2008). This study describes the processes underpinning embryonic and larval development in *P. penguin*, information central to the advancement of hatchery techniques for this species. Hatchery propagation of bivalves involves a series of production stages including embryo incubation, larval rearing and larval settlement. It was essential to map the approximate timing of each major stage of development before effective hatchery protocols could be developed. In addition to its commercial relevance, the description of morphological characteristics can be used to
accurately identify *P. penguin* embryos and larvae within plankton communities. The ability to correctly identify juveniles is necessary to investigate patterns of dispersal, settlement and recruitment of wild populations (Gribben and Hay, 2003; Da Costa *et al.*, 2008). This study adds to the currently deficient literature regarding early shell formation in bivalves by providing a detailed description of this process in a Pteriidae species.

The primary aim of this study was to address the paucity of information regarding protocols for hatchery culture of *P. penguin*, with particular emphasis on the major issues currently limiting hatchery production. This study addressed the issue of excessive mortality during embryo incubation, a common problem faced when undertaking hatchery culture of Pteriidae species (Southgate, 2008). An analysis of the results provided two sets of recommendations depending on whether tank space or the number of available eggs is the factor limiting production during the incubation phase. Hatchery production will be enhanced by knowing how the adverse effects of increased egg stocking density on survival can be ameliorated by applying an appropriate antibiotic treatment that does not compromise larval development by hindering shell formation.

The findings of this study provide evidence for successful larval rearing of *P. penguin* using concentrated algal paste as a food source rather than live algae. This will encourage hatchery propagation of *P. penguin* in facilities that lack the technical capacity for the culture of live microalgae as a food source for larvae (Southgate *et al.*, 2006; Teitelbaum and Ngalaufe, 2008). The results of this research identify the procedures required to maximise both survival and growth during hatchery culture of *P. penguin* larvae. Recommendations are provided for the larval stocking densities and feed rations that promote larval production per volume of culture medium. The cost of concentrated algae can be minimised by ensuring that the necessary food ration is not exceeded, which will in turn ensure that water quality within rearing tanks does not deteriorate due to the decomposition of unconsumed food.
The final component of this research addressed the issue of sub-optimal recruitment of *P. penguin* larvae to artificial substrates. Pearl oysters are known to show an increased rate of settlement in response to suitable physical and chemical stimuli (Doroudi and Southgate, 2002; Zhao *et al.*, 2003; Saucedo *et al.*, 2005). The results of this research provide insight into the ability of *P. penguin* larvae to respond to water-bourne and surface-bound chemical cues through alteration of their behaviour. This study compares the efficacy of several well known bivalve settlement cues in encouraging settlement behaviour of *P. penguin* pediveliger larvae. Recommendations are provided for the combination of stimuli that enhances recruitment to artificial collectors and on that basis, this study will facilitate efficient transfer of larvae to ocean-based nursery culture.

### 8.4. Future directions

The scope of this project was in some instances restricted by the limited resources available. Opportunities exist for future research that will further enhance hatchery production of this important commercial species. The following paragraphs describe the limitations encountered during each major component of this study and propose avenues for future research aimed at optimising the productivity of hatchery and farming operations.

The Flowcam® particle analyser used in Chapter 2 was an efficient tool to count algal cells when measuring clearance rate during feeding trials of *P. penguin*. However, this instrument does not achieve the required level of accuracy when measuring low concentrations of suspended microalgae, so could not be used to provide estimates of feeding behaviour at concentrations below 10,000 cells mL\(^{-1}\). This issue did not restrict research aimed at identifying the conditions required for optimal energy uptake during brood-stock conditioning programs, but prohibited investigations regarding the scope for growth of *P. penguin* when feeding on natural seston. The aquarium system described in this study could
be utilized in conjunction with fine scale instrumentation to monitor feeding behaviour under various environmental conditions. Such research could be valuable in determining habitat characteristics preferable for efficient energy uptake by *P. penguin* during ocean based nursery culture and grow-out of hatchery bred stock.

The small number of brood-stock available for the conditioning experiment in Chapter 3 resulted in a level of replication that prevented statistical analyses of data relating to gonad maturation. This experiment was designed only to provide insight into the potential for concentrated algal paste as a food source during brood-stock conditioning programs and to test the efficacy of a unique aquarium system. The results suggested that *P. penguin* were able to effectively gain access to the nutrition required for gonad maturation. Expansion of the aquarium system and study design would facilitate a robust examination of the effects of prolonged (>40 days) exposition of female brood-stock to concentrated algae and optimal feeding conditions on the production of mature oocytes outside of the natural spawning season. This research should include a range of concentrated algae diets with various species compositions in order to identify the diet that best promotes gonad maturation.

The techniques used in Chapter 4 to preserve larval samples for examination under scanning electron microscope (SEM) caused larvae to close their shell valves. Observations were made on the internal structure of larvae, but no high resolution images were available to aid in their description. The methodology later used for sample preparation and examination did not compromise the integrity of fine biological structures, therefore offers potential for visual comparisons in the early development of organs and soft tissues in bivalve species. The description provided for embryogenesis, shell formation and development stages of *P. penguin* larvae could be expanded to include a detailed analysis of growth patterns from larval settlement through to reproductive maturity.
The existing literature regarding pearl oyster hatchery culture and feedback from industry representatives were used to prioritise the most important hatchery parameters to be included in this study. Difficulty obtaining larvae for experiments in conjunction with time constraints limited the number of treatments that could be tested, leaving avenues for future research to further improve hatchery production of *P. penguin*. For instance, the results of Chapter 5 provided recommendations for the application of antibiotics during embryo incubation; however, further trials are required to identify what specific concentrations are most effective for a range of embryo stocking densities. The priority of maximising production of larvae per tank is achieved by optimising survival rate at high egg densities. Concentrations of streptomycin-sulfate exceeding those tested in this study may ameliorate the adverse effects of a high egg stocking density on normal development by combating high levels of harmful bacteria that inhibit larval growth. It is possible that tetracycline:erythromycin could be used to boost production if the standard incubation period were extended beyond the expected time required for normal embryonic development so as to allow for the delay in shell formation caused by the antibiotic combination.

The results of Chapter 6 indicated that preserved concentrated microalgae can be successfully used as a food source during larval rearing of *P. penguin*. Further investigation is required to ascertain if survival and growth rates when using the concentrated food source are comparable with rates observed for larvae of the same spawning event fed a diet of live microalgae. There are a range of preserved and concentrated aquaculture feed products available that remain to be tested as an appropriate food source for pearl oyster larvae. Opportunities exist for future research using similar techniques as those described in this study to examine the effects of various diets and feed rations of commercial ‘off-the-shelf’ products on larval performance. Research conducted in conjunction with feed manufacturers to development species-specific diets that negate the requirement for live microalgae.
production would facilitate simple hatchery techniques for the propagation of pearl oysters in regional areas and developing countries.

The findings of Chapter 7 regarding optimisation of the settlement process could be further developed by research to underpin the effects of subtle variations in the concentration of serotonin and potassium chloride, along with different aged bio-films, on the behaviour and settlement of *P. penguin* pediveliger larvae. Furthermore, the results of this study suggest that surface and water-borne cues could be isolated from biofilms and applied in conjunction with manufactured chemical inducers to encourage larval settlement. This would remove the threat of inappropriate conditioning inhibiting settlement. Exposing pearl oyster larvae to substrates that are similar in structure to an ideal natural habitat ensures a greater proportion of larvae settle on dedicated spat collectors than on the tank surface. Research devoted to identifying the unique habitat characteristics preferred by pediveliger larvae of *P. penguin* should be conducted along with an examination of how larvae detect surface-bound cues with the aim of refining the design of spat collectors used in hatcheries.
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Appendix 1: Publication of Chapter 4
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Appendix 2: Publication of Chapter 5
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Appendix 3: Publication of Chapter 7
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Appendix 4: Preparation of samples for SEM
The following is a description of the methods used to process embryo and larval samples for scanning electron microscopy (SEM) during research conducted for Chapter 4 of this thesis. This technique was modified from that described by Turner and Boyle (1974) and is suitable for the preservation and preparation of delicate biological materials for SEM analysis. The methods described here were developed with the help of the late Professor Christopher Alexander of James Cook University.

Sample collection and preservation

- 1L of culture medium was taken from incubation or larval rearing tanks by submerging 4 x 250 mL sterilized glass beakers at various depths.

- Embryos and larvae were collected by filtering the 1 L of culture medium through a small nylon sieve. The sieve was made by stretching mesh with 25 µm pore size over one end of a narrow glass cylinder. This process was repeated periodically to monitor embryonic and larval development over time.

- Each sieve was gently rinsed with 1 µm filtered seawater (FSW) and placed upright into a 25 mL plastic vial that contained 5 mL of 2.5% gluteraldehyde in FSW with a pH of 8.15 - 8.19.

- Biological samples were allowed two weeks of fixation time, during which time they remained on the sieve surface.

Sample dehydration

- Each sieve was removed from its 25 mL plastic vial, gently submerged in a phosphate buffer and then rinsed with distilled water.
A series of 10 x 50 mL plastic vials were filled with 30 mL of high-grade ethanol and distilled water, whereby each consecutive vial contained an increasing concentration of ethanol in the following order.

- 5% ethanol / 95% distilled water
- 10% ethanol / 90% distilled water
- 20% ethanol / 80% distilled water
- 30% ethanol / 70% distilled water
- 40% ethanol / 60% distilled water
- 50% ethanol / 50% distilled water
- 70% ethanol / 30% distilled water
- 90% ethanol / 10% distilled water
- 100% ethanol

The samples on each sieve were dehydrated by submerging the sieve for 10 minutes in each of the ethanol concentrations until reaching 100%, requiring a total time of 100 mins per sample.

**Sample drying** *(NOTE: The following must be conducted in a fume hood)*

- After dehydration, each sieve was placed in concentrated chloroform for a period of 20 seconds.
- Upon being removed from the chloroform the contents of each sieve was carefully scraped onto a separate 10 mm diameter round glass microscope cover slip.
- Glass cover slips where transferred to Petri dishes and placed on filter paper soaked in concentrated chloroform.
• Petri dishes were partially sealed so that the samples could dry within a vaporous environment over a period of 24 hours.

• Once dry, samples were mounted on aluminium stubs and gold coated for examination under scanning electron microscope (SEM).