Broodstock Management and Egg Quality of the Pearl Oysters *Pinctada margaritifera* and *Pinctada fucata*

Thesis submitted by
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for the degree of Doctor of Philosophy in Aquaculture of the
School of Marine Biology and Aquaculture
James Cook University.
June 2004

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Abstract

Marine pearl culture is one of the most valuable aquaculture industries in the world with a total estimated value of about US$500 million. The major pearl producing nations are Australia and French Polynesia and, although reliable methods for hatchery culture of pearl oysters were developed in the 1980's and 1990's, pearl production in these countries still relies primarily on oysters collected from the wild. Generally, the cultured pearl industry, particularly the 'black' pearl industry, has been slow to adopt important advances in aquaculture and other relevant disciplines and is still based on 'traditional' methods. Nevertheless, over recent years there has been increasing interest in research relating to general culture methods for pearl oysters and pearl oyster genetics (particularly relating to pearl quality), and there has been increasing reliance on hatchery production. This study addressed important issues relating to broodstock selection, breeding cycle and egg quality in pearl oysters.

Pinctada margaritifera and *P. fucata* were exposed to propylene phenoxetol at a concentration of 2.5 mL L^{-1} and benzocaine at concentrations of 250, 500 and 1200 mg L^{-1}. Once relaxed, oysters were observed every 5 minutes to evaluate the condition of the mantle and gills. Oysters were classified as either 'suitable saibo' or 'non-suitable saibo' depending on their suitability for use as saibo donors for pearl production. Survival of oysters in all treatments was 100%. With the exception of oysters relaxed with 250 mg L^{-1} of benzocaine, where no relaxation was recorded, oysters exposed to all other treatments became relaxed and showed good condition and acceptable characteristics to be used as saibo donors.

To determine whether mantle tissue could be removed from oysters without mortality, *P. margaritifera* and *P. fucata* were anaesthetised with 500 mg L^{-1} of

benzocaine and had the ventral margin of either their left, right or both left and right mantle lobes removed. Survival after 4 weeks was 100% for all treatments and oysters showed regeneration of excised mantle tissue. Mantle border grew back to almost its original extent within 60 days after excision. Muscular fibres within the new tissue were not seen until 30 days after mantle excision. Functional (secretory) abilities were presumably recovered before day 15 when conchiolin secretions and secretory cells were seen in the newly regenerated epithelia. Mantle regeneration in *P. margaritifera* up to 90 days after mantle excision was similar to that for *P. fucata*. Anaesthetised oysters can provide mantle tissue for pearl seeding and be kept alive for future uses which may include receptors for pearl production (following seeding season) as broodstock (only those providing mantle that produced high quality pearls) and possibly as multiple saibo donors.

A biopsy technique to obtain gonad tissue was assessed in *P. margaritifera*. Prior to biopsy, oysters were anaesthetised with 2 mL L^{-1} of propylene phenoxetol. Three different 9 cm long biopsy needles (16, 18 and 20 gauge) with a 10 mm sample notch, were compared as a means of obtaining gonad tissue from 20 oysters. Samples were removed from each oyster using each of the 3 biopsy needles. Following the biopsy procedure, each oyster was killed and the gonad sectioned for standard histological preparation. Samples were observed microscopically to assess gonad condition and to compare samples taken using biopsy with those taken using destructive sampling. Oysters showed 100% recovery from the anaesthetic and biopsy procedure after 2 weeks. Non-destructive biopsy sampling was an accurate means of assessing gonad condition in male pearl oysters. However, the use of thicker biopsy needles (e.g. 14 or 12 gauge) may allow better interpretation of gonad stage,

particularly in female oysters for which results showed that 16 gauge biopsy needles (the thickest used in this study) were unsatisfactory.

Collection of oysters from culture stock held at Magnetic Island and Orpheus Island was conducted from August 2003 to February 2004 to describe biochemical and histological changes associated to gamete development. Six oysters from each site were collected every month for seven months. Samples of mantle, adductor muscle, gonad and digestive gland were obtained for biochemical analyses. Two spawning peaks (winter and summer) were confirmed for *P. margaritifera* in north Queensland. The adductor muscle played an important role in storage of protein and carbohydrate during gonad development.

To analyse changes in micronutrient composition during embryological development of *P. margaritifera*, samples of eggs, embryos and larvae were taken for determination of carotenoid, ascorbic acid (vitamin C), α-tocopherol (vitamin E) and fatty acid content 0, 4, 8, 12, 16, 24 and 46 h after fertilisation. Carotenoids were found only in trace amounts in *P. margaritifera* eggs and probably do not have an important role in embryo development. The vitamin C and vitamin E contents of *P. margaritifera* embryos increased with time and may not be limiting nutrients for embryological development. The fatty acids 14:0, 16:0 and most unsaturated C18s were highly utilised during embryological development of *P. margaritifera* as demonstrated by their decline during development. These fatty acids may be good indicators of egg quality.

Much of the research in this study was conducted for the first time with pearl oysters. This study describes new and novel information relating to the breeding cycle, broodstock selection and egg quality of pearl oysters. The results of this study provide a basis for more efficient culture methods and may facilitate significant changes to traditional aspects of pearl oyster culture and pearl production.

Statements of contribution of others

The following people in addition to my supervisor provided support, which aided in the preparation of my thesis:

The project team led by Dr. Malcolm Brown (CSIRO, Marine Research Laboratories, Hobart, Tasmania) undertook the analyses of micronutrients reported in Chapter 8 of this thesis. Sections of the scientific report prepared by Dr. Brown were used in the writing of Chapter 8 of this thesis.

Erika Martinez-Fernandez provided technical cooperation in a number of field trips and during the biochemical analyses reported in Chapter 7 of this thesis.

Héctor Acosta-Salmón Date

Acknowledgements

This thesis would not have been possible without the supervision and support of Assoc. Prof. Paul Southgate. His guidance and assistance during this study is very much appreciated.

Thanks to the technical and administrative staff of the School of Marine Biology and Aquaculture, James Cook University, for their support, especially to Savita Francis (Laboratories), Sue Reilly (Histology) and Gordon Bailey (Computing). Special thanks to Laura Castell (Overseas Student Advisor) for her support and very valuable guidance on scientific writing as well as critical reviews of early drafts of this thesis. Thanks to Prof. James Burnell and Dr. Moira McCann for their early advise on biochemical methods. Josiah Pit provided important technical assistant on field trips and the *Pinctada fucata* oysters used throughout this study.

The financial support of my sponsor CONACyT (México) is greatly appreciated. Thanks to all personnel from the *Departamento de Becas al Extranjero* for their kind support. Financial support of the School of Marine Biology & Aquaculture is also acknowledged.

Thanks to the JCU's Marine and Aquaculture Research Facilities Unit (MARFU) staff at the Aquarium Complex and Orpheus Island Research Station in particular to John Morrison and Peter Wruck. Thanks as well to the permanent staff at "Magnetic Island Research Station" Keith Bryson.

I want to thank all my friends in Mexico and Australia for their support; especial thanks to Peter and Amy for making us feel at home.

Finally, I want to thank all my family in México, especially my parents, sister and *suegrita* for their moral support. Very special thanks to my wife Erika for all her help, support and understanding during this period.

This thesis is dedicated to *Don Héctor, 'La Chula' Salmon, Ana and Erika.*

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Statement of sources

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Héctor Acosta-Salmón Date

Chapter 1

General Introduction

Marine pearl culture is one of the most valuable aquaculture industries in the world (Fassler, 1997). Three pearl oyster species, *Pinctada maxima*, *P. margaritifera* and *P. fucata*, support the majority of commercial marine cultured pearl production in a number of countries (Gervis and Sims, 1992; Martínez-Fernández et al., 2003). Until recently, the principal pearl producing countries were Japan, French Polynesia, Australia and China (Fassler, 1997). However, countries such as Indonesia and the Philippines have made significant contributions to world pearl production in recent years. Australian pearl production is valued at around AUS$200 million per annum and it is the second most valuable aquaculture industry in the country (Tisdell and Poirine, 2000; O'Sullivan and Savage, 2003).

This chapter deals briefly with general aspects of the biology of pearl oysters and factors associated with broodstock management and egg quality, pertinent to this study.

1.1 Pearl production

Modern pearl culture began in the early 1900s in Japan with the Akoya pearl oyster, *Pinctada fucata*. Japan controlled the entire pearl industry for most of the 20th century (culture and marketing) even after pearl grafting techniques were made available to other countries after the Second World War (Cahn, 1949). Grafting techniques remained secret thanks to the Japanese 'diamond policy' which stated that pearl culturing techniques were to remain secret to all but Japanese; that all pearl production was to be exported to Japan; and that production objectives were to be

controlled and regulated to safeguard home pearl production (George, 1978 cited by Gervis and Sims, 1992). Nowadays the same pearl production technique with few (if any) variations is still used.

Most cultured round pearls are produced by implanting a round nucleus (usually made from the shells of freshwater mussels) and a piece of mantle tissue or 'saibo' from a sacrificed donor pearl oyster into the gonad of a recipient (host) pearl oyster. Between 20 and 80 pieces of saibo tissue (3 mm square) are usually obtained from each donor oyster, depending on the species (size) of oyster used and the perogative of the grafting technician. On this basis, a pearl farm grafting 100,000 oysters per year must sacrifice up to 5,000 individuals to provide saibo tissue.

Pearl oysters are removed from the water and placed into trays (usually lying on their hinge) to induce them to open their shells. Oysters to be used as mantle tissue donors are carefully selected on the basis of shell coloration (as this is an indication of the resulting pearl color), shape and general fitness and they are then sacrificed to obtain a strip of mantle tissue. This strip of mantle is cut from the outer (or ventral) edge (section 1.2.3) and is placed on a wooden or plastic board with the shell secreting epithelial cells facing upwards (this varies with the technician preferences). The marginal zone (external, middle and internal lobes) is removed and finally the strip is cut into small squares of approximately 3 mm (Kafuku and Ikenoue, 1983; Wada, 1991; Gervis and Sims, 1992).

Once grafting materials (nuclei and mantle tissue) are ready a recipient pearl oyster is placed on a stand. Mantle and gills are gently pushed aside to allow a clear view of the gonad and the grafting site. An incision is made in the gonad close to the foot and a channel is cut inside the gonad through which a square of mantle tissue and a nucleus are inserted. Once the grafting operation has finished, oysters are returned to

culture conditions. The mantle tissue graft will heal and grow within the gonad, to surround the nucleus. After a few days it begins to secrete shell material on top of the nucleus (Kafuku and Ikenoue, 1983; Wada, 1991; Gervis and Sims, 1992).

There are five major factors determining pearl quality: lustre, colour, shape, size and surface texture. These factors themselves are influenced by both environmental and biological parameters including the physiology of the recipient pearl oyster (e.g. body nutrient and energy management), food availability, water temperature and husbandry practices (e.g. culture depth, stocking density). The quality of the mantle tissue graft is perhaps the most important factors influencing pearl quality. The selection process for donors is therefore a key step in the grafting process (Taylor, 2002). The value of entire pearl crops will depend of the quality of the selected mantle donors.

Some donor oysters provide mantle tissue that produces excellent quality pearls. If adequate records were kept, each pearl could be traced back to the oyster that provided the saibo tissue for its production, and it would be possible to identify individual donor oysters that produced only high quality pearls. It would be desirable to breed these high quality oysters to obtain high proportions of juveniles with similar characteristics, which would increase the proportion of high quality pearls. However, under normal circumstances this is not possible because donor oysters are sacrificed when their mantle tissue is excised. In this regard, the cultured pearl industry is perhaps the only livestock industry that sacrifices the best individuals instead of using them as breeding stock. The artificial breeding of such oysters would provide a means of obtaining high quality pearl-producing progeny. If mantle tissue could be removed without killing the oyster, then donors producing high quality pearls could be used as future broodstock. A potential problem with this approach is that the mantle tissue of

live oysters contracts when touched and this makes excision of mantle tissue from live oysters extremely difficult.

1.2 General biology

1.2.1 Distribution and taxonomy

The blacklip pearl oyster, *Pinctada margaritifera*, is mainly distributed in the western Pacific Ocean, from French Polynesia to the Cook Islands and along the northern coast of Australia (Gervis and Sims, 1992) although populations of this species can be found as far west as India, the eastern coast of Africa and the Mediterranean Sea (Shirai, 1994). The Akoya pearl oyster, *P. fucata*, has a truly Indo-Pacific distribution, from Korea and southern China to the southeast coast of Australia, as well as in the Indian Ocean and the Red Sea (Shirai, 1994). These two species are taxonomically classified as follows:

Phyllum: Mollusca

Class: Bivalvia (=Lamellibranchia)

Order: Pterioida

Family: Pteriidae

Genus: *Pinctada*

Species: *Pinctada margaritifera* (Linnaeus, 1758)

Pinctada fucata (Gould, 1850) (=*P. imbricata*, *P. radiata*)

There has been some conjecture about the taxonomic classification of *P. fucata*, which is frequently referred to as *P. imbricata* or *P. radiata* (Colgan and Ponder, 2002). Despite some studies and taxonomic reviews, this confusion remains. *P. fucata* (not *P. imbricata*) was recognised in a major recent review of Australian bivalve molluscs (Lamprell and Healy, 1998) and, on this basis *P. fucata* is used throughout this study.

1.2.2 External Anatomy

Only organs and structures of importance to this thesis are described in this section. The shell is composed of two valves joined by an elastic ligament or hinge. Shell valves are composed of three layers: the first and outermost or periostracum is formed by conchiolin. The second is the middle or prismatic layer formed by calcite crystals. The third layer is the internal nacre layer composed of aragonite crystals joined in a protein matrix. The hinge corresponds to the dorsal side of the animal and the shell opening to the ventral side. The byssal notch identifies the anterior side (Fig. 1.1) (Fougerouse-Tsing and Herbaut, 1994).

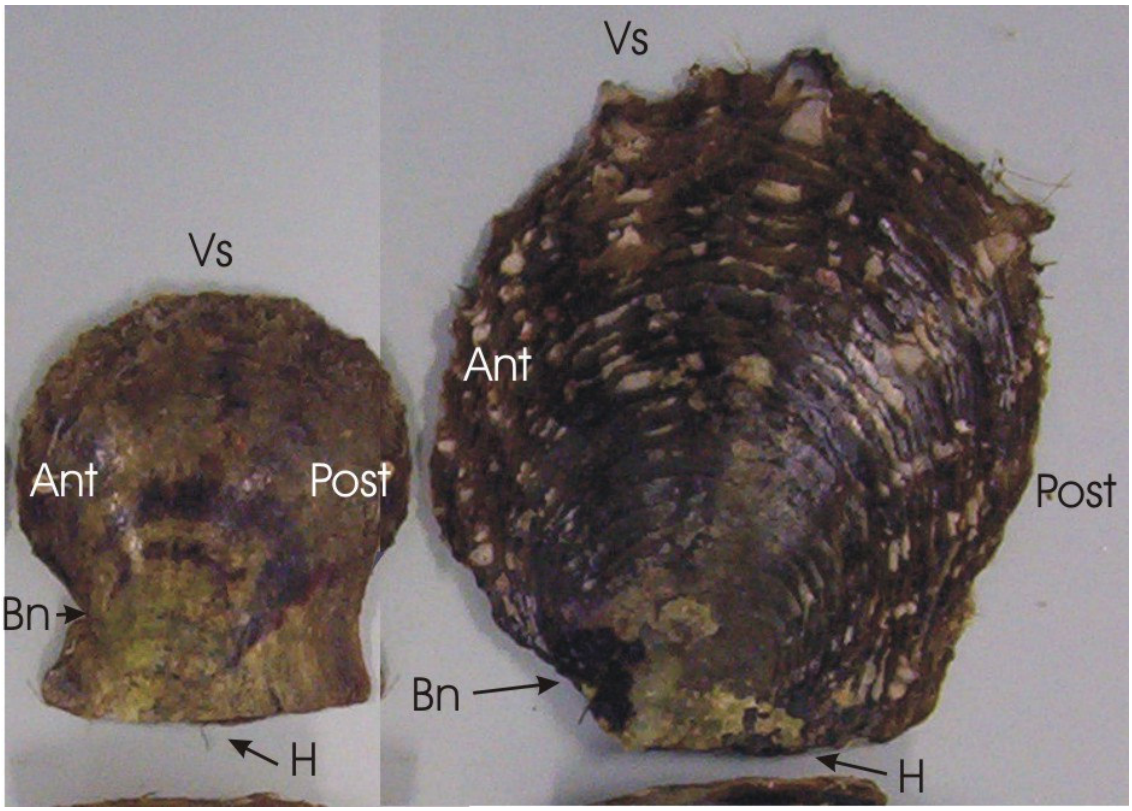

Fig. 1.1. Orientation of *P. fucata* (left) and *P. margaritifera* (right) shells showing the anterior side (Ant), byssal notch (Bn), hinge (H), posterior side (Post) and ventral side (Vs).

1.2.3 Internal anatomy

By removing one of the valves, three main structures can be observed: the mantle, the gills and the byssus. After removing the mantle tissue it is possible to observe the following major tissue components: adductor muscle, gonad (visceral mass) and foot (Figs. 1.2 and 1.3).

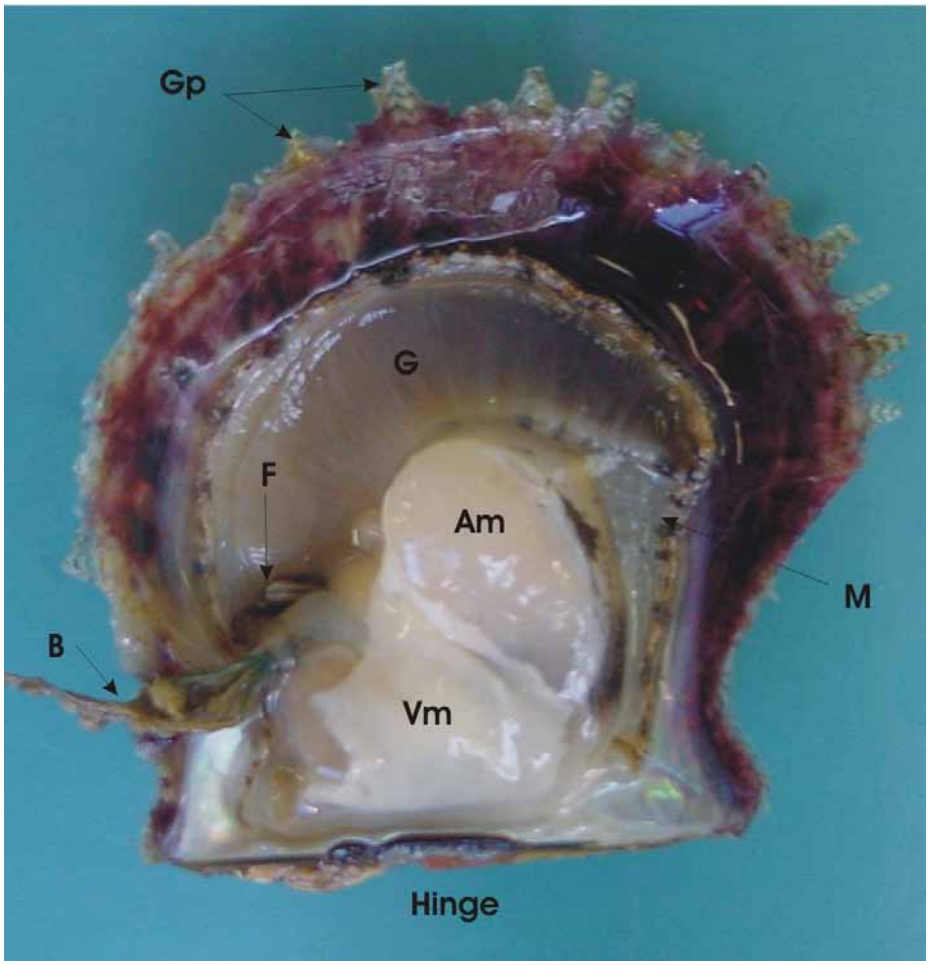

Fig. 1.2. Internal anatomy of *P. fucata* with the right shell valve removed showing the adductor muscle (Am), byssus (B), foot (F), gills (G), growth processes (Gp), mantle (M) and visceral mass (Vm).

1.2.3.1 The mantle

Lining the inside of the shell valves, the mantle encloses the visceral mass and secretes the shell. It is divided in four main zones (Fig. 1.3):

- The outermost or marginal zone that contains three lobes and the periostracal groove.
- The pallial zone, characterised by well-defined muscular threads, the pallial nerve and the pallial artery. This zone is the preferred tissue to obtain saibo for pearl seeding operations.
- The central zone covering the soft tissues, and
- The isthmus on the internal side of the hinge.

As well as shell secretion, mantle tissue may also have an important role in nutrient storage for some bivalves (section 1.5)

1.2.3.2 The gonad

The gonad is absent as an anatomically discrete organ in pearl oysters (Saucedo et al., 2002b). When the gonad is spent or in indifferent stages it is of a translucid colour and the intestine, digestive gland and retractor muscles can be seen through the gonad wall. Once the gonad develops as part of the visceral mass it assumes a white colour and internal organs are hidden by the intermingling developing gametes (Fougerouse-Tsing and Herbaut, 1994; Saucedo et al., 2002b).

The gonad requires a great amount of energy and nutrients during gametogenesis. For many bivalve species such nutrients are mobilised from stores in the adductor muscle, digestive gland and mantle (section 1.5).

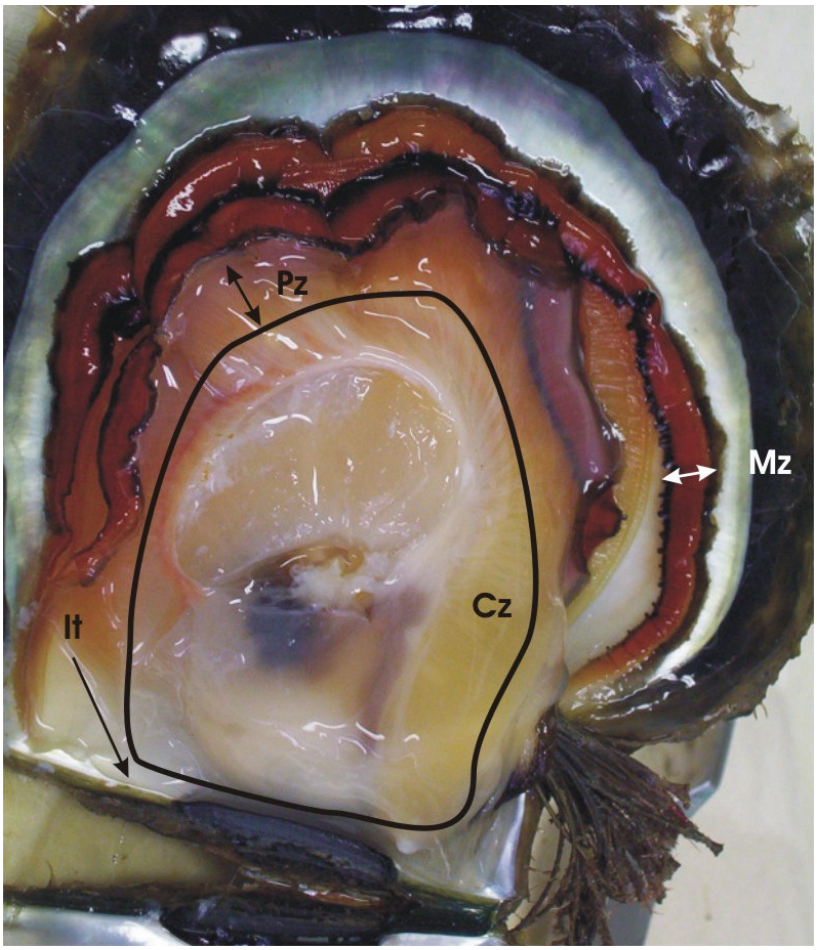

Fig. 1.3. Photograph of *P. margaritifera* with the left shell valve removed to show the central zone (Cz), marginal zone (Mz), pallial zone (Pz) and the isthmus (It) of the mantle tissue.

1.2.3.3 The adductor muscle

A single well-developed adductor muscle controls valve opening in pearl oysters. The adductor muscle is one of the major sites of nutrient storage for gonad development in bivalves (section 1.5)

1.2.3.4 The digestive gland

The digestive gland is located in the dorsal region of the visceral mass and is surrounded by the gonad. This organ also plays an important role in the nutrient mobilisation during gamete development (section 1.5)

1.2.4 Life cycle and reproduction

Reproductive biology of cultured and wild pearl oysters has been studied in all the commercially important species (Tranter, 1958a, b, c, d, 1959; Rose et al., 1990; Behzadi et al., 1997; Saucedo and Monteforte, 1997; Saucedo et al., 2002a, b; O'Connor and Lawler, 2004).

Pearl oysters are dioecious. The gonad surrounds the digestive gland and intestines. In pearl oysters protandry is observed after oysters reach a particular age or shell height (Gervis and Sims, 1992). Sexual maturity is usually reached at the age of approximately one year (Wada, 1991). Spawning season for *P. margaritifera* in Australia falls predominantly in summer and winter but may continue, at a reduced intensity during the rest of the year (Tranter, 1958d). Similar to *P. margaritifera*, the spawning season for *P. fucata* is relatively constant throughout the year and shows two periods of maximum intensity (summer and autumn) (Tranter, 1959).

External fertilization takes place after spawning. Egg size ranges between 40 and 50 μm at the moment of release. Five hours after fertilisation the gastrula first appear and 4-8 h later the trochophore develops. First D-stage veliger larvae (65-85 μm) appear between 20 and 24 h after fertilisation (Doroudi and Southgate, 2003). Embryo development up to D-stage is dependent on endogenous energy reserves originally contained within the egg. D-stage larvae start feeding at approximately 24 h after fertilisation. The ciliated velum of the veliger is the main structure for locomotion and

feeding. Later larval development stages include the umbo veliger (100-140 μm) and eye-spot or 'eyed' veliger larvae (210 μm) that appear after 11 and 20 days, respectively. After approximately 3-4 weeks pediveliger larvae (220-260 μm) begin crawling and searching for a suitable substrate to settle. Approximately 28-34 days after spawning metamorphosis occurs where the velum reabsorbs and post-larvae develop gills and mantle (Fig. 1.4).

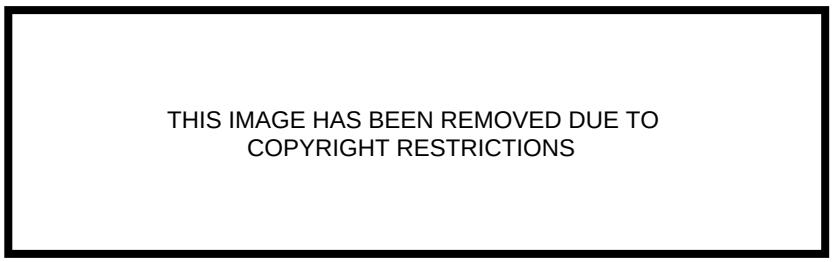

Fig. 1.4. Generalised life cycle of cultured pearl oysters (modified from Southgate and Lucas, 2003).

1.3 Physiological factors influencing gametogenesis

Gonad development has been used as a means of evaluating physiological and/or biochemical responses of bivalves under thermal and nutritional stress (Bayne, 1975). For example, gamete resorption in the mussel, *Mytilus edulis*, occurred when stress (increased water temperature) was applied at a late stage of gonad development while early development of the gonads continued in spite of a fall in body weight (Gabbott and Bayne, 1973). Also, lower lipid, protein and carbohydrate content have been observed in eggs of stressed *M. edulis* compared to those from non-stressed females (Bayne, 1975; Bayne et al., 1978) demonstrating the influence of this factor on reproductive output.

Nutritive stress significantly affects reproductive performance of bivalves, and in adult *M. edulis* had a maximum effect on early embryonic development and a minimum effect on the main larval growth phase (Bayne et al., 1975). This is explained by the fact that embryogenesis is a stage of intense morphogenetic activity at the expense of stored reserves; however, in the following stages (larval growth) there is less reliance in stored energy as larvae feed exogenously.

The effect of different temperature regimes on gonad conditioning of the pearl oyster *P. mazatlanica* was studied under laboratory conditions (Saucedo et al., 2001a). Although differences in gamete development and condition indices were observed in the different treatments, the overall results showed experimental conditions reduced general oyster health. Temperature had an effect on gamete development. However, as suggested previously, as a result of nutritional stress (low ration or poor diet quality) gamete development probably occurred at the expense of previously stored nutrients. There are no other studies about factors affecting gamete development in pearl oysters.

1.4 Broodstock management and egg quality

Pearl oyster broodstock are adults selected for particular traits such as high growth rate, nacre coloration, shape or fecundity. They are used as a source of gametes with the aim of producing high quality progeny. The quality of the progeny obtained will greatly depend on the selection of appropriate broodstock oysters. Broodstock are one of the most valuable assets of any pearl culture venture. Broodstock must provide eggs and larvae with all nutrients required for successful development to the exogenous feeding stage (~ 24 h post-fertilization). Oysters use their own reserves and nutrients derived from their diet to provide the gametes (especially oocytes) with the optimum nutrient profile even in the absence of an external source of food (Gabbott and Bayne, 1973; Bayne, 1975; Bayne et al., 1978). In general, bivalves store nutrients in the adductor muscle, mantle and digestive gland and mobilise these reserves to fuel gonad development during the early stages of the breeding season (Barber and Blake, 1981; Pazos et al., 1996, 1997; Saucedo et al., 2002a; sections 1.3 and 1.5).

Reproduction in marine bivalves has been the focus of much research and many studies have reported on breeding cycles (Tranter, 1958a, b, c, d, 1959; Lango-Reynoso et al., 2000), gonad development (Gabbott and Bayne, 1973; Helm et al., 1973; Mann, 1979; Utting and Doyou, 1992; Heasman et al., 1996; Soudant et al., 1997; Navarro et al., 2000), larval development (Kraeuter et al., 1982; Gallager and Mann, 1986; Gallager et al., 1986) and biochemical changes associated with reproduction (Bayne et al., 1975; Bayne et al., 1978; Whyte et al., 1987, 1989, 1990, 1991). There are two general ways of obtaining gametes and larvae for commercial operations and research activities. The most widely used method is to collect ripe oysters from the wild during the main spawning season and induce spawning. The second is to obtain oysters at any other gametogenic stage and culture them under specific conditions (e.g. water

temperature, food ration and quality) to induce gonad ripening, a process known as broodstock 'conditioning'.

Studies on the reproductive biology of bivalve broodstock have focused mainly on mussels (Gabbott and Bayne, 1973; Bayne et al., 1975; Bayne et al., 1978), oysters and clams (Helm et al., 1973; Mann, 1979; Gallager and Mann, 1986; Gallager et al., 1986; Utting and Doyou, 1992; Lango-Reynoso et al., 2000) and scallops (Kraeuter et al., 1982; Whyte et al., 1987, 1989, 1990, 1991; Heasman et al., 1996; Soudant et al., 1997; Navarro et al., 2000). Regarding pearl oysters, the past decade has seen increasing reliance by cultured pearl industries on hatchery-cultured spat (Gervis and Sims, 1992; Southgate and Beer, 1997). However, there is little published information relating to the quality of pearl oyster broodstock and that of gametes and larvae.

1.5 Biochemical changes associated with gonad development

There have been a number of studies on nutrient storage and mobilisation associated to gametogenic cycles in bivalves (e.g. Mathieu and Lubet, 1993; Pazos et al., 1997; Lodeiros et al., 2001; Saucedo et al., 2002a). Body organs such as the adductor muscle, digestive gland and mantle play an important role in storage and transfer of nutrients to the gonad at different stages of the gametogenic cycle. In the scallop, *Argopecten irradians concentricus*, for example, the adductor muscle was the major storage site for protein and glycogen, while the digestive gland stored lipid and the mantle tissue was composed mainly of proteins (Barber and Blake, 1981).

A similar pattern of nutrient management was observed in the pearl oyster, *Pinctada mazatlanica*. Proteins were stored in the adductor muscle and were translocated to the gonad during gametogenesis while lipid content of the digestive gland showed a close relationship to gonad development (Saucedo et al., 2002a).

1.6 Nutritional factors influencing egg quality

1.6.1 Major nutrients

The importance of lipids on egg quality has been widely documented for a number of bivalve species. For example, a direct relationship was found between initial larval lipid content (specifically neutral lipids) at the time of release and maximal viability and yield of cultured European oyster, *O. edulis*, larvae (Helm et al., 1973; Holland and Spencer, 1973). A minimum amount of lipid must be provided in the eggs for embryos to achieve successful development into first-feeding D-stage veliger larvae. For example, about 20% of the neutral lipid content of the eggs was consumed during embryo development of the blue mussel *M. edulis* (Bayne et al., 1975, 1978) and good survival of the clam, *Mercenaria mercenaria*, from egg to pediveliger was not obtained unless egg lipid content exceeded 18% (Gallager and Mann, 1986; Gallager et al., 1986).

Nutrient content is also related to egg size (Gallager et al., 1986) and this has been shown to influence larval survival in a number of species. For example, a correlation between egg size and subsequent 48 h larval survival was observed in the scallop, *Argopecten irradians*, and the clam, *Mercenaria mercenaria*, (Kraeuter et al., 1982). This may be explained by the geometric relationship of an oocyte (sphere) where a small decrease in diameter brings about a significant decrease in volume (nutrient quantity). Although nutrient proportion is maintained, lower amounts of each component are included in each oocyte, which results in a smaller egg, with a limited amount of available energy (Bayne et al., 1975, 1978).

The importance of proteins as a means of determining bivalve egg quality has been suggested for a number of species. For example, in the scallop, *Patinopecten*

yessoensis, and the oyster, *O. edulis*, egg protein was catabolized during embryogenesis at a similar rate and provided almost the same amount of energy as lipids (Helm et al., 1973; Holland and Spencer, 1973; Whyte et al., 1991).

1.6.2 Energy

Lipids and protein are the main nutrients providing energy for embryogenesis in bivalve species. For example, total egg energy expended during embryonic development of the scallops, *Crassadoma gigantea* and *Patinopecten yessoensis*, were 11.8 and 10.2 kJ g^{-1}, respectively. Lipid and protein contributed approximately 47% and 44% of the expended energy, respectively (Whyte et al., 1990, 1991).

There are no studies on energy requirements during pearl oyster embryo development. However, a related study showed that early *P. margaritifera* larvae consumed 56% and 40% of their lipid and protein content between days 1 and 4 after fertilisation (Strugnell and Southgate, 2003). Even when larvae had begun feeding, overall nutrient loses were still recorded indicating that larvae still relied to some extent on parentally derived nutrients at this stage.

1.6.3 Micro-nutrients

Micronutrients are essential components present in small quantities in eggs. Successful embryo development depends on the particular amount and ratios of each micronutrient.

1.6.3.1 Fatty acids

Fatty acid content is an important factor in defining egg quality in bivalves. For example, edible oysters, *Crassostrea* spp., were found to assimilate dietary fatty acids

into tissue lipids prior to or during oogenesis (Chu, 1996). To achieve reproductive success eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), together with other fatty acids, must be supplied by the diet, as oysters cannot elongate them from shorter chain fatty acids (Chu, 1996). Similarly, seasonal variation in the total n-3 fatty acids in the gonad of *P. maximus* was observed to correlate with its reproductive cycle (Pazos et al., 1997).

Fatty acids may play different roles in embryo development. For example, a linear rate of catabolism of EPA and a constant level of DHA were observed during embryonic development of the scallops, *Crassadoma gigantea* (Whyte et al., 1990, 1991) and *Pecten maximus* (Soudant et al., 2000). This indicated that EPA played an energy-provider role, while DHA played a structural role. In a similar study, gametogenic, embryonic and metamorphosis impairments in *P. maximus* were found to be caused by a deficiency in dietary DHA (Soudant et al., 2000).

1.6.3.2 Carotenoids and vitamins

Carotenoids and vitamins are micronutrients whose nutritional roles in broodstock diets and during larval development have recently been studied in some invertebrates. It is now generally accepted that they play an important role in biological defence and reproduction in sea urchins (e.g. Kawakami et al., 1998; Matsuno and Tsushima, 2001). In eggs of the sea urchin, *Strongylocentrotus intermedius*, the content of the pigment echinenone dropped by 50% of its initial content in the first 20 h of embryo development (Vershinin and Lukyanova, 1993). This indicates that echinenone, a carotenoid, plays an antioxidative role as it is during the early stages of development when the intensity of oxygen metabolism is higher (Vershinin and Lukyanova, 1993).

Artificial diets enriched with β-carotene have been shown to increase gonad growth and were proposed as useful for gonad conditioning in the sea urchin, *S. droebachensis* (DeJong-Westman et al., 1995a,b). In another species of sea urchin, *Pseudocentrotus depressus*, broodstock diets containing astaxanthin, β-echinenone, β,β-carotene, vitamin A and vitamin E were studied (Tsushima et al., 1997). β-echinenone and vitamin E had a significant effect on the number of ovulated eggs; the lowest malformation rates and best survival were obtained with diets containing β-echinenone and β,β-carotene while vitamin E had no effect on larval survival. β-echinenone in particular was concluded as exerting a major influence on embryo development

Dietary carotenoids have also been found to play an important role in the reproduction of the sea urchin, *Lytechinus variegatus*, (George et al., 2001). The level of carotenoids in diets fed to adult urchins had an effect on the number of spawned eggs, larval size and survival and juvenile production; best results were obtained by feeding the xanthophylls, lutein and zeaxanthin.

No similar research has been done with pearl oysters; however, given the high pigment content of microalgal species (the main source of nutrients for pearl oysters), these nutrients may play an important role defining egg quality and successful embryo development in pearl oysters.

1.7 Broodstock selection

Broodstock management practices are based on selecting the best traits or characteristics of the parents, which will hopefully be passed to the progeny. Selection for nacre colour, shell form and shape are some important traits used for selecting pearl oyster broodstock (Wada, 1993). However, selection for nacre colour is made on the

basis of inspecting the coloration of the internal or external faces of the shell itself and not the pearls produced (section 1.1). As such it is an inaccurate means of assessing potential broodstock quality. There is currently no means of selecting pearl oyster broodstock based on the pearl quality produced by individual mantle donors.

In Japan, white shell coloured pearl oysters were selected as broodstock to obtain donor oysters, as these produced higher proportions of desirable white coloured pearls (Wada, 1986). Three groups of *P. fucata martensi* (Dunker) donor oysters were obtained from different crosses between oysters with white and brown coloured shells (e.g white:white, white:brown and brown:white). Mantle donor oysters from the white:white coloured breedings group yielded a higher proportion of non-yellow (white) pearls (more valuable) compared to donors with one brown coloured parent (Wada & Komaru, 1996). Furthermore, in the silver- or gold-lip pearl oyster, *P. maxima*, silver nacre donors have been shown to produce mostly silver coloured pearls while golden nacre donors produced mainly low-value creamy-yellow coloured pearls; however, some golden-nacre *P. maxima* produced only high-value gold pearls (Taylor, 2002).

1.8 Anaesthetics

In general, the use of anaesthetics to handle broodstock is commonly applied in fish culture industries, as fish are more susceptible to stress caused by handling, crowding or transportation. In contrast, relaxing chemicals are seldom used for bivalve broodstock management, as most bivalves can withstand prolonged periods of desiccation if adequate conditions of temperature and humidity are provided. However, a number of chemicals (e.g. benzocaine, propylene phenoxetol or tricaine methanesulfonate) have been used to relax pearl oysters and facilitate internal

inspection and grafting operations (Norton et al., 1996, 2000; Acosta-Salmón and Rangel-Dávalos, 1997; Saucedo et al., 2001b). Despite these studies, the pearl industry is still to embrace the use of relaxing chemicals in the pearl production process arguing several reasons including mantle collapse, oyster 'weakness', lower weight of pearls (though no difference in quality) and mucus production (Norton et al., 1996, 2000; Acosta-Salmón and Rangel-Dávalos, 1997; O'Connor and Lawler, 2003). The rate of success using the current grafting technique seems to be acceptable for pearl farms and the potential benefits of using relaxants may not compensate for the increased labour, costs (time) or logistic disadvantages involved.

Anaesthetics may also be used to obtain gonad, mantle or other organ samples for genetic or histologic examination with the same objective of reducing stress and damage as well as making the sample taking more efficient if the oyster is relaxed and not opposing such procedure.

1.9 Major objectives of this study

Though pearl oyster culture dates from the late 1800s, most research with them has been carried out during the past 20 years. Topics such as reproductive biology, larval production and grow-out (nursery and pearl) culture have received special attention and nowadays are some of the most advanced topics in pearl oyster research. However, other areas of importance such as broodstock management and pearl quality have received relatively little research attention. In general pearl production processes have hardly changed since its early beginnings and the original techniques are still widely used.

There are no studies on the utilisation of micronutrients (fatty acids, carotenoids or vitamins) by pearl oyster larvae. It is important to determine which compounds are

involved in providing energy to the developing embryo until it reaches the exogenous feeding stage and to understand which compounds determine egg quality in pearl oysters. In turn this would reflect important nutrients for broodstock diets.

The specific aims of this study were:

- To identify suitable relaxants and concentrations to allow internal inspections, sex determination and excision of mantle tissue (saibo) without sacrificing the oysters (Chapter 3).
- To evaluate a biopsy technique as a potential non-destructive means of obtaining gonad tissue from *P. margaritifera* to assess gonad condition (Chapter 4).
- To assess the feasibility of keeping donor pearl oysters alive as potential broodstock by assessing oyster mortality following removal of mantle and their ability to repair and regenerate mantle tissue following this procedure (Chapter 5 and 6).
- To determine biochemical changes and energy storage in different body tissues in relation to the breeding season (Chapter 7).
- To analyse micronutrient changes during embryogenesis of *P. margaritifera* to identify nutrients with particular influence on egg quality (Chapter 8).

Results from this study will provide important new information related to broodstock management and egg quality in pearl oysters and will help to improve current pearl oyster broodstock management techniques. Important nutritional elements affecting egg quality will also be identified, for the first time, for pearl oysters, and will help in the further development of both, broodstock conditioning and larval rearing techniques.

Chapter 2

General methods

2.1 Research sites

Experiments were carried out at three sites. Field experiments were conducted at either James Cook University's Orpheus Island Research Station (OIRS) (18°35' S, 146°29' E) (Figs. 2.1 and 2.2) or at Horseshoe Bay, Magnetic Island (19°12' S, 146°52' E) (Figs. 2.1 and 2.3). Laboratory experiments were conducted at the Aquarium Complex of the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University's main campus in Townsville, north Queensland, Australia.

North Queensland is located in tropical Australia and experiences a characteristic weather pattern with marked dry (winter) and wet (summer) seasons. Seawater temperature off Townsville ranges between 22°C in August and 30°C in February (Beer and Southgate, 2000). Surface salinity ranges from 27 ‰ in March to 36‰ in August (Beer and Southgate, 2000) but remains constant at 3-4 m depth (35-36‰) (Pit, 1998).

2.2 Oyster stock

Individuals of *Pinctada margaritifera* (Fig. 2.4) used in this study were either hatchery produced or collected as adults from the wild. The *P. fucata* (Fig. 2.4) used in this study were hatchery produced. All oysters were maintained in panel (pocket) nets (Taylor et al., 1998; Southgate and Beer, 2000) on a 120 m length, long-line (Gervis and Sims, 1992) at OIRS, and were transferred to the long-line (75 m length) at Horseshoe Bay when required for experiments. Long-lines consisted of a 25 mm 'mainline' maintained in suspension by plastic floats positioned at 2 m intervals. Long-

lines were anchored with 3 concrete anchors at each end. ‘Dropper’ lines attached to the mainline at 1 m intervals were used to attach culture nets. Before experimental work, oysters were transferred from the long-lines to the adjacent laboratories where they were cleaned.

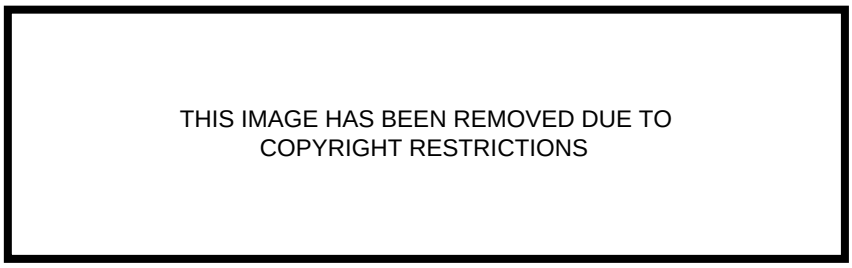

Fig. 2.1. Location of research sites used during this study. 1: Orpheus Island; 2: Magnetic Island.

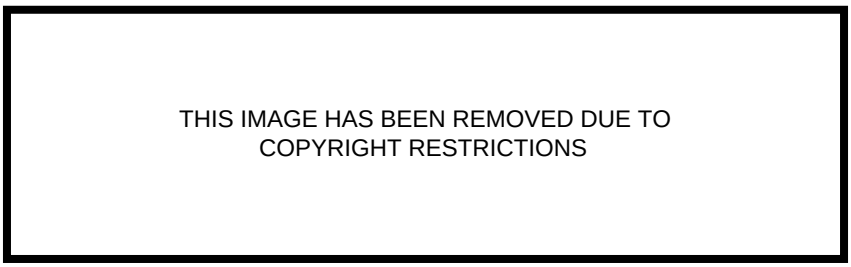

Fig. 2.2. Orpheus Island Research Station: Location of the culture long-line at Pioneer Bay, Orpheus Island (▲).

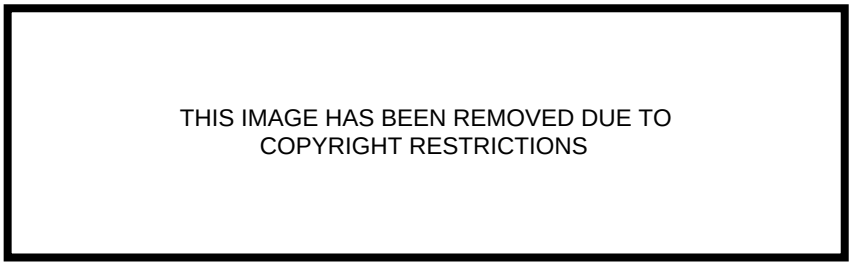

Fig. 2.3. Horseshoe Bay at Magnetic Island: Location of the culture long-line (▲).

Fig. 2.4. Pearl oyster species used for this study. A, blacklip pearl oyster *Pinctada margaritifera* (Linnaeus); B, Akoya pearl oyster *Pinctada fucata* (Gould). Photos not at scale.

2.3 Spawning and larval culture

Samples of eggs, embryos and larvae up to 48 h old were required for biochemical analysis during this study (Chapter 8). Oysters were transported from the culture long-line to the laboratory where they were cleaned of fouling organisms and sediment before spawning induction (Southgate and Beer, 1997). Two tanks filled with

1 µm filtered seawater at temperatures of 21 and 31°C were used for spawning induction. Oysters were kept at each temperature for 30 minutes at a time. Once oysters began to spawn they were separated into individual containers to identify sex and evaluate gamete viability. Eggs were pooled into a 20 L bucket and aliquots of sperm solution were used to fertilise the eggs. Fifteen minutes after fertilisation eggs were washed with filtered seawater on a 25 µm mesh sieve to remove debris and excess sperm. Eggs were dispensed at a density of 10 mL^{-1} into replicate 500 L fibreglass tanks containing gently aerated, 1 µm filtered seawater at 30°C. When required, samples were taken from the 500 L tanks by draining eggs and larvae onto a 25 µm mesh size sieve. Eggs and larvae were concentrated in a 1 L plastic measuring cylinder where 1 mL aliquots were obtained for counting. Once samples were obtained the remaining eggs/larvae were returned to the culture tank. Larval rearing was terminated 2 days after spawning, therefore food was never provided to larvae.

2.4 Measurements

Diameter was measured for eggs and embryos, as they are spherical. Larval length (antero-posterior measure) was obtained by measuring the longest axis parallel to the hinge. Shell height (dorso-ventral measure) was used to describe adult oysters size by recording the longest distance between the hinge and the ventral edge of the shells (Gervis and Sims, 1992) (Fig. 2.5). Counting of eggs and larvae was done using plastic Sedgewick-Rafter 1 mL counting chambers observing under a binocular microscope.

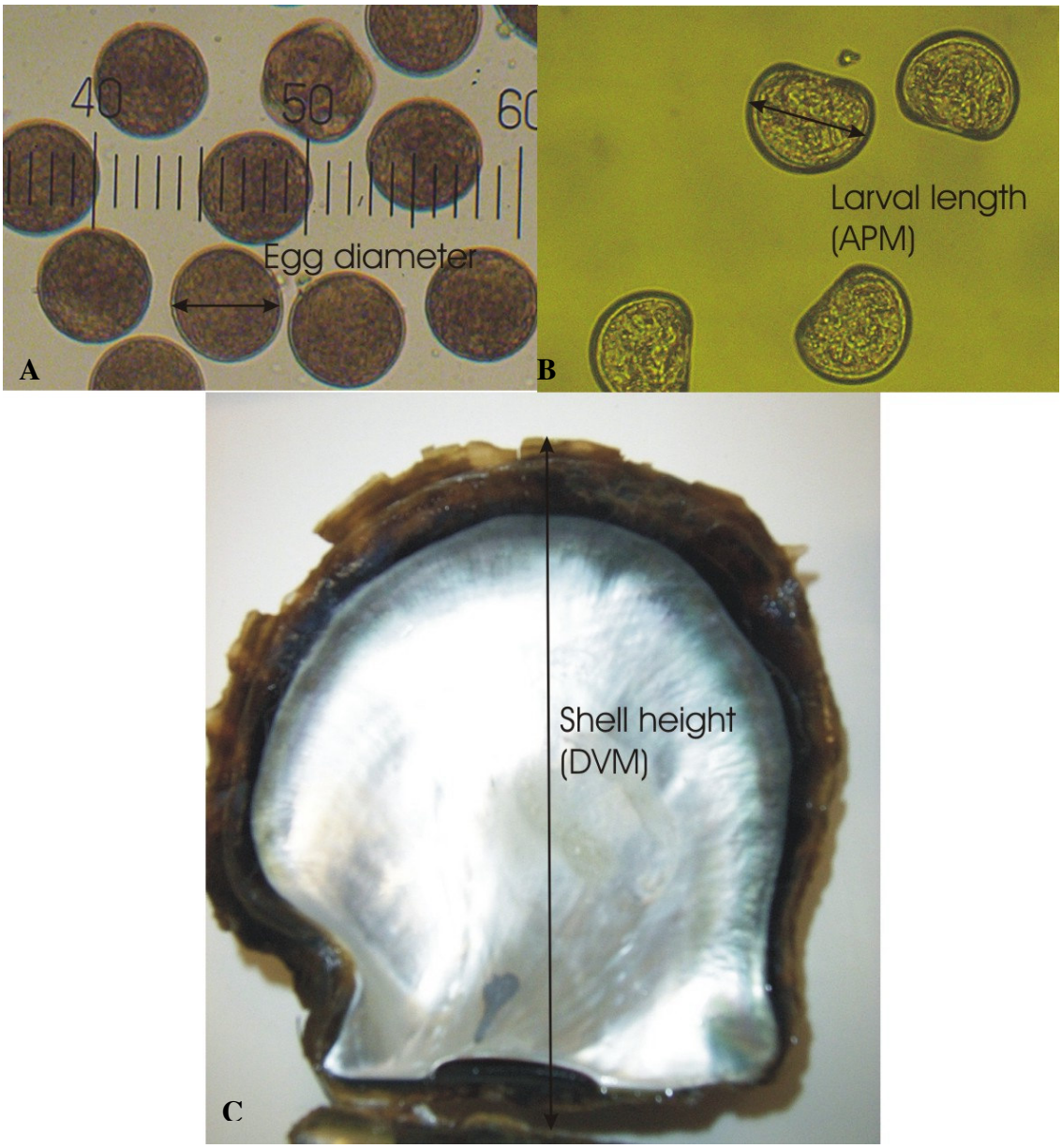

Fig. 2.5. Photograph of (A) pearl oyster eggs, (B) D-stage larvae and (C) adult shell. Arrows indicate measures taken for each development stage.

2.5 General histology

Histological analyses were conducted at the JCU Biological Sciences Histology Laboratory following standard techniques (Bradbury and Gordon, 1977; Culling et al., 1985). Gonad tissue was preserved with Formaldehyde-Acetic Acid-Calcium Carbonate (FAACC) gonad fixative; mantle tissue was fixed in 10% formaldehyde. Dehydration

of tissue samples was performed in an alcohol series dilution after which they were embedded in paraffin. All samples were sectioned to 5 μm using a manual rotary Leitz microtome. Tissue sections were stained with Mayer's Haematoxilin and Young's Eosin-Erythrosin. Microphotographs were obtained using an Olympus BH-12 microscope equipped with an Olympus DP12 digital camera. For detailed procedures for particular stains see Appendix 1.

2.6 General biochemical analyses

Analyses of dry weight, ash weight, protein, lipid and carbohydrate were conducted following methods described by Holland and Gabbott (1971) and Mann and Gallager (1985).

Samples were freeze dried. Each pre-dried and pre-weighed sample was placed in a labelled micro-centrifuge tube and homogenised with an IKA UltraTurrax T25 homogeniser with 1 mL of distilled water. For the lipid assay 300 μL of the homogenate was transferred to 5 mL polypropylene centrifuge tubes. Lipids were extracted in 1:2 v/v chloroform:methanol (Bligh and Dyer, 1959) followed by a second extraction in 2:1 v/v chloroform:methanol and purification by the addition of 0.7% w/v NaCl solution (Folch et al., 1957). Aliquots of lipid-containing chloroform were oven dried at 60°C until all chloroform evaporated. Concentrated sulphuric acid was added to the lipid residue and heated in a boiling water bath for 10 minutes. Lipid quantification followed the sulphophosphovanillin method (Barnes and Blackstock, 1973) using cholesterol (Ajax LabChem 1729-100G) as standard.

For carbohydrate and protein determinations, 500 μL of homogenate was transferred to 1.7 mL microcentrifuge tubes. Carbohydrates were extracted using 5%

w/v trichloroacetic acid and allowed to stand overnight. After centrifugation, carbohydrate-containing supernatant was removed for carbohydrate assay and the precipitate was used for protein analysis (Holland and Gabbot, 1971; Mann and Gallagher 1985). Carbohydrate content was quantified following the phenol-sulphuric acid method of Dubois et al., (1956) using D-glucose (AnalaR 10117) as standard. Protein-containing precipitate was dissolved in 0.1 N NaOH and heating at 60°C for 1 h (modified from Holland and Gabbott, 1971). Protein determination followed the method of Bradford (1976) using BioRad® protein assay dye reagent concentrate, using bovine serum albumin (Sigma A-7888) as standard. For detailed procedures of biochemical analyses see Appendix 2.

Chapter 3

Use of anaesthetics with pearl oysters

3.1 Introduction

Anaesthetics with pearl oysters may be used to facilitate internal inspection and grafting operations and when obtaining tissue samples from broodstock (Norton et al., 1996, 2000; Acosta-Salmón and Rangel-Dávalos, 1997; O'Connor and Lawler, 2003). It is important to work within safe limits for both oysters and the personnel involved in sample taking. Experimental work during this project required the relaxation of oysters on a continuous basis to obtain biopsy samples of gonad tissue for histological or biochemical analyses (Chapter 4) and to facilitate excision of mantle tissue (Chapter 5 and 6). Other uses included determination of oyster sex by gently puncturing the gonad with a sharp-ended Pasteur pipette while the oyster is relaxed (Chapter 7). An ideal relaxant for pearl oysters must meet particular requirements such as rapid and 100% effectiveness, fast and complete recovery as well as being non-toxic for both oyster and operator.

A number of chemicals (e.g. benzocaine and propylene phenoxetol) have been used to relax pearl oysters and appropriate concentrations have already been suggested. For example, Benzocaine at concentrations of 250 and 500 mg L^{-1} produced relaxation in the pearl oyster, *Pteria sterna* (Acosta-Salmon and Rangel-Davalos, 1997). A concentration of 1,200 mg L^{-1} of benzocaine was also successfully used to relax *P. albina* (Norton et al., 1996). Propylene phenoxetol at concentrations between 2 and 3 mL L^{-1} has been successfully used to relax *P. margaritifera*, *P. maxima* and *P. fucata* (Norton et al., 1996, 2000; Mills, 1997; O'Connor and Lawler, 2003). The aim of this Chapter was to follow-up previous research on this field and to evaluate the suitability

of oysters to function as saibo donors when exposed to such relaxants. A number of problems related to the use of anaesthesia with pearl oysters such as mantle collapse (Norton et al., 1996, 2000; O'Connor and Lawler, 2003), 'weakness' or body collapse (O'Connor and Lawler, 2003) and mucus production (Acosta-Salmón and Rangel-Dávalos, 1997) have previously been reported. Oysters showing these symptoms are unsuitable for pearl seeding and these characteristic responses to anaesthetics were used as evaluation criteria in this study. This Chapter assesses effects of anaesthetics on *Pinctada margaritifera* and *P. fucata* to determine the period when oysters can provide saibo before any of these problems appear.

3.2 Materials and methods

Pinctada margaritifera and *P. fucata* used in this study were maintained on the long-line at Magnetic Island (Chapter 2). Oysters were transferred from the long-line to the laboratory where they were cleaned prior to experiments.

Sixty oysters of each species (dorso-ventral measure (DVM ± SD) = 112.53 ± 10.98 mm and 79.1 ± 4.97 mm for *P. margaritifera* and *P. fucata*, respectively) were used to evaluate the effects of relaxants on the general conditions of oysters for grafting (whether or not the relaxed oysters could be used as saibo donors). Four groups of 15 oysters for each species were exposed to the following treatments:

1. Group 1, was exposed to 2.5 mL L^{-1} of propylene phenoxetol (Norton et al., 1996);
2. Group 2, was exposed to 250 mg L^{-1} of benzocaine (Acosta-Salmón & Rangel-Dávalos, 1997);
3. Group 3, was exposed to 500 mg L^{-1} of benzocaine (Acosta-Salmón & Rangel-Dávalos, 1997);

4. Group 4, was exposed to 1200 mg L^{-1} of benzocaine (Hahn, 1989; Norton et al., 1996).

The necessary amount of propylene phenoxetol was stirred with seawater in a small container to disperse the chemical into small droplets before adding to a larger container (Norton et al., 1996). To prepare the concentrations of 250 and 500 mg L^{-1}, a working solution 1:4 w/v of benzocaine-methanol was made previously (Acosta-Salmón & Rangel-Dávalos, 1997) then, the desired amount of this solution was poured into hot seawater (92-96 °C) to ensure that the benzocaine dissolved. This solution was then poured into a large container with seawater to reach the desired concentration. For the benzocaine concentration of 1200 mg L^{-1}, a solution in ethanol of 100 g L^{-1} was prepared previously and then slowly added directly to seawater (Hahn, 1989; Norton et al., 1996).

Four 20 L plastic aquaria containing 1 μm filtered seawater at 26°C with no aeration were used to test the different anaesthetics. Oysters were monitored for full relaxation by observing their responses when the mantle was touched. Oysters that did not respond to this stimulus were classified as relaxed. Evaluation criteria to identify particular stages observed during anaesthesia are shown in Table 3.1. After becoming relaxed, oysters were observed every 5 min for up to 1 h to evaluate the condition of the mantle and gills. At each observation, oysters were classified as either 'suitable-saibo' (SS) or 'non-suitable saibo' (NS); NS oysters were placed into clean seawater for recovery. To determine the longest time of exposure for which oysters remained suitable as saibo donors, SS oysters were returned to the relaxant up to a maximum time of 30 minutes. After treatments, oysters were returned to the long-line and survival was recorded 1 and 4 weeks after treatments. To identify differences between relaxants, a

one-way ANOVA was performed to determine whether there were differences between treatments for the time required for oysters to relax and the time they remained relaxed.

Table 3.1. Evaluation criteria to define stages of relaxation in oysters treated with different anaesthetics.

Stage	Shells	Body	Observations
Normal (not relaxed)	Narrow (enough to feed and breath)	Strong	With the exception of mantle and gills, no other organs can be seen
Relaxed	Wider Opened	Firm	All organs can be seen without difficulty
Mantle collapse		Feeble	Mantle falls away from the shells. Gills easily damaged with manipulation
Body collapse	Widest	Very Feeble	No muscular strength in mantle, adductor muscle or body

3.3 Results

There were some difficulties while preparing and using the relaxant chemicals in this experiment. Propylene phenoxetol was not easily dissolved and drops of the chemical remained undissolved for a long period of time. Preparation of 250 and 500 mg L^{-1} of benzocaine also required time for heating seawater and care had to be taken when dispensing the methanol-based solution into hot seawater as it evaporates immediately on contact with the hot seawater. The application of the ethanol-

benzocaine solution used to treat oysters in Group 4 (1200 mg L^{-1} of benzocaine) caused separation of the solution as ethanol dissolved in seawater and benzocaine crystallised and precipitated.

Survival of oysters was 100% after 4 weeks under culture conditions for all groups. Both *P. margaritifera* and *P. fucata* in Group 1 (propylene phenoxetol 2 mL L^{-1}), Group 3 (Benzocaine 500 mg L^{-1}) and Group 4 (Benzocaine 1200 mg L^{-1}) showed complete relaxation. Group 2 (Benzocaine 250 mg L^{-1}) oysters did not relax during the time of exposure since animals showed the valves widely open and closed them quickly when the mantle was touched.

Unfortunately, data for the effects of the different anaesthetics on *P. fucata* is incomplete. However, relaxation times for *P. fucata* are shown in Table 3.2.

Results for *P. margaritifera* in Groups 1 (2.5 mL L^{-1} propylene phenoxetol) and 3 (500 mg^{-1} L benzocaine) were similar. NS oysters appeared within 5 min of exposure to propylene phenoxetol (Fig. 3.1) and benzocaine (Fig. 3.2). Before 30 min of exposure, these groups showed no significant difference ($p<0.05$) between SS and NS oysters. Mean (± S.D.) time required to reach relaxation for *P. margaritifera* exposed to 2.5 mL L^{-1} propylene phenoxetol was 9.6 ± 4.3 min and for oysters exposed to 500 mg L^{-1} was 11 ± 7.3 min. The mean times (± S.D.) that oysters remained as suitable saibo were 14.8 ± 11.5 min and 17.8 ± 10.5 min for Group 1 and 3, respectively.

Benzocaine at a concentration of 1200 mg L^{-1} (Group 4) was the best treatment in terms of the minimum time required to reach relaxation as well as time the *P. margaritifera* remained relaxed. Benzocaine at a concentration of 1200 mg L^{-1} induced rapid relaxation and most oysters remained as SS for at least 15 min (Fig. 3.3). Mean relaxation time (± S.D.) for *P. margaritifera* exposed to 1200 mg L^{-1} benzocaine was

9.0 ± 4 min. Mean time (± S.D.) oysters remained as suitable saibo was 24.6 ± 9.7 min for Group 4.

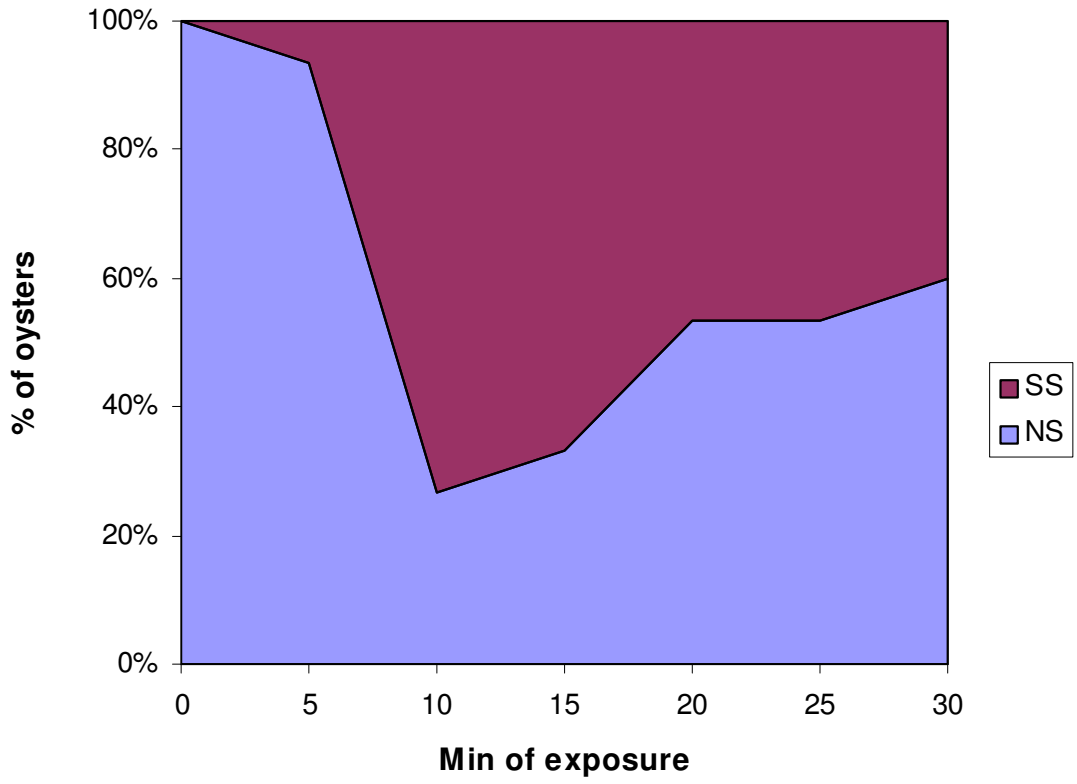

Fig. 3.1. Percentage of non-suitable saibo (NS) and suitable saibo (SS) *P. margaritifera* at different times after exposure to 2.5 mL L^{-1} propylene phenoxetol.

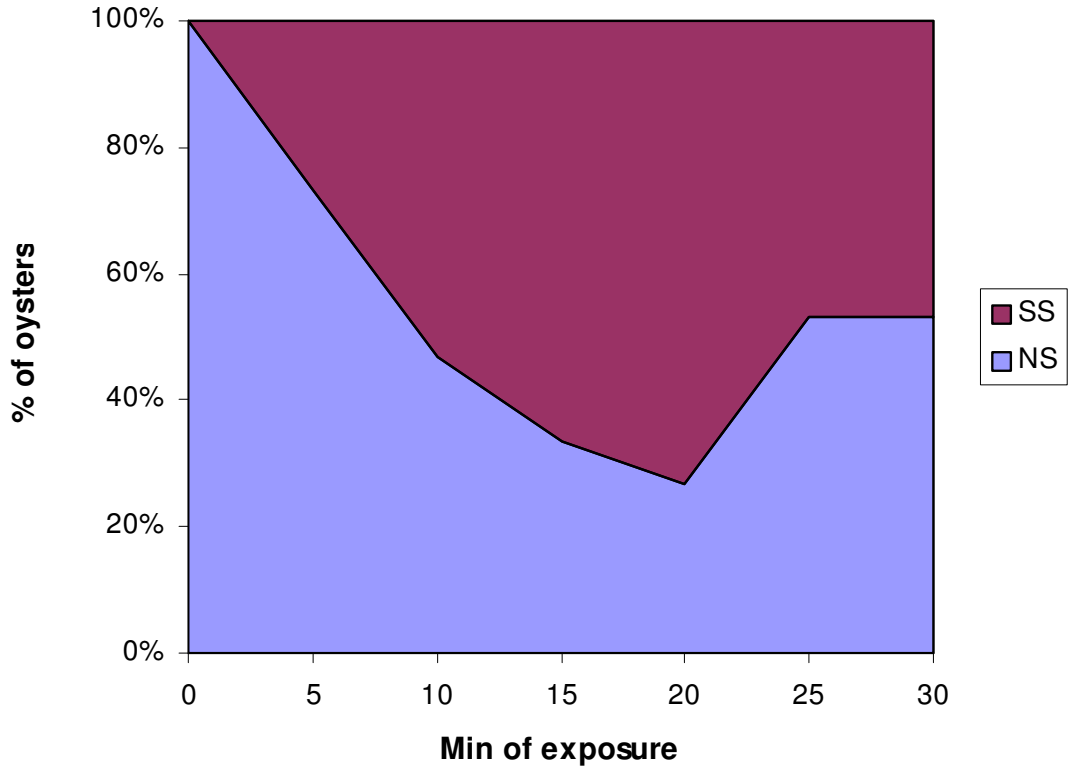

Fig. 3.2. Percentage of non-suitable saibo (NS) and suitable saibo (SS) *P. margaritifera* at different times after exposure to 500 mg L^{-1} benzocaine.

Table 3.2. Mean (± S.D.) times of relaxation and time *P. fucata* remained as suitable saibo when exposed to 2.5 mL L^{-1} propylene phenoxetol (PP), 500 (B500) and 1,200 (B1200) mg L^{-1} benzocaine. ND: Not determined due to technical difficulties.

	PP	B500	B1200
Time to relax	ND	ND	$10.27^{\pm 4.41}$
Suitable saibo	$9.67^{\pm 5.76}$	$21.5^{\pm 10.11}$	$16.75^{\pm 9.26}$

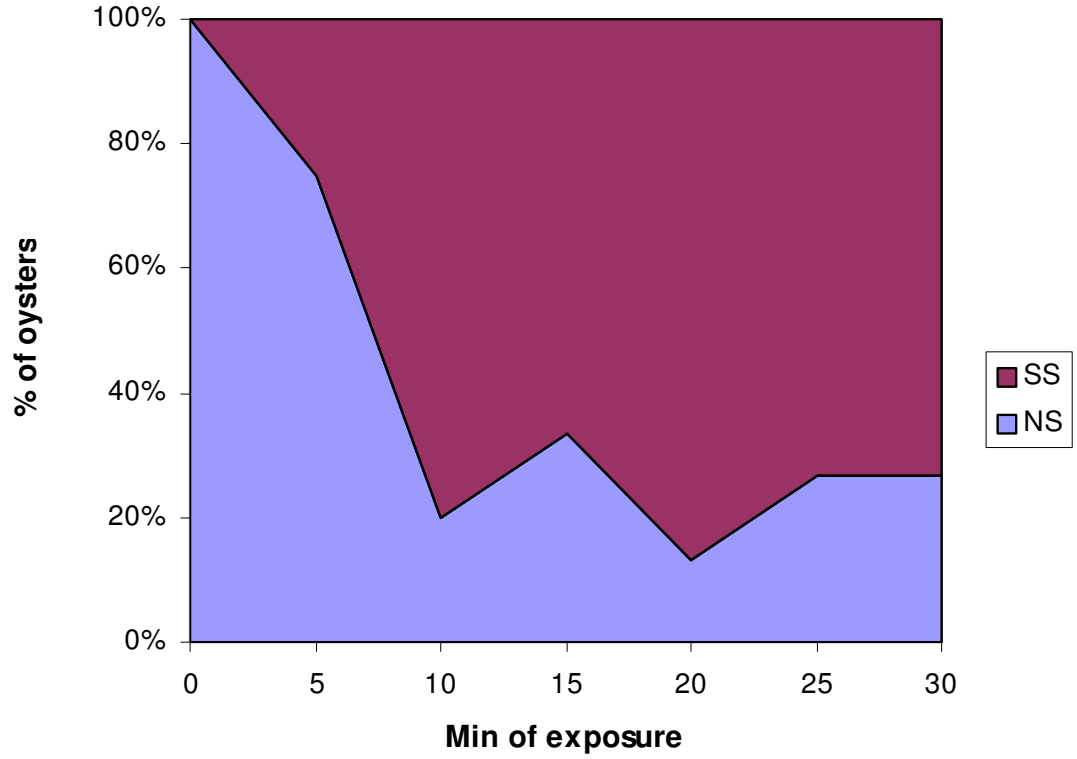

Fig. 3.3. Percentage of non-suitable saibo (NS) and suitable saibo (SS) *P. margaritifera* at different times after exposure to 1,200 mg L^{-1} benzocaine.

3.4 Discussion

The 100% survival obtained for oysters exposed to benzocaine and propylene phenoxetol in this study supports previous results for different pearl oyster species using these anaesthetics at similar concentrations (Norton et al., 1996, 2000; Acosta-Salmón & Rangel-Dávalos, 1997; O'Connor and Lawler, 2003). Both benzocaine and propylene phenoxetol gave satisfactory results especially during the first 15 to 30 min of exposure.

The best results were obtained with 1200 mg L^{-1} of benzocaine, as most of the oysters relaxed within the first five minutes of exposure and remained as SS for an average time of 30 min. However this treatment poses some inconveniences such as

potential health risks for the persons involved in handling the oysters (e.g. breathing in vapour) as well as uncertainty that the whole 1200 mg of benzocaine are dissolved into the seawater and causing the relaxing effect. Undissolved benzocaine crystals could be seen on the bottom of the container as well as on the water's surface. For these reasons, this treatment was not used in subsequent Chapters of this thesis. It may be useful to test this concentration of benzocaine by heating a small amount of seawater and then dissolving the benzocaine as proposed by Acosta-Salmón and Rangel-Dávalos (1997). Many of the oysters classified as NS in this treatment showed body collapse but did not show mantle collapse (Table 3.1), which may indicate that benzocaine may act differently to propylene phenoxetol. This condition was characterised by a wider than normal shell opening and flaccid body tissue. To know what causes mantle and body collapse, research is needed to understand the mechanisms of action for both chemicals, benzocaine and propylene phenoxetol, on the pearl oysters.

Benzocaine at a concentration of 500 mg L^{-1} and propylene phenoxetol at a concentration of 2.5 mL L^{-1} can be used safely to some extent for up to 15 to 30 min after relaxation occurs. In some relaxed oysters, the body appearance changed from relaxed to body collapse (Table 3.1) after some time of exposure. The evaluation for saibo oysters was made following criteria to select recipient oysters. This is a valid and conservative approach as, for example, oysters showing very weak body but no mantle collapse were classified as NS even though they could still be used as saibo donors.

Norton et al. (2000) mentioned that mantle collapse may be due to a decrease in hemolymph pressure. As the mantle tissue is of the utmost importance for the pearl formation process (Taylor, 2002), oysters with collapsed mantle should be avoided as donors as it is not known if the healing and developing capacity of the collapsed mantle

is the same as that of normal mantle. In this regard, more research is needed to assess the viability of the mantle treated with the different chemicals (Chapters 5 and 6).

Preparing the benzocaine at a concentration of 1200 mg L^{-1} by heating a small amount of sea water (as used to prepare the benzocaine concentrations of 250 and 500 mg L^{-1}) may optimise this method. However, further research is needed to test this assumption. If successful, this would be the preferred relaxing method to obtain saibo tissue from pearl oysters.

This Chapter has shown that *P. margaritifera* and *P. fucata* can be easily relaxed using both benzocaine and propylene phenoxetol. If relaxation is needed to obtain biopsy cores of gonad tissue, to determine oyster sex or just to evaluate oyster health, times of exposure to the chemicals and their concentrations are not a problem as oysters recover completely in a short period of time and mortality is nil. However, if anaesthesia is used to facilitate excision of saibo tissue (Chapters 5 and 6), then care must be taken to avoid undesirable conditions (e.g. mantle collapse), which appear after short (5-15 min) time of exposure and that affect the mantle tissue in particular.

Chapter 4

Use of a biopsy technique to obtain gonad tissue

4.1 Introduction

Aquaculture research often requires samples of various animal tissues to be obtained for analysis and observation. An example of this is the sampling of gonad tissue to assess reproductive seasonality or the effectiveness of broodstock conditioning protocols in studies with bivalve molluscs. In many cases, this process is destructive and involves sacrificing large numbers of animals. While this is not a problem with lower value aquaculture species, such as rock oysters, it is often prohibitive with higher value bivalves such as pearl oysters, which have the potential to produce pearls worth many hundreds or thousands of dollars. The reproductive cycle of the silver- or gold-lip pearl oyster (*Pinctada maxima* Jameson), for example, was described by Rose et al. (1990) and required the sacrifice of 1,340 oysters with a value of approximately US$10-15 each.

Biopsy sampling uses specialized needles that allow small tissue samples to be taken from a target tissue without sacrificing the animal. Biopsies are primarily used for human tissue collection but have also been used to obtain tissue samples from invertebrates for various analyses. Biopsies have previously been used to obtain mantle and gonad tissue from bivalves (Crawford et al., 1986; Benzie & Ballment 1994; Berg et al., 1995). This study evaluated a biopsy technique as a potential non-destructive means of obtaining gonad tissue from *P. margaritifera* to assess gonad condition.

4.2 Materials and methods

The *P. margaritifera* used in this part of the study were maintained on the long-line at Orpheus Island Research Station (Chapter 2).

Sixty oysters with mean (± SD) dorso-ventral height (DVH) of 109.8 ± 21.3 mm were used to evaluate the impact of the biopsy sampling technique on oyster mortality. Oysters were transferred from the long-line to the laboratory where they were cleaned prior to biopsies being taken. Forty-eight oysters were anaesthetised using 2 mL of propylene phenoxetol per L of 1 µm filtered seawater (Chapter 3). The remaining 12 oysters were kept without anaesthesia as controls. Three groups of 12 anaesthetised oysters were sampled using one of three different biopsy needles (16, 18 and 20 gauge). Each oyster was biopsied once. Biopsy samples were taken from the central portion of the gonad between the foot retractor muscles (Fig. 4.1). All needles were 9 cm long with a 10 mm sample notch. The 12 remaining anaesthetised oysters were not sampled and were kept as a survival control group. Oysters were maintained under culture conditions at Orpheus Island (Chapter 2) for two months to assess survival.

To assess the effectiveness of the biopsy technique for taking gonad samples that can be used to assess gonad condition, a further 25 oysters were cleaned and anaesthetised as described above. Each of the 16 gauge, 18 gauge and 20 gauge biopsy needles (GBN) were used to obtain gonad tissue from each oyster. After the biopsy procedure, each oyster was killed and the gonad sectioned for standard histological preparation. All samples were preserved with Formaldehyde-Acetic Acid-Calcium Carbonate (FAACC) gonad fixative, dehydrated in an alcohol series dilution, embedded in paraffin, sectioned to 5 µm and stained with Mayer's Haematoxilin and Young's Eosin-Erythrosin. Samples were examined microscopically to assess gonad condition and to compare samples taken using biopsies with those taken using destructive

sampling. Structures within the gonad sections and gonad development stages were identified based on previous studies with pearl oysters and other bivalves (Rose et al. 1990; De Gaujelac et al., 1995; Saucedo & Monteforte 1997; Saucedo et al., 2002b).

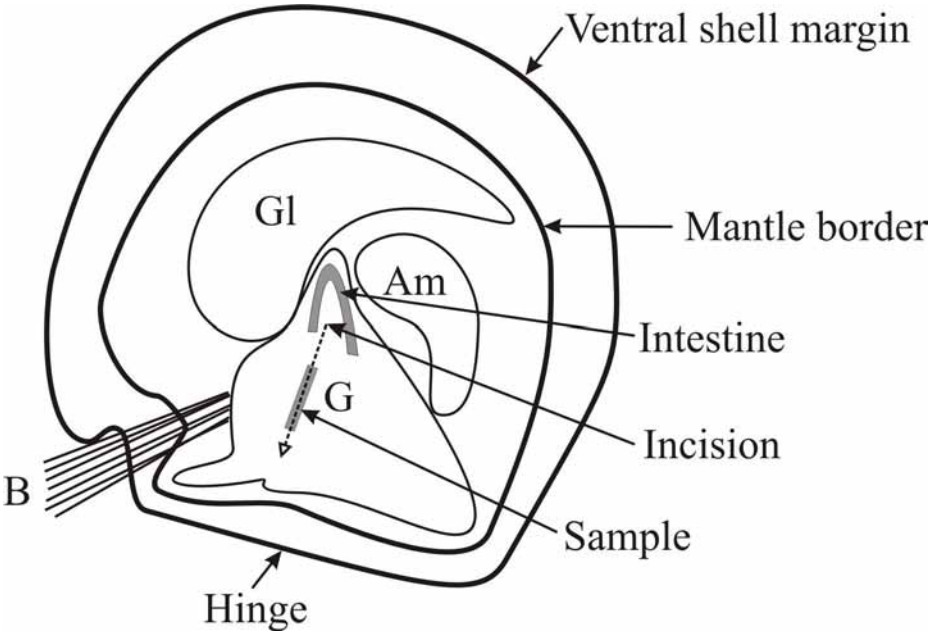

Fig. 4.1. Diagram showing the position at which biopsy samples were taken from the gonad of *Pinctada margaritifera* with the dotted arrow showing the direction of the incision. *Am,* adductor muscle; *B,* byssus; *G,* gonad; *Gl,* gills.

4.3 Results

Survival from the anaesthetic and biopsy procedure after 2 months was 100% in all groups. Histological examination of samples taken using standard destructive sampling showed that pearl oysters gonads were in a stage of mid development or 'mid gametogenesis' (Behzadi et al., 1997; Saucedo et al. 2002b). Acini appeared partially full and all developmental phases of the gametes were present.

In males, spermatogonia were present at the periphery of the acini, followed by spermatocytes and spermatids deeper into the lumen of the acini. Ripe spermatozoa were densely packed with acidophilic (eosinophilic) (pink) tails radiating from the center of lumen. In females, oogonia and previtellogenic oocytes were present at the periphery of the acini. Pedunculate (vitellogenic) oocytes still attached to the acini contained a large nucleus and 1 or 2 nucleoli and some postvitellogenic oocytes (round in shape) appeared at the centre of the lumen. (Figs. 4.2a, b).

The thickest biopsy needle (16 GBN) provided the greatest amount of gonad tissue for analysis. The samples obtained with the 3 different needles contained enough tissue to determine sex and gonad stage in males (Figs. 4.3a, b, c). Again, all developmental stages were evident in the samples obtained with the 16 GBN, arranged in the same way as described for the samples obtained using standard destructive sampling. Samples taken using the 18 and 20 GBN allowed identification of all structures and their quantification; however, there was some disruption to the organisation of the tissue samples. For example, there was no clear acini wall and ripe spermatozoa were scattered throughout the sample. In females the core contained enough material to define sex; however, there was not sufficient to identify gonad stages (Figs. 4.4a, b, c). Cores obtained with the 16 GBN contained mainly vitellogenic and post-vitellogenic oocytes but less developed forms (oogonia) were absent from the

core. In samples taken with the 18 and 20 GBN, developed oocytes were scarce and identification of gonad stage was not possible.

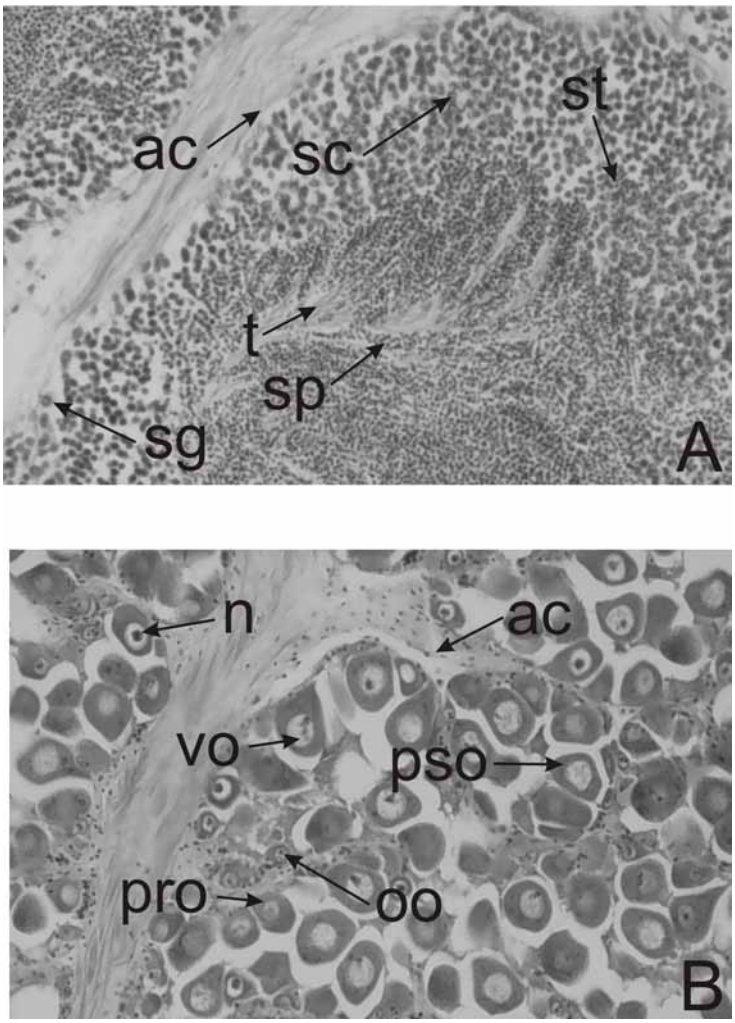

Fig. 4.2. Histological view of a portion of *Pinctada margaritifera* gonad. A: Male gonad in developing stage (40X). *sg,* spermatogonia; *sc,* spermatocytes; *st,* spermatids; *sp,* spermatozoa; *t,* tails; *ac,* acinus. B: Female gonad in developing stage (20X). *oo,* oogonia; *pro,* previtellogenic oocyte; *vo,* vitellogenic oocyte; *pso,* postvitellogenic oocyte; *n,* nuclei.

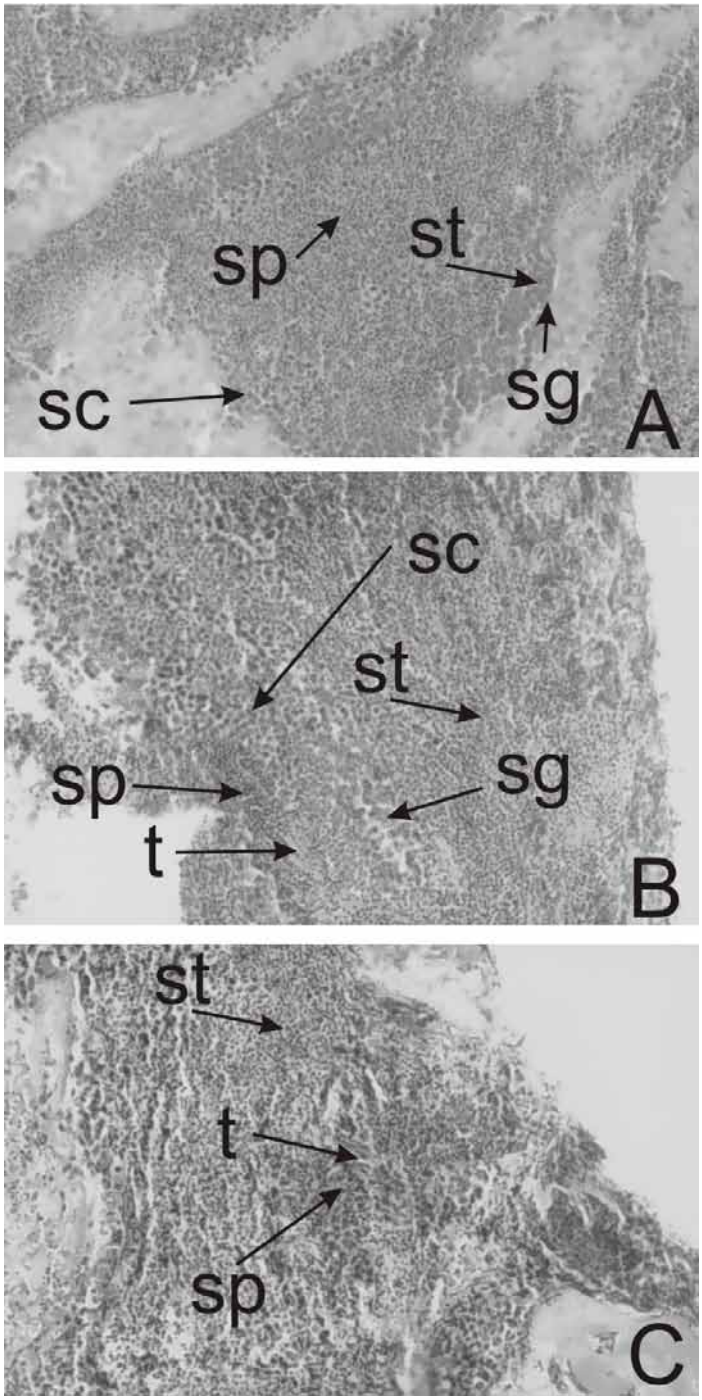

Fig. 4.3. Samples obtained from male *P. margaritifera* using the different biopsy needles (40X). A: 16 gauge biopsy needle (GBN); B: 18 GBN; C: 20 GBN. *sg,* spermatogonia; *sc,* spermatocytes; *st,* spermatids; *sp,* spermatozoa; *t,* tails.

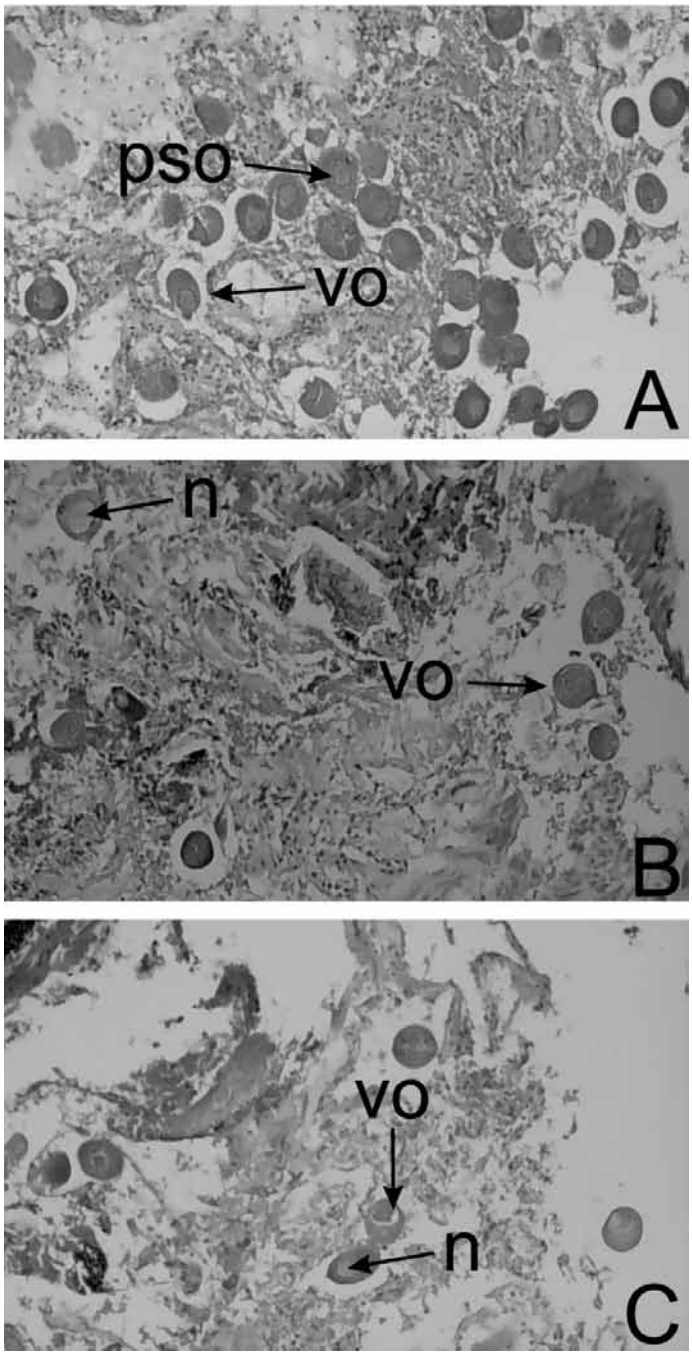

Fig. 4.4. Samples obtained from female *P. margaritifera* using the 3 different biopsy needles (20X). A: 16 GBN; B: 18 GBN; C: 20 GBN. *vo,* vitellogenic oocyte; *pso,* postvitellogenic oocyte; *n,* nuclei.4.

4.4 Discussion

No mortality of oysters anaesthetised with propylene phenoxetol and sampled with the 3 biopsy needles was seen up to 8 weeks after the procedure. In contrast, exposure to propylene phenoxetol and insertion of a bead into the gonad of *P. margaritifera* for cultured pearl production was reported to result in significant oyster mortality after 6 weeks (Norton et al., 2000). This difference may result from greater tissue damage caused by the bead insertion process compared to biopsy sampling; however, long exposure to propylene phenoxetol seems to have a negative effect on oyster recovery, which beyond a threshold limit, may cause an irreversible sedative effect. Though propylene phenoxetol did not affect oyster survival in this study, it affected the general condition of oyster tissue causing collapse of mantle and gills. These effects were not quantified but increased the time required to take samples with the biopsy needles.

Tranter (1958d) reported on the reproductive seasonality of *P. margaritifera* from the Torres Strait, north Queensland, Australia. The oysters used in this study were in a stage of mid-gametogenesis that corresponded to Tranter's findings for oysters in late spring when this study took place. Subsequent use of the biopsy technique described here to assess reproductive seasonality in *P. margaritifera* has shown that more mature gonads are easier to sample and analyse than poorly developed gonads. The greater tissue mass and more tightly packed structure of the former allowed biopsy samples to be taken with a higher mass of gametes. This, in turn, allowed easier identification of gonad stage. Cores from poorly developed gonads, which contained a greater amount of connective tissue, generally loose their uniform structure, making processing and analysis more difficult.

Gonad development in pearl oysters is usually divided into five stages: (1) indeterminate; (2) gametogenesis; (3) ripe; (4) spawned; and (5) spent (Rose et al. 1990; Garcia-Dominguez et al., 1996; Behzadi et al. 1997). As mentioned above, the earlier stages of development (indeterminate and spent) may be difficult to determine from cores extracted using biopsy needles; however, structures specific to each gametogenic stage present in the samples could allow identification of particular stages.

The results presented here indicate that non-destructive biopsy sampling could be a valuable means of assessing gonad condition in pearl oysters. The use of thicker biopsy needles (14 or 12 gauge) may allow better interpretation of gonad stage, particularly in female oysters for which results showed that 16 GBN were unsatisfactory. This aspect requires further study. A combination of visual examination of the intact gonad through the shell gape and histological examination of cores obtained with the biopsy needles, will allow more accurate non-destructive assessment of the gonad stage of pearl oysters.

This Chapter reports on a useful technique, novel to pearl oysters, which can be used to obtain gonad samples to assess factors such as reproductive seasonality or broodstock conditioning. This technique allows such research to be implemented without the need to kill valuable oysters.

Chapter 5 Excision of mantle tissue

5.1 Introduction

In the pearl oyster industry, broodstock are frequently selected on the basis of nacre colour as well as shell form and shape (Wada, 1993). Similar criteria are used for selection of *saibo* donors before sacrificing, where shells and tissue are inspected to assess general health and nacre quality and only the best oysters are sacrificed to be used as donors (Wada, 1991; Taylor, 2002). The quality of resulting pearls depends greatly on selection of adequate donor oysters (Taylor, 2002). In the pearl oyster industry, unlike most livestock industries, the best individuals (hence, the more valuable) are not used as broodstock but are sacrificed to obtain the highest quality pearls possible. However, if high quality saibo donors could be used without being sacrificed, they could potentially be used as future broodstock. This approach would ensure that only oysters producing high quality pearls are used as broodstock resulting in high quality progeny and improvement in the quality of the farm stock. This in turn would bring noticeable benefit to the pearling industry on the basis of improvements in pearl quality.

A potential problem with the hypothesis outlined above is that the mantle tissue of live pearl oysters contracts when touched and this makes excision of mantle tissue from live oysters extremely difficult. However the use of suitable relaxants (Chapter 3) may facilitate removal of saibo tissue from live oysters without killing the oyster. This Chapter investigates the feasibility of keeping saibo donor pearl oysters alive as potential broodstock. It assesses survival of anaesthetised *P. fucata* following mantle excision and their ability to heal the wound site.

5.2 Materials and methods

The *P. fucata* used in this part of the study were maintained on the long-line at Magnetic Island (Chapter 2). Three groups of 15 *P. fucata* with mean (± SD) dorso-ventral measurement (DVM) of 67.1 ± 3.7 mm were used to assess the effect of removing one of either or both mantle lobes on survival following relaxation using 500 mg L^{-1} Benzocaine (Chapter 3).

Once oysters were relaxed, strips of mantle tissue (length = 32.1 ± 3.34 mm) from either the left or the right mantle lobe were removed from oysters in Groups 1 and 2, respectively, and strips were removed from both mantle lobes of oysters in Group 3 (Fig. 5.1). Oysters were returned to culture conditions and survival was determined 1 and 4 weeks after this procedure. A control group without mantle excision was run simultaneously. After 3 further months five oysters from each group were relaxed and sacrificed for histological determination of mantle healing. Sections of mantle were removed from both the 'normal' and the healed sections (Fig. 5.1). Tissues were histologically processed as described in Chapter 2. Tissues were stained with Mayer's Haematoxilin and Young's Eosin–Erythrosin or Masson's Trichrome to observe general morphology and Alcian Blue-PAS to observe acid and neutral mucopolysaccharides (secretory cells) (Culling et al., 1985). Samples were observed microscopically to identify any differences between normal and healed mantle tissues.

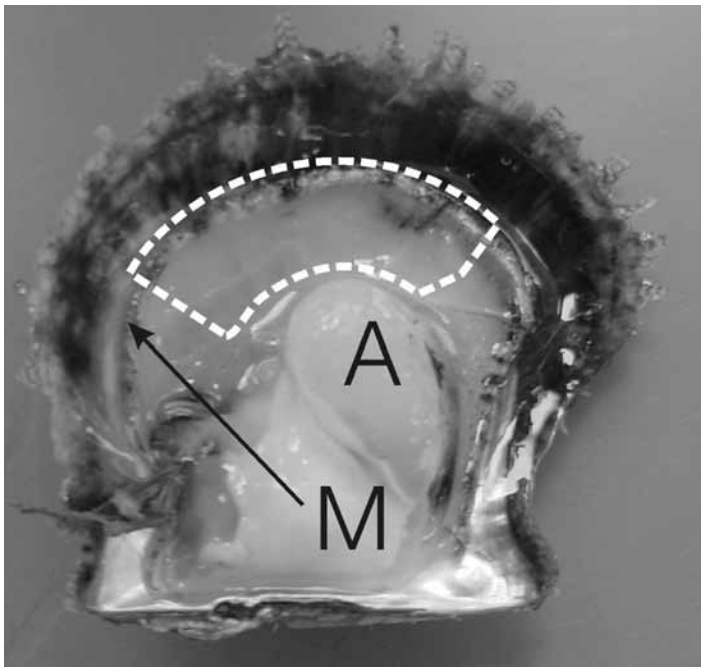

Fig. 5.1. Photograph of *P. fucata* with one shell valve removed to show A (adductor muscle), M (mantle edge). Area included within the dotted line indicates the section of mantle tissue excised.

5.3 Results

Survival of *P. fucata* from all treatment groups was 100% at the end of the 4th week following mantle excision. Byssal threads had been secreted by many of the oysters from all groups 7 days after this procedure. Histological analysis showed that excised mantle had not only healed but showed complete regeneration 3 months after mantle removal. Both, normal (NT) and regenerated mantle tissues (RT) (Fig. 5.2a, b, c) showed the typical morphology of bivalve mantle with the marginal (including the 3 folds), pallial and central zones; the pallial artery, pallial nerve and secretions of conchiolin were observed in both NT and RT.

There was no difference in the outer low columnar epithelium of the pallial zone between NT and RT (Fig. 5.3a, b); the Alcian Blue-PAS technique showed the different

secretory cells in both inner and outer epitheliums of the pallial zone for NT and RT (Fig. 5.4a, b). In the internal face of the pallial zone, the ciliated columnar epithelium and the sets of longitudinal and radial muscles were well developed in both NT and RT (Fig. 5.5a, b). On the shell, new nacre laid down by regenerated mantle tissue appeared with good lustre and colour but had an irregular, not smooth, surface (Fig. 5.6); new periostracum laid down by regenerated mantle appeared darker than shell laid down by normal mantle tissue.

5.4 Discussion

Relaxants are not generally used to prepare oysters for grafting in large-scale operations because of the large number of oysters used and the extra time and labour that would be required. However, for the relatively small numbers of oysters used as saibo donors this extra effort (cost) may be justified on the basis that saibo donors can be kept alive and used as future broodstock.

One week after removing the mantle lobes, oyster survival was 100% and they had already attached with some byssal threads, indicating a rapid recovery from the surgery. *P. fucata* not only healed the wound site but were able to complete regenerate excised mantle tissue within three months. Regeneration of mantle tissue in pearl oysters has only previously been reported at the level of pearl-sac development or epithelial cell proliferation of a section of the mantle (Suzuki et al, 1991; Suzuki, 1992; Awaji and Suzuki, 1995, 1998) following grafting. This study is the first to report on regeneration of pearl oyster mantle tissue *in vivo*. Normal mantle appeared more pigmented than regenerated mantle when observed with the naked eye, particularly the inner (larger) fold. However, histological analyses showed no differentiation in the pallial zones; the outer epithelial cells in this part of the mantle did not show

differences between NT and RT; large secretory basophilic cells believed to play an important role in nacre layering (Garcia-Gasca et al., 1994) were also present in both tissues as well as the mucous secretory cells in the inner side of the pallial zone. As this part of the mantle is chosen by seeding technicians as saibo, the ability of pearl oysters to regenerate mantle tissue may lead to the possibility of obtaining saibo tissue from high quality donor oysters on more than one occasion.

Norton et al. (2000) evaluated the quality of pearls produced using relaxed oysters although they did not use relaxed mantle tissue as saibo. The results presented here have shown that oysters used as saibo donors need not be killed for pearl production and, as such, they could be used a future broodstock and possibly for repeated saibo donation(s). These findings may have major significance for the cultured pearl industry. However, fundamental to this process is the relaxation of saibo donors and, by implication, the use of relaxed saibo for pearl production. Further research is required to determine the influence of relaxed and/or regenerated saibo tissue on pearl-sac development, pearl formation and pearl quality.

This Chapter has demonstrated the feasibility of obtaining mantle tissue from selected oysters without killing them. Furthermore, it has shown that pearl oysters can regenerate the excised section of the mantle in less than 3 months under culture conditions. On this basis, the pearls produced by individual donor oysters could be evaluated and those producing high-quality pearls could be used as future broodstock. Furthermore, it may be possible to reach a point where specific saibo donors, which produce pearls with specific colours, can be identified and maintained as distinct breeding lines. However, further histological and histochemical studies are required to fully understand the mantle regeneration process in pearl oysters (Chapter 6) and it effects (if any) on further oyster growth and nacre quality.

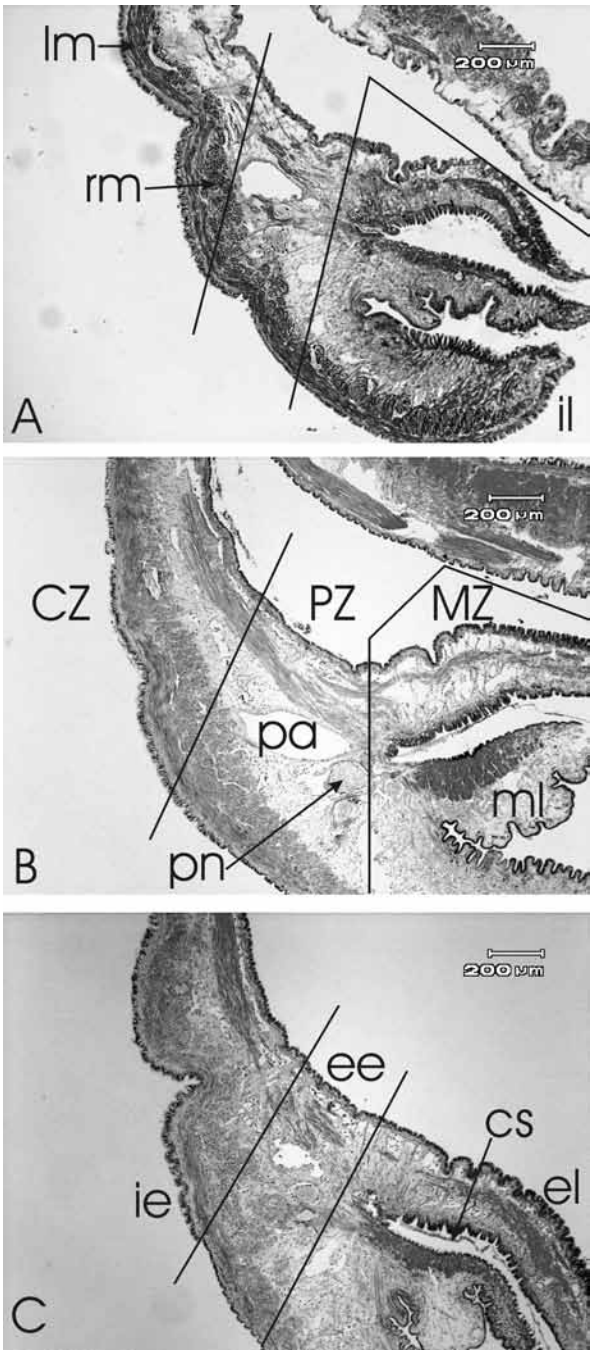

Fig. 5.2. Histological view of the mantle border of *P. fucata*. A: regenerated tissue; B: normal tissue; C: regenerated tissue; cs: conchiolin secretion; CZ: Central Zone; ee: external epithelium; el: external lobe; ie: internal epithelium; il: internal lobe; lm: longitudinal muscles; ml: middle lobe; MZ: Marginal Zone; pa: pallial artery; pn: pallial nerve; PZ: Pallial Zone; rm: radial muscles. (A: Masson Trichrome; B and C: Haematoxilin eosin-erithrosin technique).

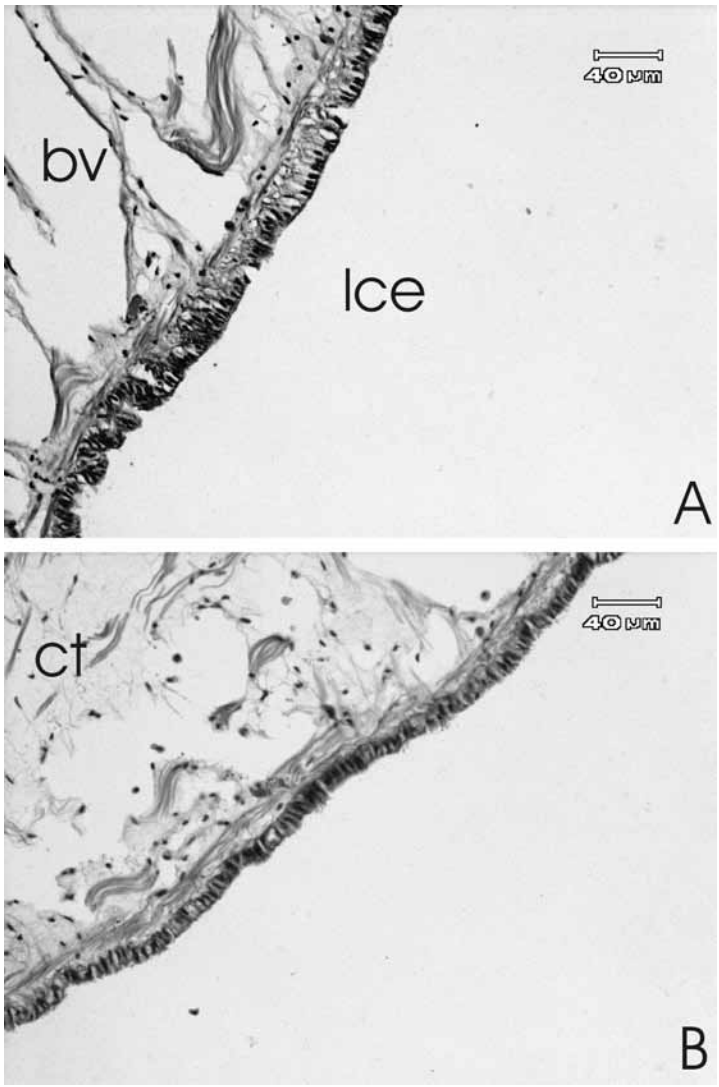

Fig. 5.3. Histological view of the pallial external epithelium (used for pearl production) of the mantle border of *P. fucata*. A: normal tissue; B: regenerated tissue. bv: blood vessel; ct: connective tissue; lce: low columnar epithelium. Haematoxilin eosin-erithrosin technique.

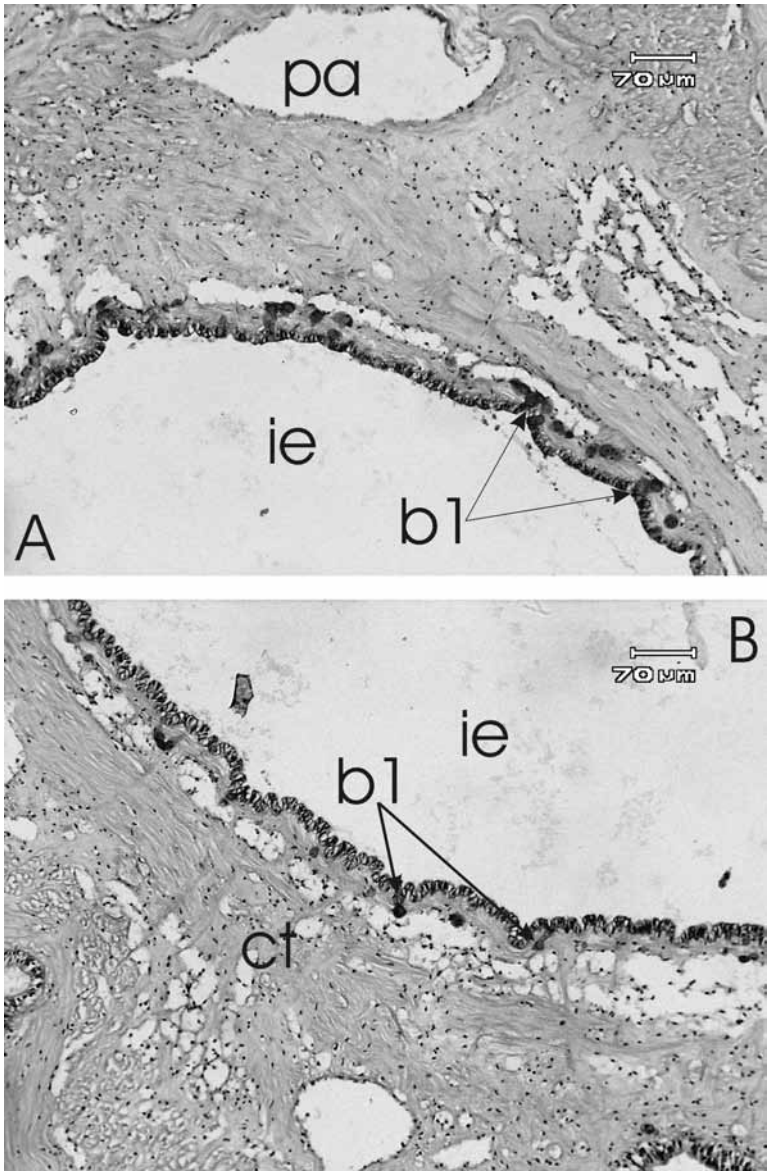

Fig. 5.4. Histological view of the pallial external epithelium of the mantle border of *P. fucata*. A: normal tissue; B: regenerated tissue. pa: pallial artery; ct: connective tissue; ee: external epithelium; b1: type b1 secretory cells. Alcian Blue-Periodic acid-Shiffer (PAS) technique.

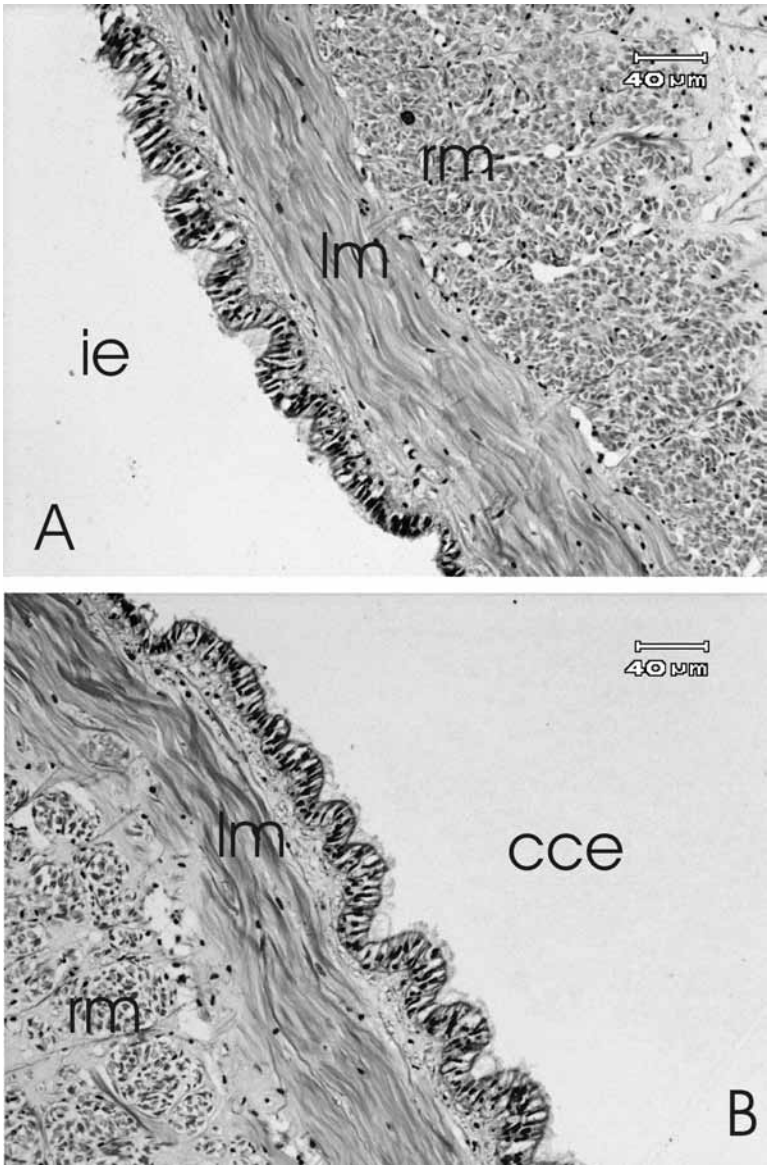

Fig. 5.5. Histological view of the internal side of the pallial zone of mantle border of *P. fucata*. A: normal tissue; B: regenerated tissue; cce: cliliated columnar epithelium; ie: internal epithelium; lm: longitudinal muscles; rm: radial muscles. Haematoxilin eosin-erithrosin technique.

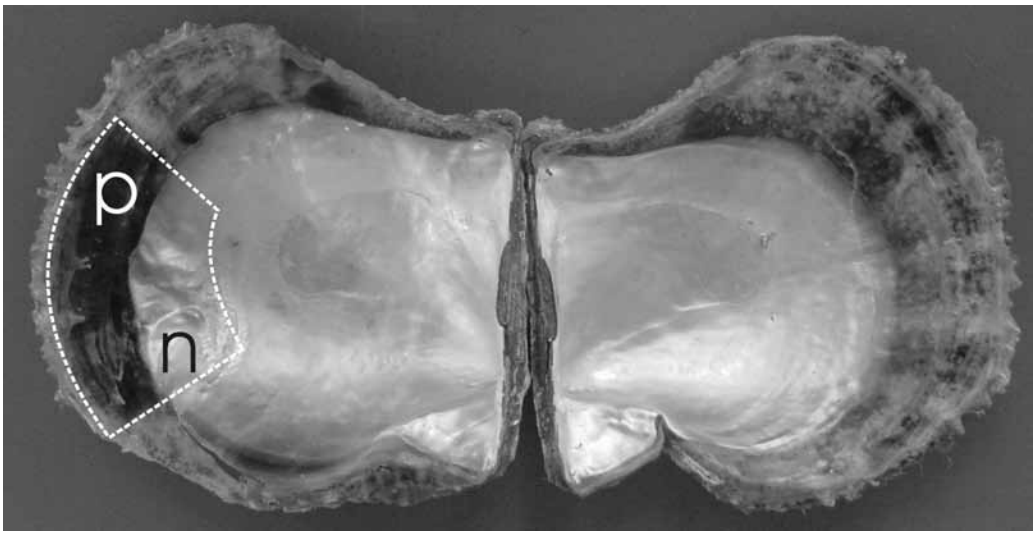

Fig. 5.6. Shell valves from *P. fucata* from which mantle tissue was excised on the left valve and left intact on the right valve. The left valve shows new nacre and periostracum laid down by regenerated mantle tissue (p, periostracum; n, nacre). The shell valve on the right is 'normal'.

Chapter 6

Regeneration of mantle tissue

6.1 Introduction

Regeneration is an essential process for all animals and is regarded as a device by which functional competence is recovered (Goss, 1969). For mantle tissue in pearl oysters, shell production (periostracum, prismatic and nacreous layers) and sensorial abilities are the most important functions. A number of studies have reported on regeneration of tissue by bivalve molluscs. In the species *Macoma baltica*, *Donax hanleyanus*, *Prothotaca staminea* and *Scrobicularia plana*, for example, siphonal tissues are quickly regenerated after being lost to predators or during laboratory experiments (Hodgson, 1982; Peterson and Quammen, 1982; Pekkarinen, 1984; De Vlas, 1985; Luzzatto and Penchaszadeh, 2001).

Given the value of the cultured pearl industry and the importance of mantle tissue in determining pearl quality (Taylor, 2002), it is remarkable that after more than two centuries of studies on animal regeneration (Goss, 1969), and more than a century of studies into cultured round pearl propagation (Saville-Kent, 1893; George, 1966), there is a complete lack of studies on in vivo mantle regeneration in pearl oysters. Chapter 5 showed that anaesthetised pearl oysters not only survived excision of mantle tissue and healed the mantle wound but also regenerate this section of the mantle. The aims of this Chapter were to describe by histological means the progressive regeneration of mantle tissue in *P. fucata* following excision and to determine whether *P. margaritifera* has similar ability to regenerate excised mantle tissue.

6.2 Materials and methods

Fifty *P. fucata* and 10 *P. margaritifera* were relaxed with 2 mL L^{-1} propylene phenoxetol (Norton et al., 2000) (Chapter 3) and sections of mantle tissue were removed randomly from either the left or the right mantle lobe (Chapter 5). After mantle excision, oysters were returned to the long-line at Magnetic Island (Chapter 2) for 3 months during which sub-samples were removed at regular intervals to assess mantle regeneration. *P. fucata* were relaxed (Chapter 3) and sacrificed on days 3, 6, 9, 12, 15, 20, 30, 45, 60 and 90 after mantle excision when regenerating mantle tissue was obtained and sectioned for standard histological preparation (Chapter 2, Appendix 1). Tissues were stained with H-E, MSB trichrome or Alcian blue-PAS. *P. margaritifera* were relaxed and sacrificed at the end of the three month period and regenerated mantle tissue was processed as described above.

6.3 Results

Survival was 100% at the end of the experiment for *P. fucata*. One *P. margaritifera* died before day 20 and two further oysters died between days 60 and 90. The shell of the dead oysters showed secretions of new prismatic layer on top of the old nacreous layer. However, all shells of dead *P. margaritifera* were fouled in the shell margin adjacent to the wound site.

Progressive regeneration of excised mantle tissue in *P. fucata* is shown in Fig. 6.1. Rounding of the square ends of the wound occurred within 6 days (Fig 6.1b). The length of the lesion was reduced by the anterior and posterior ends of the wound drawing closer together (Fig. 6.1c) to a point where further regeneration of the mantle was accomplished through extension of the central zone and formation of marginal

tissue at the wound site to a point where it was continuous (Figs. 6.1d and 6.1e). The three lobes of the marginal zone were clearly visible by day 45 and complete regeneration of mantle tissue to its original extent was seen by day 60 (Fig. 6.1f)

Histological analyses showed that by day 3, a healing zone packed with what are presumably haemocytes had developed on the distal end of the mantle (Fig. 6.2a). As early as 6 days after mantle excision, what will later become the pallial artery had already formed between the muscular tissue and the healing zone (Fig. 6.2b). After 9 and 12 days, elongation of both inner and outer epithelia and of new connective tissue had taken place and the pallial artery was embedded within the connective tissue (Fig. 6.2c). The marginal zone and the internal, middle and external lobes were evident 15 days after mantle excision and secretions of conchiolin could be seen on the periostracal groove (Fig. 6.2d). Epithelial cells were well defined on the inner and outer surfaces of the mantle. After 20 days the lobes had grown more evident but there was little muscular tissue on the regenerated section of the mantle (Fig. 6.2e). Pigments appeared on the marginal zone by day 30 (Fig. 6.2f) when there was significant muscle mass present. Elongation of the regenerated section continued until day 60 with more muscular development.

Ninety days after mantle excision, *P. margaritifera* showed complete regeneration of mantle tissue to its original extent. Macroscopic observations showed a well pigmented marginal zone of the mantle and new nacre and prismatic secretions on the shell. Histological observations of the regenerated mantle showed the marginal zone (including the outer, middle and inner folds as well as secretions of conchiolin), the pallial zone (showing the pallial artery and the pallial nerve) and the central zone (Fig. 6.3).

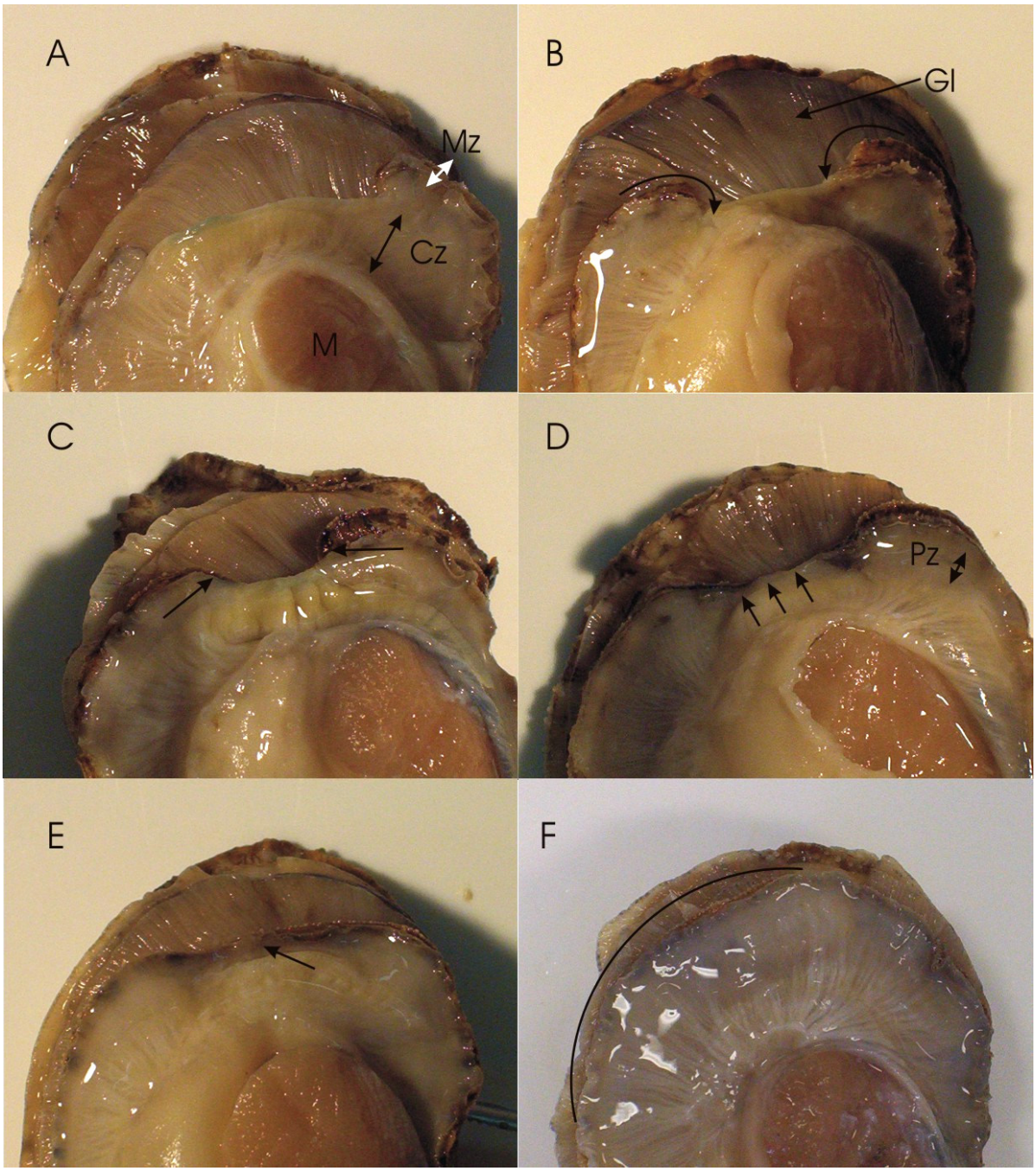

Fig. 6.1. Macroscopic progression of mantle regeneration in *P. fucata* at A: 3 days; B: 6 days; C: 12 days; D: 20 days; E: 30 days; F: 60 days after mantle excision. The black line in F indicates the extent of normal fully extended mantle lobe. Arrows indicate assumed directions of mantle re-growth Cz: central zone; Gl: gills; M: adductor muscle; Mz: marginal zone.

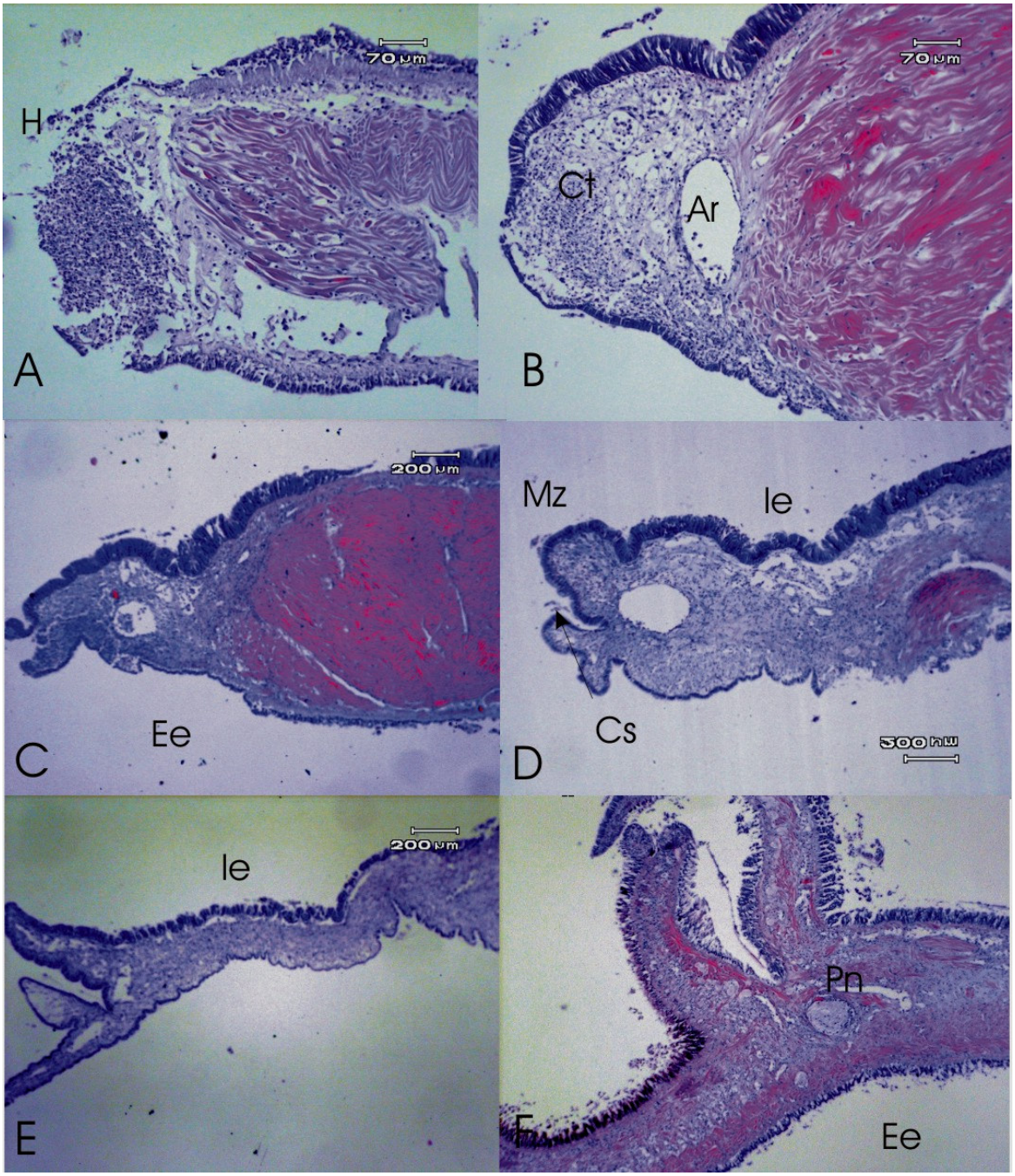

Fig. 6.2. Histological views of mantle regeneration in *P. fucata* at A: 3 days; B: 6 d; C: 12 d; D: 15 d; E: 20 d; F: 30 d; after mantle excision. Ar: artery; Cs: conchiolin secretion; Ct: connective tissue; Ee: external epithelium; H: presumably haemocytes; Ie: internal epithelium; Mz: marginal zone; Pn: pallial nerve.

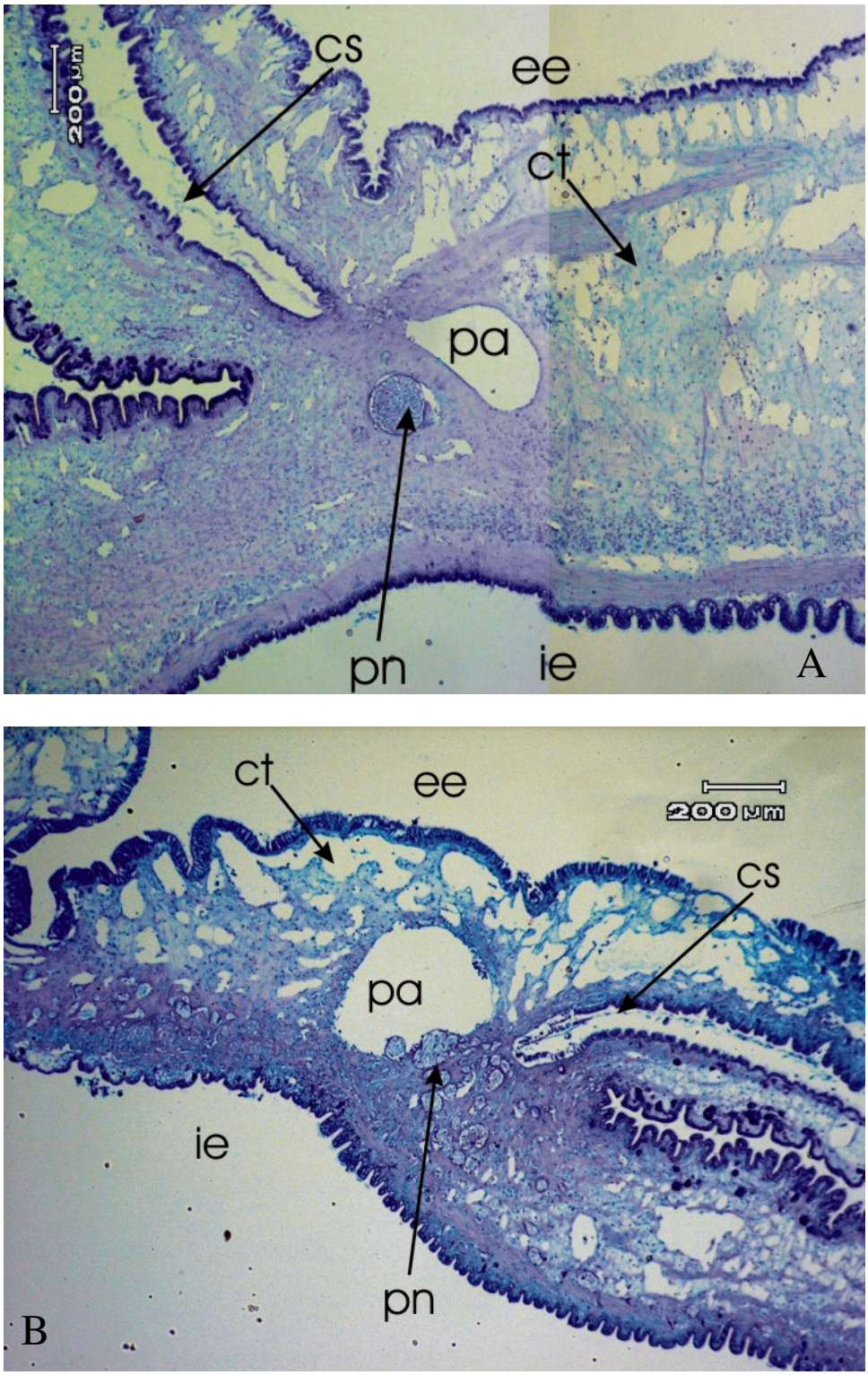

Fig. 6.3. Microphotograph of normal (A) and regenerated (B) mantle tissue of *P. margaritifera* three months after mantle excision. cs: conchiolin secretions; ct: connective tissue; ee: external epithelium; ie: internal epithelium; pa: pallial artery; pn: pallial nerve. Stained using Alcian blue-PAS technique.

6.4 Discussion

Oyster survival was similar to that obtained using this same technique in Chapter 5. It is notable that there was some mortality of *P. margaritifera* during this study in the later stages of the mantle regeneration process. *P. margaritifera* used in this study were older than the *P. fucata* used, and the difference in oyster age may have influenced the results obtained; in particular, the speed of mantle regeneration. Fouling was evident on the margins of *P. margaritifera* shells adjacent to the mantle wound site. Relatively slow mantle regeneration could have allowed fouling organisms to colonize the shells of *P. margaritifera* exerting further stress on the oysters. However, overall survival of *P. margaritifera* was high supporting the hypothesis that excision of saibo tissue from live *P. margaritifera* for pearl production is possible with minimal mortality.

Older pearl oysters are frequently selected in preference to young ones to be used as saibo donors because older oysters show a slower growth rate. It is believed that saibo tissue from these oysters will secrete nacre at a slower rate resulting in better quality pearls (Gervis and Sims, 1992). There is likely to be an optimum age for saibo excision where nacre quality is maximal. Further research is required into the influence of oyster age on quality of nacre and the rate of mantle regeneration in pearl oysters. Regular cleaning of oyster shells following mantle excision may contain biofouling and minimise oyster mortality. Stress resulting from this procedure could also be reduced by excision of a smaller piece of mantle tissue. This would reduce wound size, allowing more rapid healing and as such, reduce the opportunity for biofouling to colonise the inside of oyster shells, however care must be taken to ensure sufficient saibo pieces can be obtained to allow for accurate evaluation of the pearl production ability of such oysters.

Mantle borders of *P. fucata* rolled inwards at the site of excision possibly to contain the haemorrhage caused when the pallial artery was severed. Hodgson (1982) reported a similar pattern while observing siphon regeneration in *Scrobicularia plana* where the lesion width was reduced by muscular action to provide the siphon with a mechanical seal to prevent blood loss. Similarly, *P. fucata* seemed to mechanically act to contain haemorrhage and reduce the size of the wound thus it could be sealed quickly using smaller numbers of haemocytes and less connective tissue. Mantle tissue surrounding the wound site grew in two directions: (1) ventrally towards the shell margin from the mantle central zone forming the pallial and marginal zones; and (2) laterally with the ends of the wound healing and growing towards each other reducing the length of the lesion. Further studies on the initial wound sealing process (Hodgson, 1982) immediately following mantle excision in pearl oysters are required for a greater understanding of the healing process

Presumably, the period immediately following mantle excision is when oysters are more likely to be susceptible to death. However, despite a significant loss of body (mantle) tissue, the healing process was quick enough to contain haemorrhaging and avoid death. Once the lesion had sealed (by day 3), regeneration of mantle tissue was rapid and complete. Observations on byssus production from Chapter 5 (one week after mantle excision) could be then considered as normal, and indicates that the damage caused when excising mantle tissue and the healing and regeneration process do not interfere with other body functions.

Although the mantle border grew back to almost its original extent within 60 days after excision, it was not until day 30 that muscular fibres were seen within the new tissue. Functional (secretory) abilities were presumably recovered before day 15 when conchiolin secretions and secretory cells were seen in the newly regenerated

epithelia. Mantle regeneration up to day 90 after mantle excision in *P. margaritifera* was similar to that described above for *P. fucata*.

Mantle regeneration after excision should be an energetically expensive process for pearl oysters. Seasonal patterns of nutrient storage and utilisation in relation to the reproductive cycle have been described for many marine bivalves and have shown that mantle tissue functions as a site for storage of nutrients in some species (Zandee et al., 1980; Barber and Blake, 1981; Mathieu and Lubet, 1993). Pearl oysters also show seasonal patterns of energy storage in different body tissues (Saucedo et al., 2002a). Chapter 7 of this thesis examined nutrient and energy management in body tissues (including mantle tissue) of *P. margaritifera* related to the reproductive season. These processes may provide the nutrients and energy sources required for mantle regeneration in pearl oysters. Seasonal fluctuations in the biochemical and energy contents of the major tissues of pearl oysters (Chapter 7) however, may influence the rate of mantle regeneration.

No previous studies have reported on the regeneration of the whole mantle tissue in pearl oysters as it is not a common practice for pearl farms to obtain mantle tissue without killing the donor pearl oysters. In the 'autograft' method of cultured pearl production, recipient oysters are implanted with a relatively small piece of their own mantle tissue and, on this basis, the mantle donor (which is also the recipient) is kept alive for pearl production. A slight modification to this method is still used for pearl production from *P. maxima* in Myanmar (J. Taylor, pers. comm.) where the donor (plus six to seven oysters) are grafted with its mantle. Similar to the 'autograft' method, donor oysters used in this study were also kept alive. However, the technique described here provides the basis for a single oyster to provide saibo tissue for a number of recipient oysters and the donor oyster is not seeded for pearl production.

Given the many advantages of keeping saibo donors alive, it is anticipated that within a few years the cultured pearl industry will obtain saibo tissue from donor pearl oysters without killing them. Techniques for removal of saibo and husbandry of saibo donors can be further developed through a greater understanding of the processes involved in mantle healing and regeneration.

The excision of mantle tissue seems not to have any negative effect on oyster growth. Furthermore, pearl seeding is normally conducted in cooler months where the growth rate is slower. Protocols for long-term culture (broodstock culture) can remain the same with minor modifications at the early stages after mantle excision when oysters are more vulnerable.

This Chapter provided new important information to improve current broodstock management techniques. Up until now, oysters have been sacrificed to obtain mantle tissue. With the development of the excision technique (Chapter 5) and the information provided in this Chapter it is possible to develop specific maintenance protocols for oysters that have provided saibo.

Chapter 7

Nutrient management during breeding season

7.1 Introduction

There have been numerous studies on nutrient storage and mobilisation associated with gametogenic cycles in bivalves (e.g. Mathieu and Lubet, 1993; Pazos et al., 1997; Lodeiros et al., 2001; Saucedo et al., 2002a). Tissues such as the adductor muscle, digestive gland and mantle play important roles in storage and translocation of nutrients used for gonad maturation at different stages of the gametogenic cycle. In the scallop, *Argopecten irradians concentricus*, for example, a seasonal cycle of energy storage prior to gonad development was observed, where the adductor muscle was the main site for storage of protein and glycogen, while the digestive gland stored lipid reserves and the mantle tissue was composed mainly of proteins (Barber and Blake, 1981). Late gonad development and spawning occurred mainly at the expense of the protein and lipid stored in the adductor muscle (Barber and Blake, 1981).

A similar pattern of nutrient management was reported in the pearl oyster, *P. mazatlanica*. Major spawnings were recorded in spring and summer. Proteins were stored in the adductor muscle and were translocated to the gonad during gametogenesis while lipid content of the digestive gland showed peaks coinciding with the major spawnings (Saucedo et al., 2002a).

The gametogenic cycle for wild *P. margaritifera* from the Torres Strait, north Queensland (Australia) was reported by Tranter (1958d). Breeding activity was found to be constant throughout the year with major spawnings occurring in January and July. Sex ratio in younger oysters was in favour of males and this stabilised during later breeding seasons until reaching a 1:1 (male:female) ratio (Tranter, 1958d). There are no

other studies, for the rest of the Queensland coast, reporting on the breeding season of wild *P. margaritifera* or on the reproductive cycle of cultured *P. margaritifera*. However there is indication of similar winter and summer spawnings. Peaks of *P. margaritifera* spat recruitment were recorded at Orpheus Island (north Queensland) for example, during October-December and February-March (Southgate and Beer, 2000). Considering a larval period of 28 days and a spat collector immersion time of 4 weeks (Southgate and Beer, 1997), pearl oyster spawnings should have occurred during August-October and December-January.

The objective of this study was to determine the pattern of nutrient management during gametogenesis of the blacklip pearl oyster, *P. margaritifera*, at two different locations in north Queensland. Samples of mantle, adductor muscle, gonad and digestive gland were examined for proximate biochemical content for a period of seven months and these data were related to the histological development of the gonad.

7.2 Material and methods

Collection of oysters from culture stock held at Magnetic Island and Orpheus Island (Chapter 2) was conducted from August 2003 to February 2004. Six oysters from each site were collected monthly for seven months. One month prior to the experiment, six female oysters from Orpheus Island were identified by puncturing the gonad with a micropipette to identify gametes. They were then moved to Magnetic Island to assure the presence of females at this site. Oysters were opened by cutting the adductor muscle and samples of mantle, adductor muscle, gonad and digestive gland were obtained and stored at –80°C until biochemical analyses were performed (Chapter 2; Appendix 1). A section of the gonad was fixed in FAACC to determine gonad stage by histological means (Chapters 2 and 4; Appendix 2). Identification of six gonad development stages

(indeterminate, early development, late development, ripe, partially spawned and spent) was based on previous studies with pearl oysters (Tranter, 1958b; Garcia-Dominguez et al., 1996; Saucedo & Monteforte 1997; Saucedo et al., 2002b).

Water temperature at both collection sites was recorded continually at 2 h intervals using an automatic YSI 6600 water quality sonde (John Morris Scientific, Brisbane, Australia). All water temperature readings for each month were used to calculate the average monthly water temperature.

7.3 Results

Water temperature data are shown in Fig. 7.1. Water temperature gradually increased at Orpheus Island from a low of 22.7°C in August 2003 to a maximum of 29.4°C in January 2004. Maximum water temperature at Magnetic Island occurred in December 2003 (29.3°C) and remained relatively constant until February 2004. Water temperature at Magnetic Island was, on average, 0.6°C higher than at Orpheus Island from the months of August to December 2003.

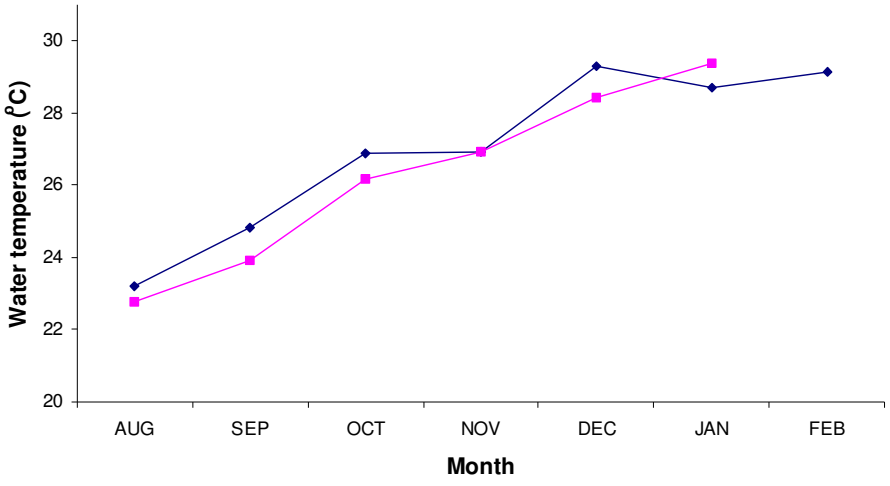

Fig. 7.1. Mean monthly water temperature at Magnetic Island (◆) and Orpheus Island (■).

7.3.1 Histological analysis

Males outnumbered females at both sites. A total of 55 males and 22 females were recorded during the 7 months of the experiment giving an overall sex ratio of 2.5:1 (male:female). The sex ratio of oysters held at Magnetic Island was 5.2:1 (male:female), while at Orpheus Island was 1.5:1 (male:female). The only females observed at Magnetic Island were those transferred from Orpheus Island prior to the experiment. Four oysters for which sex could not be determined (three at Magnetic Island and one at Orpheus Island) and one hermaphrodite (Magnetic Island) were also observed.

Spawning events were observed in October-November 2003 and January 2004 for oysters held at Magnetic Island and in September 2003 and December 2003-January 2004 for oysters held at Orpheus Island when the proportion of partially spawned oysters increased (Figs. 7.2 and 7.3). Oysters in early and late development stages were observed in all months at both sites. The highest proportion of ripe oysters was recorded in September and December 2003 and in February 2004 at Magnetic Island (Fig. 7.2). The highest proportion of ripe oysters at Orpheus Island was recorded in February 2004 (Fig. 7.3). One indeterminate oyster (for which reproductive stage could not be determined) appeared in August 2003 at Orpheus Island. Three indeterminate oysters appeared in November 2003 (two oysters) and January 2004 (one oyster) at Magnetic Island. Spent oysters were observed in January and February 2004 at Orpheus Island and Magnetic Island, respectively (Figs. 7.2 and 7.3).

7.3.2 Biochemical analysis

There was a pattern for nutrient storage and utilisation within oysters maintained at Orpheus Island and Magnetic Island with lipid and carbohydrate

accumulating with time towards the end of the sampling period in most tissues (Figs. 7.4 to 7.15). Tissue nutrient content for the first 4 to 5 months was, in most cases, lower in oysters maintained at Magnetic Island than in those from Orpheus Island (Tables 7.1 to 7.3).

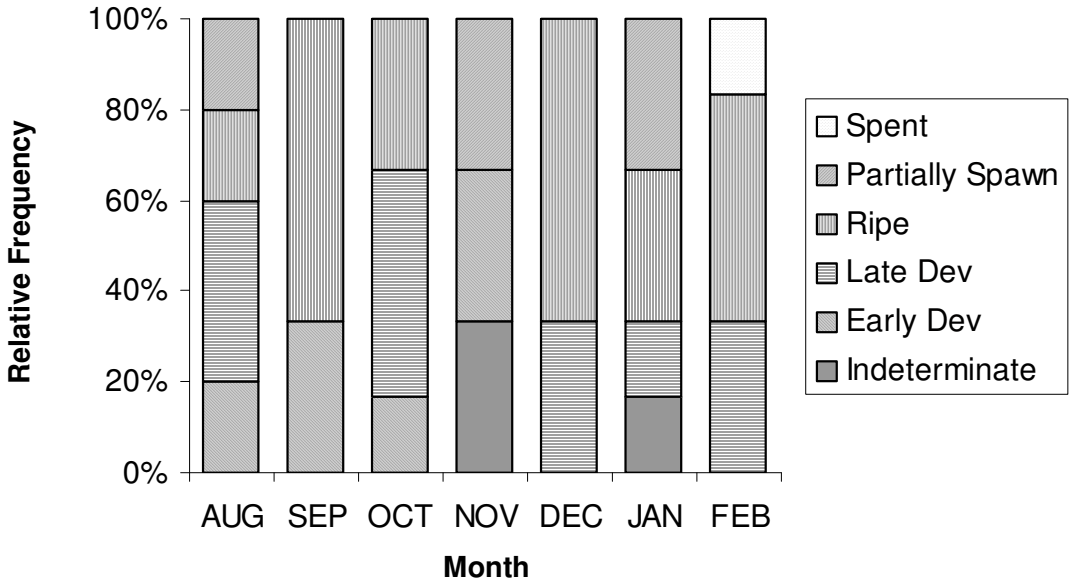

Fig. 7.2. Relative frequency (%) of gonad stages for *P. margaritifera* held at Magnetic Island between August 2003 and February 2004.

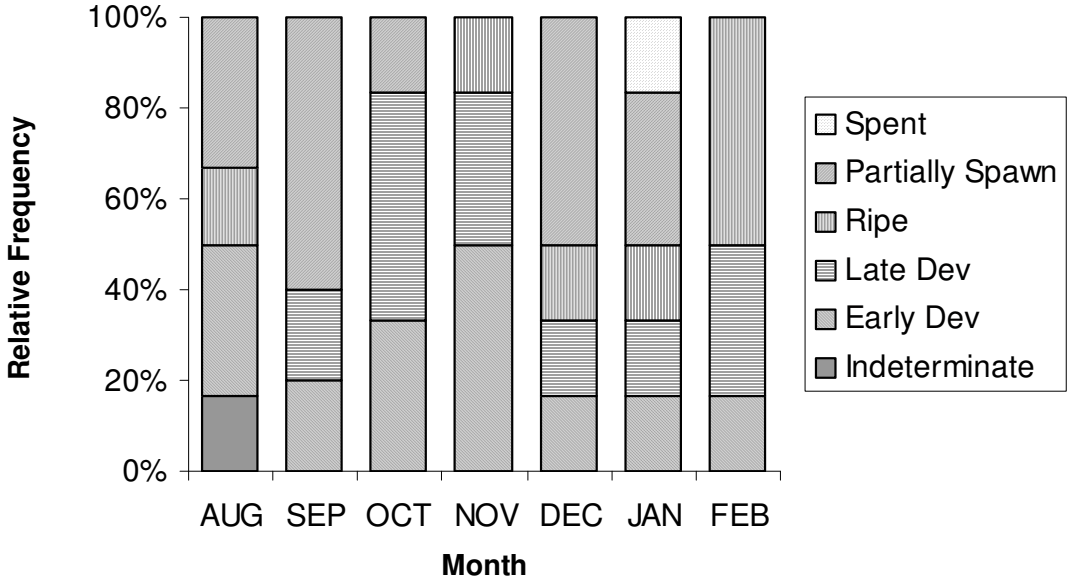

Fig. 7.3. Relative frequency (%) of gonad stages for *P. margaritifera* held at Orpheus Island between August 2003 and February 2004.

7.3.2.1 Gonad tissue

Changes in protein content of gonad tissue showed a different pattern for oysters held at Magnetic Island to those maintained at Orpheus Island where protein content remained relatively constant at around 164.0 mg g^{-1}. Protein content of gonad tissue showed two peaks for oysters held at Magnetic Island in the months of September 2003 and January 2004 of 249.99 ± 126.4 mg g^{-1} and 337.3 ± 22.1 mg g^{-1}, respectively. The lowest gonad protein content in oysters at Magnetic Island was recorded in November 2003 with a mean content of 93.1 ± 43.2 mg g^{-1} (Table 7.1; Fig. 7.4).

Lipid content of gonad tissue for oysters at both sites remained fairly constant around a level of 90 mg g^{-1} from August 2003 to January 2004. There was a rise in the lipid content of oysters held at Magnetic Island from the lowest content in October 2003 (74.5 ± 22.7 mg g^{-1}) to a maximum of 162.4 ± 73.8 mg g^{-1} in February 2004. Oysters maintained at Orpheus Island showed a minimum gonad lipid content of 79.6 ± 22.3 mg g^{-1} in August 2003 increasing steadily to a maximum of 114.5 ± 38.2 mg g^{-1} in December 2003 and decreasing again to 73.8 ± 29.5mg g^{-1} in February 2004 (Table 7.2; Fig. 7.5).

Carbohydrate content of gonad tissue remained relatively constant in oysters at both sites at around 18 mg g^{-1}. However, a pattern of accumulation of gonad carbohydrate was observed from November 2003 (12.4 ± 2.9 mg g^{-1}) to January 2004 (23.1 ± 3.6 mg g^{-1}) for oysters held at Magnetic Island (Table 7.3; Fig. 7.6).

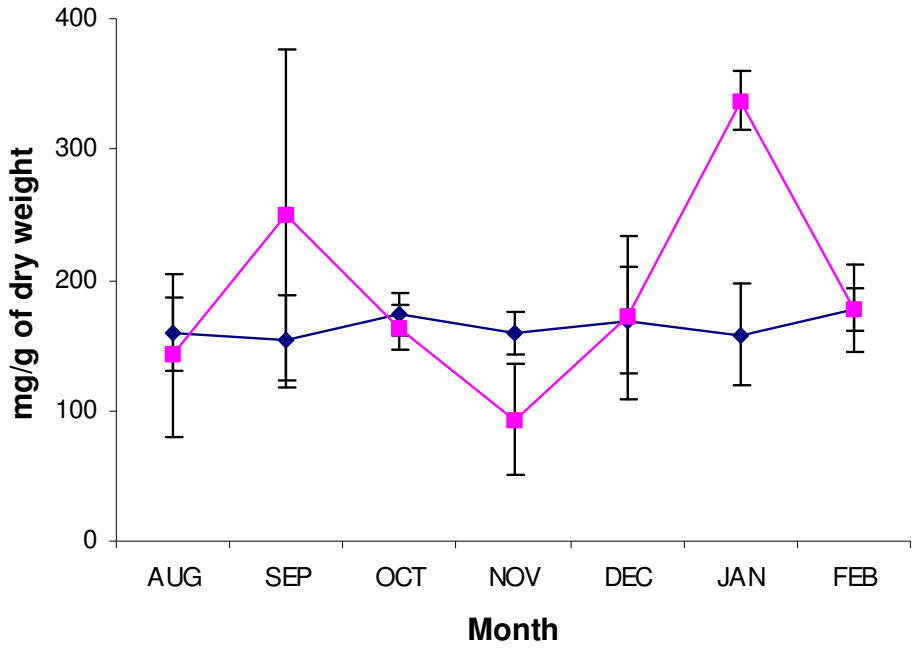

Fig. 7.4. Mean (±SD) monthly protein content of the gonad of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

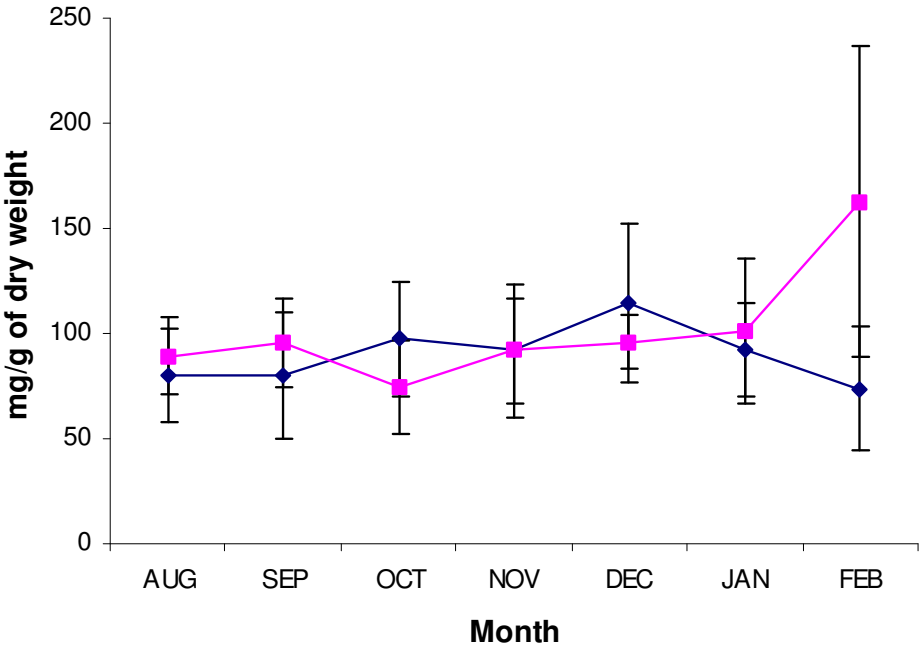

Fig. 7.5. Mean (±SD) monthly lipid content of the gonad of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

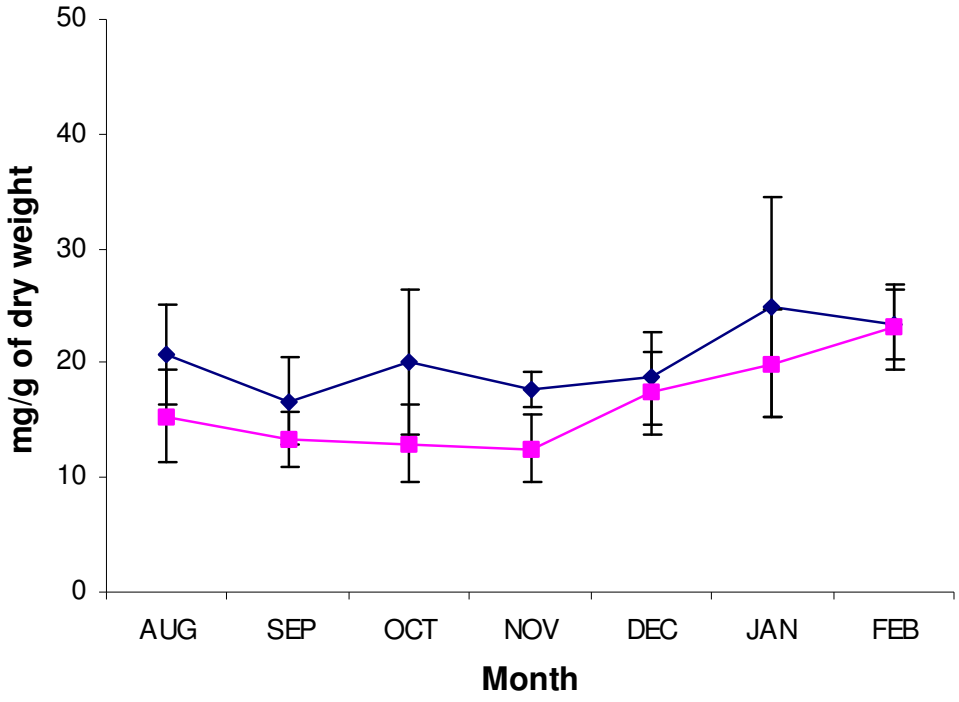

Fig. 7.6. Mean (±SD) monthly carbohydrate content of the gonad of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

7.3.2.2 Digestive gland tissue

Relatively constant protein contents (around 130 mg g^{-1}) were observed for digestive gland tissue of oysters from both Magnetic Island and Orpheus Island. There was no pattern evident of either storage or utilisation of protein in the digestive gland (Table 7.1; Fig. 7.7) although there was a slight increase in oysters held at Orpheus Island from a low of 124.1 ± 11.9 mg g^{-1} in October 2003 to a maximum of 157.3 ± 26.8 mg g^{-1} in January 2004. For oysters held at Magnetic Island protein content in the digestive gland approximately doubled from December 2003 (91.1 ± 15.8 mg g^{-1}) to January 2004 (193.3 ± 51.4 mg g^{-1}).

Lipid content of the digestive gland tissue of oysters held at Magnetic Island showed a minimum value of 55.8 ± 14.8 mg g^{-1} in October 2003 but accumulated in the

following months to reach a maximum of 129.2 ± 12.7 mg g^{-1} in February 2004. Oysters held at Orpheus Island showed accumulated lipid in the digestive gland tissue from September (102.3 ± 33.7 mg g^{-1}) to January 2004 (131.9 ± 30 mg g^{-1}) followed by a sharp decrease to 88.4 ± 22.6 mg g^{-1} in February 2004 (Table 7.2; Fig. 7.8).

Carbohydrate content of the digestive gland of oysters held at Orpheus Island remained relatively constant at around 29 mg g^{-1} with maximum content in August 2003 (33.7 ± 1.9 mg g^{-1}) and January 2004 (32.1 ± 6.4 mg g^{-1}). Oysters held at Magnetic Island showed the minimum carbohydrate content in August 2003 (19.9 ± 3.0 mg g^{-1}) with the highest level recorded in February 2004 (38 ± 8.2 mg g^{-1}) (Table 7.3; Fig. 7.9)

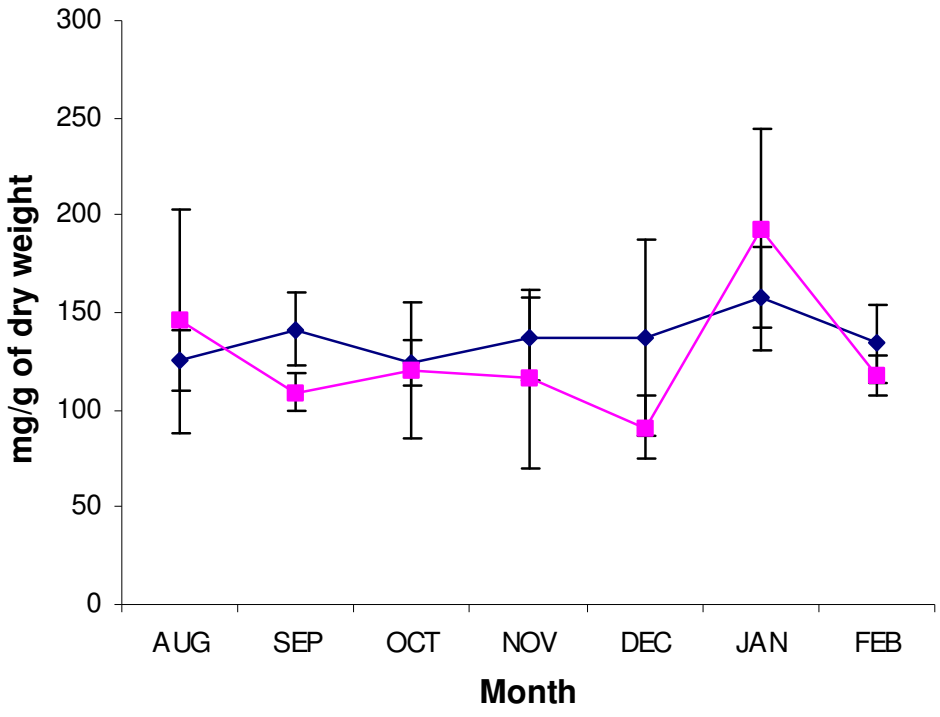

Fig. 7.7. Mean (±SD) monthly protein content in the digestive gland of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

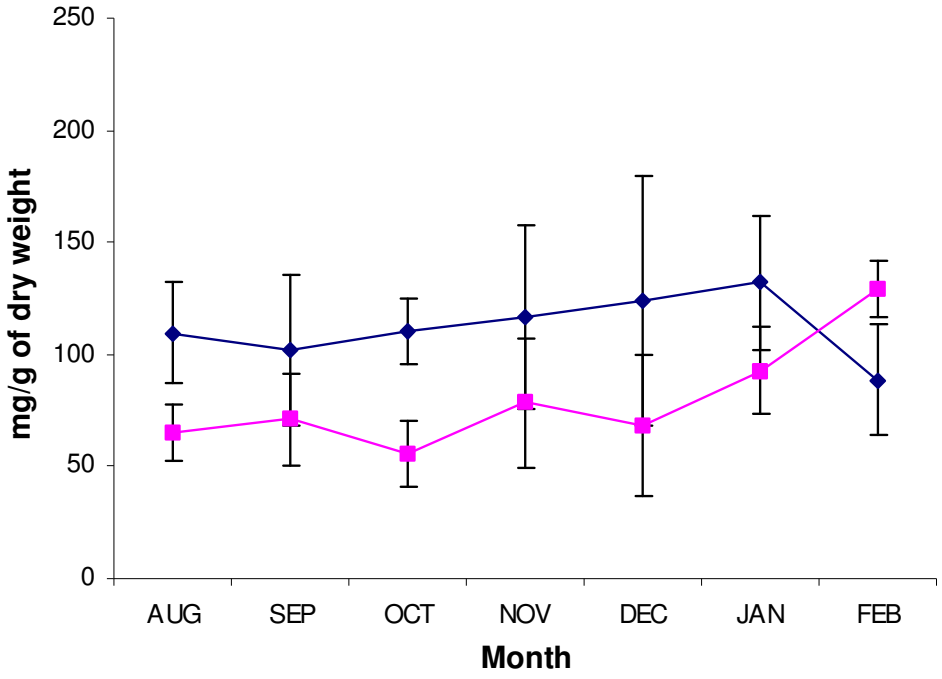

Fig. 7.8. Mean (±SD) monthly lipid content in the digestive gland of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

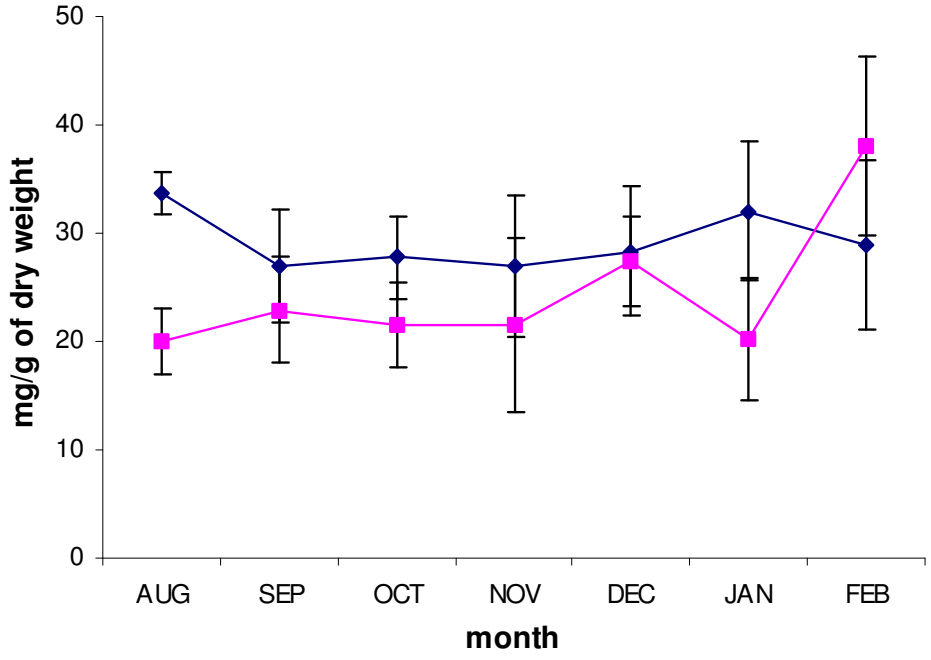

Fig. 7.9. Mean (±SD) monthly carbohydrate content in the digestive gland of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

7.3.2.3 Mantle tissue

A similar pattern of protein content was observed in the mantle tissue of oysters held at both sites from October 2003 to February 2004. Protein content of mantle tissue for oysters held at Orpheus Island remained within a relatively narrow range throughout the seven month study period. Protein content varied from a maximum of 107 ± 43.4 mg g^{-1} in August 2003 and 109 ± 28.9 mg g^{-1} in November 2003 to a minimum of 70.2 ± 12.9 mg g^{-1} in February 2004 (Table 7.1; Fig. 7.10). Lipid content of mantle tissue of oysters held at Orpheus Island increased from 65.2 ± 14.5 mg g^{-1} in September 2003 to a maximum of 80.9 ± 24.3 mg g^{-1} in November 2003 followed by a sharp decrease in December 2003 (50.3 ± 5.9 mg g^{-1}) and a further increase to 75.6 ± 23.6 mg g^{-1} in January 2004 (Table 7.2; Fig. 7.11). Carbohydrate content in the mantle tissue of oysters held at Orpheus Island remained relatively constant at around 13 mg g^{-1} from August 2003 to November 2003. Accumulation of carbohydrate in the mantle tissue was observed from December 2003 (11.1 ± 2.2 mg g^{-1}) to February 2004 (16.4 ± 4.1 mg g^{-1}) (Table 7.3; Fig. 7.12).

Oysters held at Magnetic Island showed 2 peaks of protein content in mantle tissue in September 2003 (147.9 ± 84.3 mg g^{-1}) and January 2004 (152.6 ± 71 mg g^{-1}) while the lowest protein content was observed in October 2003 (58.2 ± 14.4 mg g^{-1}) (Table 7.1; Fig. 7.10). Lipid content in mantle tissue remained relatively steady from August to December 2003 at an average of 50 mg g^{-1} and then increased up to a maximum of 85.7 ± 29.5 mg g^{-1} in February 2004 (Table 7.2; Fig. 7.11). A similar pattern was observed for carbohydrates. Carbohydrate content of mantle tissue remained relatively constant at around 7 mg g^{-1} from August to December and then increased up to a maximum of 17.6 ± 3.9 mg g^{-1} in February 2004 (Table 7.3; Fig. 7.12).

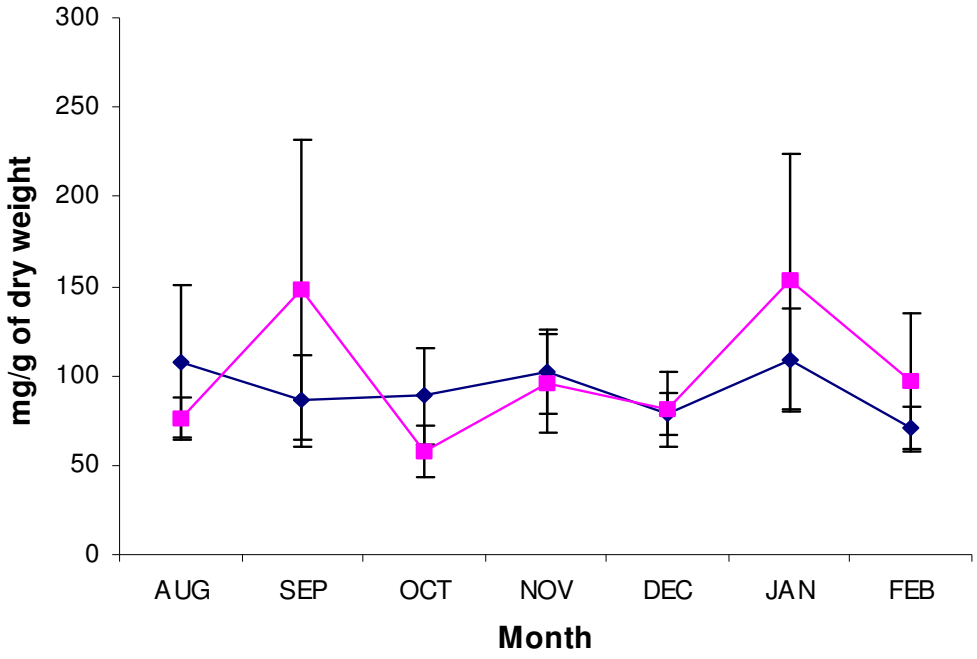

Fig. 7.10. Mean (±SD) monthly protein content in the mantle tissue of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

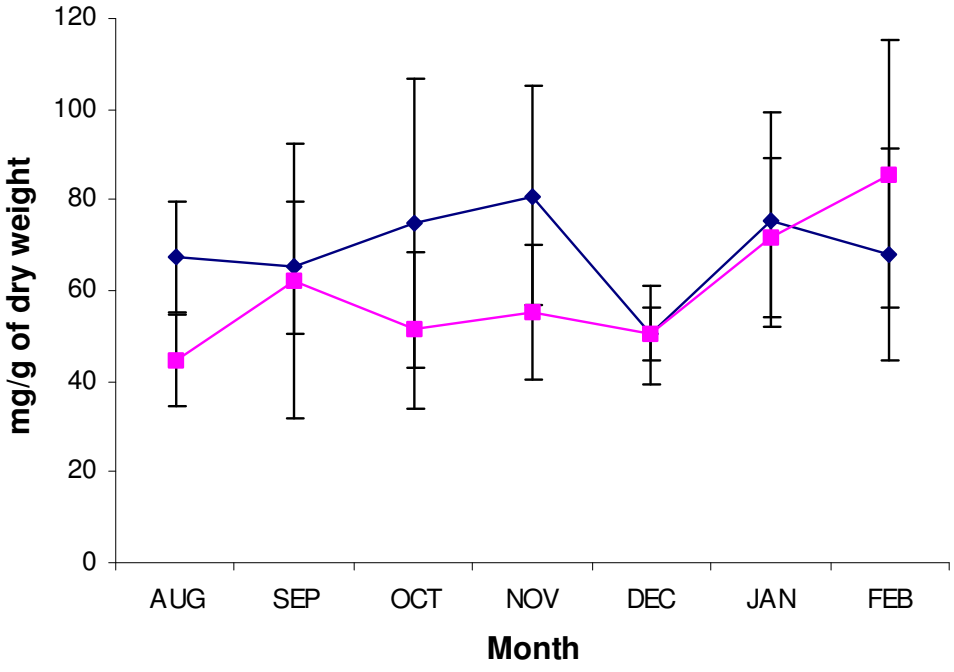

Fig. 7.11. Mean (±SD) monthly lipid content in the mantle tissue of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

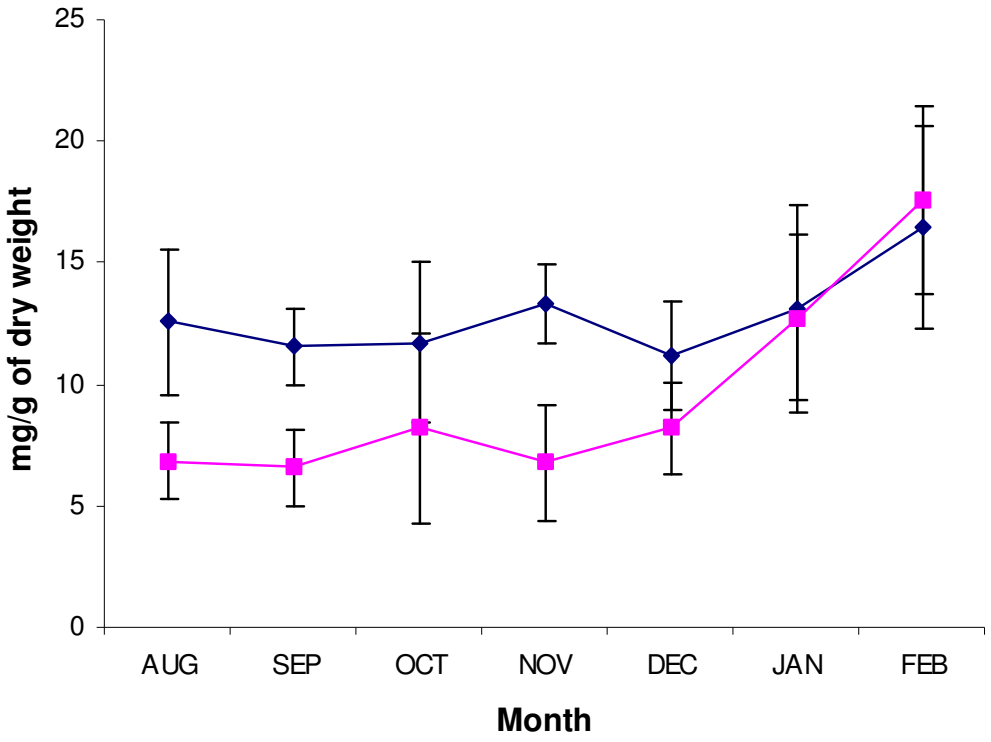

Fig. 7.12. Mean (±SD) monthly carbohydrate content in the mantle tissue of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

7.3.2.4 Adductor muscle

Protein content of adductor muscle declined significantly in oysters held at Magnetic Island from a high of 406.3 ± 101.8 mg g^{-1} in August 2003 to 188.3 ± 44.5 mg g^{-1} in September 2003 with a further reduction to 137.4 ± 127 mg g^{-1} in November 2003 rising again to a secondary peak in January 2004 of 216.6 ± 135.6 mg g^{-1} (Fig. 7.13). In contrast, oysters held at Orpheus Island did not show a particular pattern of storage or mobilisation of protein. Protein content decreased from August 2003 (239.5 ± 64.4 mg g^{-1}) to October 2003 (163.8 ± 69.5 mg g^{-1}) and increased from December 2003 (126 ± 33.9 mg g^{-1}) to February 2004 (239.6 ± 74.6 mg g^{-1})(Table 7.1; Fig. 7.13).

Lipid content of adductor muscle in oysters held at Magnetic Island reduced from a high of 37.9 ± 9 mg g^{-1} in August 2003 to the lowest amount in October 2003 (20.4 ± 8.2 mg g^{-1}) and then increased to a maximum of 67.2 ± 12.9 mg g^{-1} in February

2004. The lipid content of adductor muscle of oysters held at Orpheus Island remained within a range of 40 to 60 mg g^{-1} throughout the seven month experimental period with maximum peaks in August 2003 (55.9 ± 26 mg g^{-1}) and February 2004 (59.9 ± 37.5 mg g^{-1}) and minimum content in December 2003 (35.4 ± 9.7 mg g^{-1}) (Table 7.1; Fig. 7.14). Carbohydrate content of adductor muscle showed a similar pattern between oysters held at both sites. Oysters at Magnetic Island accumulated carbohydrates from a low of 8.3 ± 3.4 mg g^{-1} in November 2003 to a maximum of 62.7 ± 24.9 mg g^{-1} in February 2004. Similarly, carbohydrate content of adductor muscle of oysters held at Orpheus Island increased from 10.5 ± 2.6 mg g^{-1} in September 2003 to 16 ± 4.8 mg g^{-1} in December 2003 and then doubled to a maximum of 39.4 ± 12.8 mg g^{-1} in February 2004 (Table 7.1; Fig. 7.15).

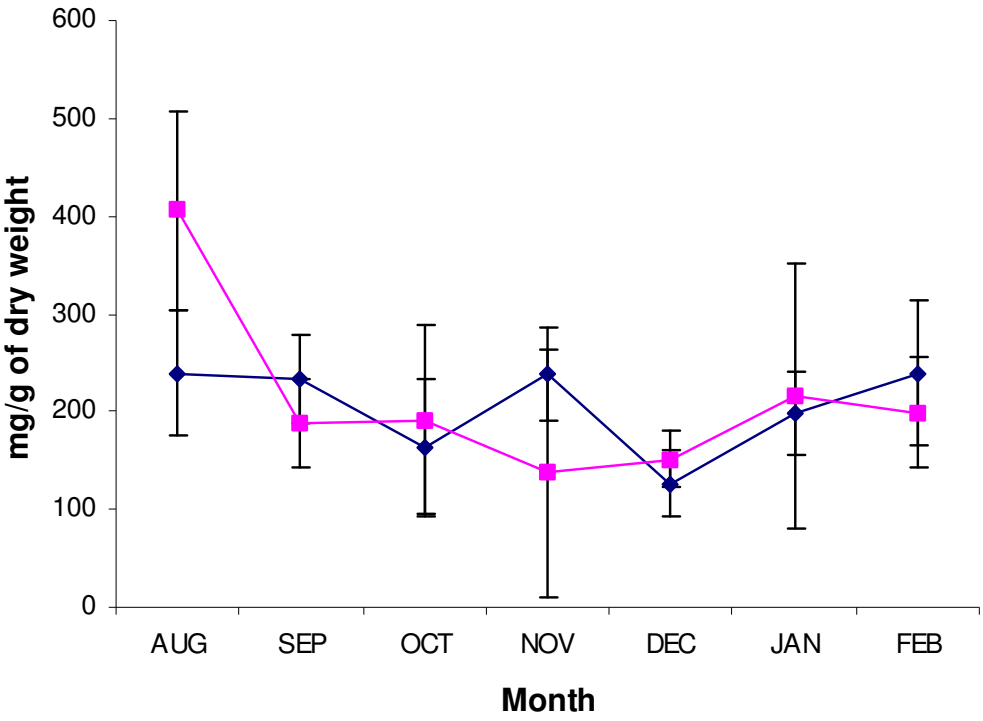

Fig. 7.13. Mean (±SD) monthly protein content in the adductor muscle of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

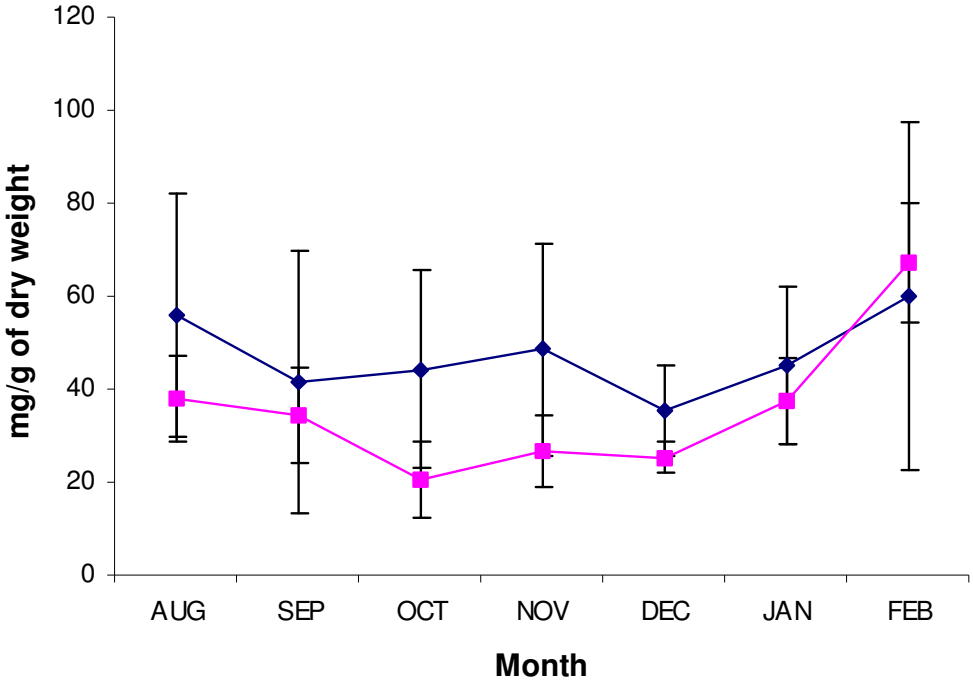

Fig. 7.14. Mean (±SD) monthly lipid content in the adductor muscle of *P. margaritifera* at at Orpheus Island (◆) and Magnetic Island (■).

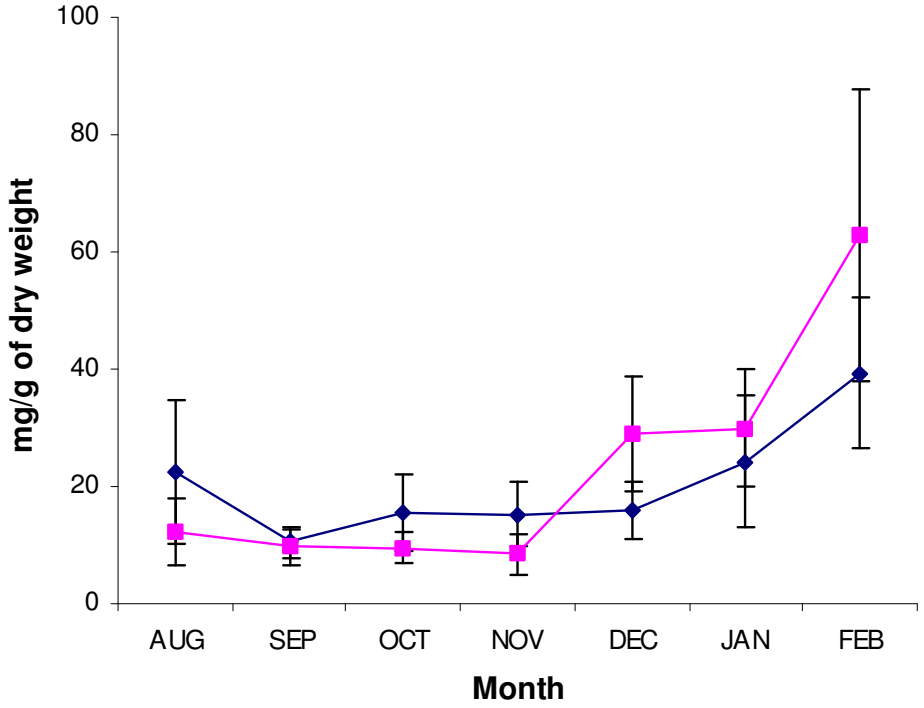

Fig. 7.15. Mean (±SD) monthly carbohydrate content in the adductor muscle of *P. margaritifera* at Orpheus Island (◆) and Magnetic Island (■).

Table 7.1. Mean (±SD) protein content of the digestive gland (D), gonad (G), mantle (M) and adductor muscle (S) of *P. margaritifera* over a seven month period from August 2003 to February 2004 at Orpheus Island (OI) and Magnetic Island (MI). Values are mg g^{-1} dry weight.

	AUG	SEP	OCT	NOV	DEC	JAN	FEB
D-OI	125.5 (±15.6)	141.2 (±18.8)	124.1 (±11.9)	136.6 (±21.5)	137.5 (±50.7)	157.3 (±26.8)	134.0 (±19.8)
D-MI	145.9 (±57.4)	109.0 (±9.7)	120.1 (±34.6)	116.0 (±45.9)	91.1 (±15.8)	193.3 (±51.4)	117.5 (±10.8)
G-OI	158.9 (±27.7)	153.0 (±36.0)	173.4 (±16.2)	159.4 (±16.6)	168.8 (±40.6)	157.6 (±38.9)	177.2 (±15.7)
G-MI	142.4 (±61.9)	250.0 (±126)	163.6 (±17.2)	93.1 (±43.2)	171.2 (±62.6)	337.3 (±22.1)	177.8 (±33.5)
M-OI	107.0 (±43.4)	86.1 (±25.7)	88.7 (±27.2)	102.1 (±23.7)	78.8 (±11.4)	109.1 (±28.9)	70.3 (±12.9)
M-MI	76.4 (±10.8)	148.0 (±84.3)	58.2 (±14.4)	96.0 (±27.7)	81.5 (±21.3)	152.6 (±71.0)	97.2 (±38.1)
S-OI	239.5 (±64.4)	233.8 (±45.9)	163.8 (±69.5)	237.4 (±47.6)	126.0 (±33.9)	198.8 (±43.0)	239.6 (±74.6)
S-MI	406.3 (±101)	188.3 (±44.5)	191.0 (±98.5)	137.2 (±127)	151.7 (±28.5)	216.7 (±135)	199.5 (±56.7)

Table 7.2. Mean (±SD) lipid content of the digestive gland (D), gonad (G), mantle (M) and adductor muscle (S) of *P. margaritifera* over a seven month period from August 2003 to February 2004 at Orpheus Island (OI) and Magnetic Island (MI). Values are mg g^{-1} dry weight.

	AUG	SEP	OCT	NOV	DEC	JAN	FEB
D-OI	109.7 (±22.1)	102.3 (±33.7)	110.5 (±14.8)	116.9 (±41.1)	124.0 (±55.8)	131.9 (±30.0)	88.4 (±24.6)
D-MI	64.9 (±12.6)	70.9 (±20.0)	55.8 (±14.8)	78.5 (±29.1)	68.8 (±31.5)	92.8 (±19.6)	129.2 (±12.7)
G-OI	79.6 (±22.3)	80.3 (±30.2)	97.3 (±27.4)	91.7 (±31.3)	114.5 (±38.2)	92.2 (±22.6)	73.8 (±29.5)
G-MI	88.9 (±18.3)	95.6 (±20.9)	74.5 (±22.7)	92.0 (±25.2)	96.1 (±13.1)	101.1 (±34.9)	162.4 (±73.8)
M-OI	67.5 (±12.3)	65.2 (±14.5)	74.8 (±32.0)	81.0 (±24.3)	50.3 (±5.9)	75.6 (±23.6)	68.2 (±23.4)
M-MI	44.5 (±10.0)	62.2 (±30.3)	51.3 (±17.2)	55.2 (±14.7)	50.2 (±11.0)	71.7 (±17.4)	85.7 (±29.5)
S-OI	55.9 (±26.0)	41.6 (±28.3)	44.2 (±21.3)	48.6 (±22.9)	35.4 (±9.7)	45.0 (±17.0)	59.9 (±37.5)
S-MI	38.0 (±9.0)	34.5 (±10.3)	20.5 (±8.2)	26.8 (±7.8)	25.2 (±3.3)	37.3 (±9.1)	67.3 (±12.9)

Table 7.3. Mean (±SD) carbohydrate content of the digestive gland (D), gonad (G), mantle (M) and adductor muscle (S) of *P. margaritifera* over a seven month period from August 2003 to February 2004 at Orpheus Island (OI) and Magnetic Island (MI). Values are mg g^{-1} dry weight.

	AUG	SEP	OCT	NOV	DEC	JAN	FEB
D-OI	33.8 (±1.9)	27.0 (±5.2)	27.8 (±3.8)	26.9 (±6.6)	28.3 (±5.9)	32.1 (±6.4)	29.0 (±7.8)
D-MI	20.0 (±3.0)	22.9 (±4.8)	21.6 (±3.9)	21.6 (±8.0)	27.4 (±4.1)	20.2 (±5.7)	38.1 (±8.2)
G-OI	20.8 (±4.4)	16.7 (±3.8)	20.2 (±6.3)	17.7 (±1.5)	18.7 (±4.1)	24.9 (±9.6)	23.4 (±3.1)
G-MI	15.4 (±4.1)	13.3 (±2.4)	12.9 (±3.4)	12.5 (±2.9)	17.4 (±3.6)	20.0 (±4.7)	23.1 (±3.6)
M-OI	12.6 (±3.0)	11.6 (±1.6)	11.7 (±3.3)	13.3 (±1.6)	11.2 (±2.2)	13.1 (±4.3)	16.5 (±4.1)
M-MI	6.8 (±1.6)	6.6 (±1.5)	8.2 (±3.9)	6.8 (±2.4)	8.2 (±1.9)	12.7 (±3.4)	17.6 (±3.9)
S-OI	22.5 (±12.1)	10.5 (±2.6)	15.6 (±6.6)	15.3 (±5.5)	16.0 (±4.8)	24.1 (±11.2)	39.4 (±12.8)
S-MI	12.3 (±5.7)	9.6 (±2.9)	9.4 (±2.6)	8.4 (±3.4)	28.9 (±9.8)	30.0 (±9.9)	62.7 (±24.9)

7.4 Discussion

The sex ratio of oysters reported in this study is similar to that reported in other studies on pearl oysters where male oysters were more abundant than females under culture conditions. Pearl oysters are protandric hermaphrodites (Gervis and Sims, 1992). Once oysters reach a particular age or size a sex change from male to female often occur (Tranter, 1958c, d; Wada, 1991; Gervis and Sims, 1992; Saucedo and Monteforte, 1997). Culture conditions are known to affect this sex change in pearl oysters. For example, sex ratios between 8.3:1 and 1.44:1 (male:female) have been reported for cultured pearl oyster *P. mazatlanica* (Saucedo and Monteforte, 1997; Saucedo et al., 2002b), while sex ratios of 1:1, 1.33:1 and 1.95:1 (female:male) have been reported for wild populations of pearl oysters (Tranter, 1958d; Garcia-Dominguez et al., 1996; Saucedo et al., 2002b). Personal observations for oysters held at Orpheus Island (while analysing data from Chapter 2) suggests a sex ratio of 10:1 (male:female) for young *P. margaritifera* oysters under 110 mm shell height and 4:1 (male:female) for older oysters above 125 mm shell height. The 'better' sex ratio found in the present Chapter for oysters held at this same site may have resulted from a reduction of culture stress caused by cleaning old-growth fouling on a significant proportion of the oyster stock prior to the start of the experiment, thus allowing better water flow and feeding.

Differences in sex ratios between culture sites are likely to be caused by environmental parameters such as nutrient availability. Magnetic Island is a near-shore island influenced by nutrient runoff which influences environmental conditions such as water temperature, salinity and suspended matter (McCulloch et al., 2003). In contrast Orpheus Island has a 'mid-shelf' location which is influenced less by coastal runoff resulting in more constant environmental conditions. Considerable variation in environmental parameters has been recorded within the Great Barrier Reef lagoon in

relation to distance from the coast and proximity to major sources of run-off (McCulloch et al., 2003).

Indication that culture or adverse environmental conditions affect sex ratios has been documented for a number of pearl oyster species (Dolgov, 1991; Gervis and Sims, 1992; Saucedo and Monteforte, 1997; Saucedo et al., 2002b). This may be influenced by factors such as nutritional stress caused by culture densities and associated fouling. Clearance rates in *P. margaritifera* can be as high as 2,400 to 2,800 L per oyster per day (Yukihira et al., 1998; Pouvreau et al., 2000a, b). On an average size pearl farm, for example, pearl oysters would deplete the phytoplankton of a significant amount of water every day. If water exchange is not sufficient, a constant (chronic) nutritive stress (aggravated by slow accumulation of suspension-feeding biofouling) may prevent pearl oysters from developing the energetically expensive female gonad, resulting in a greater proportion of males in pearl oyster populations under culture conditions.

The gametogenic cycle observed for *P. margaritifera* at Orpheus Island and Magnetic Island followed closely that reported for the same species in the Torres Strait (Queensland, Australia), approximately 1,800 km north of the present study sites (Tranter, 1958d). Oyster populations at Magnetic Island and Orpheus Island contained partially spawned individuals in August-September coinciding with the winter spawning event reported for the Torres Strait population (Tranter, 1958d). A decrease in gonad condition was observed in oysters at Orpheus Island and Magnetic Island from September to November followed by a fast development and spawning in December-January, which coincided with the spawning pattern for this species in the Torres Strait (Tranter, 1958d).

Table 7.4 describes patterns of nutrient management on specific body tissues for different marine bivalves in relation to their breeding cycle. In general, nutrients are

stored in a given body tissue(s) in the early stages of gonad development and are then used to complete late gonad development and spawning. In other bivalve species, the adductor muscle has been reported to play a very important role as a nutrient storage site and is the major source of nutrients for gonad development during the reproductive cycle of many bivalve species (Barber and Blake, 1981; Racotta et al., 1998; Martinez and Mettifogo, 1998). For example, adductor muscle protein was used by the scallop *A. irradians concentricus* to meet maintenance energy demand during late gonad development and spawning while the use of lipid and glycogen was associated with early oocyte growth (Barber and Blake, 1981).

For *P. margaritifera*, adductor muscle showed the highest protein content of the four tissues analysed. Protein content of adductor muscle in *P. margaritifera* correlated closely with reproductive stage. Maximum content of protein in the adductor muscle was observed when gonads were in late development or ripe stages (August 2003 and January-February 2004). Protein content decreased when there was a higher proportion of partially-spawned oysters (November 2003) indicating that adductor muscle protein is likely to be a major energy source for late gamete development and spawning. This pattern differs from that reported for other bivalve species including the pearl oyster, *P. mazatlanica*, where protein level in adductor muscle was at its lowest when the proportion of late development and ripe stages were more abundant (Zandee et al., 1980; Barber and Blake, 1981; Saucedo et al., 2002a).

Table 7.4. Comparison of protein (PRO), lipid (LIP), glycogen (GLY) and carbohydrate (CHO) management in different bivalve species related to their gametogenic cycles. ND: not determined.

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Changes in the gonad lipid content seemed to follow a similar pattern to that of the digestive gland. The lowest lipid content for both tissues was recorded in September-October 2003 and the maximum lipid content was recorded in January-February 2004. This coincided with the pattern reported for *P. mazatlanica* where gonad and digestive gland lipid followed the same pattern of storage and utilisation (Saucedo et al., 2002a).

Carbohydrate content in the gonad of *P. margaritifera* followed a similar pattern to the carbohydrate content of the digestive gland. This differed from the pattern in *P. mazatlanica* where an inverse relationship between carbohydrate content in both tissues was observed (Saucedo et al., 2002a). Such differences have been attributed to inter-species differences in use and storage of nutrients in other bivalve species (Barber and Blake, 1981; Pazos et al., 1997; Saucedo et al., 2002a)

This pattern (lowest values in September-October 2003 and highest in January-February 2004) was observed for carbohydrate content of the adductor muscle, gonad and mantle tissues, suggesting that these nutrients are being accumulated regardless of the stage of gonad development.

Mantle tissue seemed to contain more carbohydrate towards the summer spawning peak. In contrast, glycogen content of the mantle tissue of *M. edulis*, decreased during gonad development and the lowest content was recorded at the moment of spawning (Zandee et al., 1980).

Protein and carbohydrate content of the digestive gland were of similar magnitude to those reported for *P. mazatlanica* (Saucedo et al., 2002a). However lipid content of the digestive gland and gonad was higher for *P. margaritifera* in this study. This difference may have been influenced by the different techniques used for lipid determination in the two studies. On the other hand, carbohydrate content in the

adductor muscle of *P. margaritifera* was lower than that reported for *P. mazatlanica* (Saucedo et al., 2002a).

The progressive storage of nutrients (i.e. carbohydrates in mantle tissue, adductor muscle and gonad and lipid in the digestive gland) independent of gonad development stage may indicate that these nutrients are not being used to meet energy demands for gamete production but rather they are generally accumulated when the environment permits (i. e. in relation to food availability). Natural food supply may be sufficient to cover the energy demand of the summer spawning, while utilization of nutrient reserves may be required for gonad maturation and spawning in cooler months. The general trend of nutrient storage towards the summer spawning peak may be related to phytoplankton availability. Chlorophyll 'a' in Magnetic Island and Orpheus Island shows the highest concentration between October and December (spring- early summer) and remains relatively constant from February to June and then decreases during the coldest months (July-August) (Pit, 2004)

Major seasonal changes in nutrient composition were observed in the gonad and adductor muscle of *P. margaritifera*. These tissues could be sampled for biochemical analysis by using biopsy needles (Chapter 4) to correlate with gonad stage for a reproductive annual cycle. This would help to identify the winter spawning peak and determine whether stored nutrients are used for gonad growth during the cooler months. The results of this study showed that the adductor muscle plays an important role in energy management in *P. margaritifera*. This is similar to the findings of studies with other bivalves (Barber and Blake, 1981; Pazos et al., 1997; Racotta et al., 1998; Martinez and Mettifogo, 1998) including pearl oysters (Saucedo, et al., 2002a)

This study is the first to report on the link between histological and biochemical changes related to reproductive season in the blacklip pearl oyster *P. margaritifera*.

Results from this study suggested that *P. margaritifera* can obtain not only the necessary nutrients to achieve gonad maturation from its natural food sources but can also store excess nutrients for future use. Winter and summer reproductive peaks were confirmed for *P. margaritifera* in north Queensland. The results indicate that hatchery spawning inductions may be attempted with higher degree of success in August-September and December-January when a higher proportion of ripe oysters was recorded. These findings will be useful in developing improved broodstock management protocols for *P. margaritifera*.

Chapter 8 Micronutrient changes during embryogenesis

8.1 Introduction

Embryogenesis is a process where major anatomical changes occur at the expense of stored reserves. For these changes to occur, a minimum level of energy must be provided in the egg to fuel development to a stage where larvae can fed exogenously (Bayne et al., 1975). In molluscs this energy is provided by maternally-derived nutrient reserves.

Compositional analysis of egg and larval prior to first-feeding is often a first step in understanding the likely nutritional requirements of larvae once feeding begins. For example, ratios and percentages of key polyunsaturated fatty acids (PUFAs) in eggs are often taken as a reflection of likely dietary needs (Sargent et al., 1999). In addition, changes in the amounts and ratios (due to oxidation or conversions) of these PUFAs, as well as vitamins and major biochemical fractions (e.g. protein, carbohydrates and lipids) during larval ontogeny of bivalve molluscs have provided information on nutrient and energetic requirements of first-feeding larvae (Whyte et al., 1990; Seguineau et al., 1996).

Poor appetite and poor food conversion efficiency has been reported for other marine invertebrates such as the prawn *Penaeus monodon* when fed diets lacking the fat-soluble vitamins A, D, and E and ten water-soluble vitamins (Reddi et al., 1999). Other micronutrients such as carotenoids exert influence in the reproductive capacity of some invertebrates, such as gonad growth, fecundity, embryo malformation and development, larval size and survival and juvenile production (Vershinin and Lukyanova, 1993; DeJong-Westman et al., 1995a,b; Tsushima et al., 1997; George et al., 2001).

There are no previous studies on utilisation of carotenoids by bivalve embryos or larvae, and only a few studies have reported on their utilisation of vitamins. As pearl oysters are mostly tropical species and their breeding cycle is during the warmer months of the year (Chapter 7), carotenoids and vitamins may play an important antioxidant role during embryo development due to the high water temperatures and their rapid development rate.

The aim of this Chapter was to determine changes in levels of micronutrients in the tissues of embryos and early larvae of the blacklip pearl oyster *P. margaritifera*. The specific objective was to identify significant utilization or preservation of particular nutrients during embryological development. Clearly such nutrients would be considered important during embryological development and their identification has obvious implications for determination of egg and larval quality and selection of appropriate broodstock diets.

8.2 Materials and methods

P. margaritifera adults were induced to spawn using the technique described in Chapter 2. Once eggs were fertilised, the developing embryos were stocked into ten 500 L fibreglass tanks at a density of 50 embryos per mL. Triplicate samples for determination of micronutrient content were randomly taken from the replicate tanks at 0, 4, 8, 12, 16, 24 and 46 h after fertilisation collecting larvae through a 25 µm sieve. Samples of eggs and larvae were filtered through micro fibreglass filters (Whatman), washed with 3.2 % (w/v) ammonium formate, frozen in liquid nitrogen and stored at – 80 °C until transported to CSIRO Marine Research Laboratories, Hobart, Tasmania.

At each sampling time separate samples containing at least 100 eggs, embryos or larvae were placed in 5 mL vials with larval preservative (Culliney et al., 1973) for further measuring.

8.2.1 Analysis of carotenoids

Analysis of samples for carotenoids was undertaken using HPLC. Prior to sample analyses standard calibration curves were established by injecting 0 to 400 ng of carotenoid standards (β-carotene, astaxanthin and lutein). In an initial assessment, a single sample was extracted and analysed to assess whether carotenoids could be detected and quantified. The sample (previously stored in liquid nitrogen) was added to a 10 mL plastic test tube, together with 3.0 mL of 90% acetone at 4ºC (Wright and Jeffrey, 1997). The sample was vortexed and sonicated (Labsonic 1510 sonic probe; 30 s at 100 W) to facilitate extraction, then placed at 4ºC overnight. The sample was then filtered and carotenoid content was analysed using a Waters HPLC system with an Alltech Alltima C18 column, 4.6 x 250 mm column eluted with a ternary solvent system of: a) 80:20 methanol: 0.5 M ammonium acetate (pH 7.2); b) 90% acetonitrile; and c) ethyl acetate (Wright and Jeffrey, 1997). Carotenoids were detected using a Waters 996 photodiode array detector and quantified using Millenium software (Waters™).

8.2.2 Analysis of Ascorbic acid (AsA)

Egg and larval samples were extracted using metaphosphoric acid (3%) and acetic acid (8%) at 4°C, and the extracted Ascorbic acid (AsA) was derivatised by incubating with ascorbate oxidase and subsequently with *o*-phenylenediamine (Brown and Miller, 1992). The fluorescent derivative was analysed by reverse-phase HPLC

using an Alltech Alltima C18, 4.6 x 250 mm column eluted isocratically with 80:20 (v/v) 0.08 M potassium dihydrogen phosphate (pH 7.8) and methanol, respectively, at a flow rate of 1.0 mL min^{-1}. The derivatized product was detected using a Waters Model 475 scanning fluorescence detector with the excitation maximum set at 355 nm and emission maximum at 425 nm. The peak area was quantified using Waters Millenium software.

8.2.3 Analysis of α-Tocopherol (α-T)

Egg and larval samples were transferred to 10 mL plastic centrifuge tubes, together with 2.0 mL of methanol containing 2 mg of butylated hydroxy-toluene as antioxidant (Huo et al., 1999). Samples were sonicated, vortexed and left to extract at room temperature for 1 h. Samples were vortexed again, centrifuged (1000 g, 10 min) and the supernatant was filtered through a 0.45 μm syringe filter (Alltech). α-Tocopherol (α-T) was detected using the same HPLC system as described above (section 8.2.2), except the fluorescence detector was set for an excitation maximum 292 nm and emission maximum at 330 nm. The column used was a C18 from SGE; 4.6 x 250 mm, which was eluted isocratically with 100% methanol at a flow rate of 1.0 mL min^{-1}.

8.2.4 Analysis of fatty acids

Fatty acid methyl esters (FAME) were derived directly from the freeze-dried samples, by heating them in a solution of methanol:chloroform:hydrochloric acid (10:1:1) under nitrogen at 80ºC for 2 h. After extraction and concentration an internal standard was added and the FAME samples were analysed with a HP5890A gas chromatograph. A non-polar cross-linked 5% Ph Me Silicone (HP-5) fused-silica capillary column (50 m x 0.32 mm i.d. x 0.17 μm film thickness) was used for the

analysis (Agilent Technologies, USA). Samples were injected at 50°C, after 1 minute the oven temperature was raised to 150 °C at 30 °C per minute and then to 250°C at 2°C per minute, and finally to 300 °C at 5 °C per minute. The final temperature was maintained for 15 minutes. Helium was used as the carrier gas. The injector temperature was 290°C and detector temperature was 310°C. Peak areas were quantified with Millennium software (Waters) on an IBM-compatible computer.

8.3 Results

Dry weight per egg or larva was calculated for all samples analysed (Appendix 1). Average data from samples collected at each sampling time is shown in Fig. 8.1. From an initial mean (±SD) value of 19.8 (± 0.32) ng per egg, values showed small changes over the first 12 h post-fertilisation; thereafter values steadily increased up to 187 (± 65.8) ng per larva at 46 h after fertilisation.

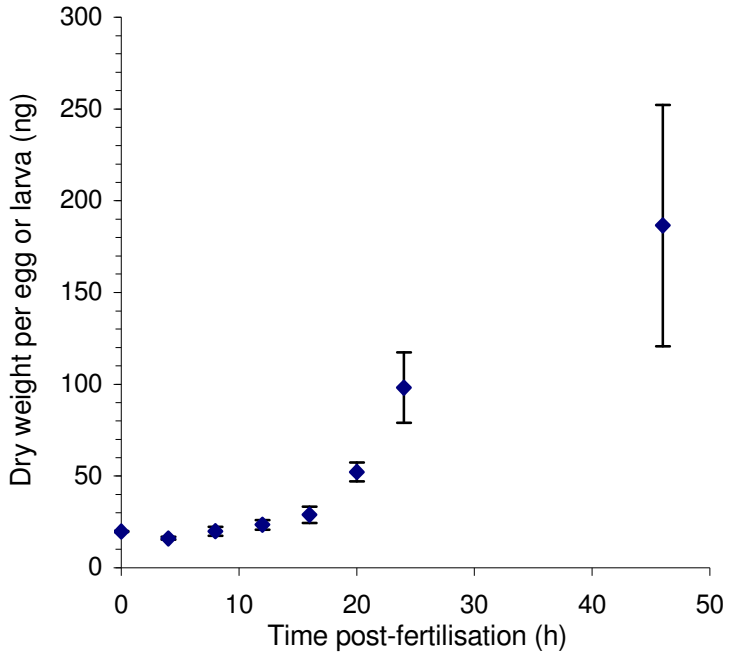

Fig. 8.1. Changes in mean (± S.D.) dry weight per egg or larva of *P. margaritifera* up to 46 h after fertilisation.

8.3.1 Analysis of carotenoids

The analysis showed several small peaks, but because of their low concentrations they could not be accurately identified or quantified. Based on this result, it was concluded that the concentration of carotenoids (i.e. at least β-carotene, astaxanthin and lutein) was beyond the limits of accurate quantitation (< 1.5 to 2 μg g^{-1} dry weight).

8.3.2 Analysis of Ascorbic acid

There was no consistent trend in the concentrations (μg g^{-1} dry weight AsA) or content (pg per egg or larva) of AsA in egg or larvae during embryo development (Table 8.1). Only trace amounts of AsA were detected in unfertilised eggs. After 8 h, AsA content apparently increased, with highest values after 24 h (19.0 pg per larva). On a weight-specific basis, larvae contained their highest level of AsA after 16 h (315 μg g^{-1}).

8.3.3 Analysis of α-Tocopherol (α-T)

There were significant differences in both the concentrations and content of α-T in egg per larvae (ANOVA $P < 0.05$). Content was not different between 0 and 20 h (range 2.9 to 3.9 pg per egg or larva); however, it approximately doubled after 46 h. Concentrations were relatively constant between 0 and 8 h (around 180 μg g^{-1}) and reduced significantly after 20 h (72.8 ± 0.3 μg g^{-1}), and further after 46 h (35.4 ± 1.0 μg g^{-1}) (Table 8.2).

Table 8.1. Mean (± S.D.) concentrations and content of AsA in eggs and larvae of *P. margaritifera.* Values within the same column sharing a common superscript are not significantly different (P > 0.05).

	AsA (μg g^{-1})	AsA (pg per egg or larva)
Sample time	**Mean ± SD**	**Mean ± SD**
0 h	4.7± 8.1 ^{d}	0.10± 0.2 ^{f}
4 h	205.1± 58.8 ^{b}	3.78± 1.9 ^{de}
8 h	96.0± 19.3 ^{c}	1.81± 0.3 ^{e}
12 h	233.5± 21.5 ^{b}	5.00± 0.2 ^{d}
16 h	315.0± 33.9 ^{a}	9.16± 2.2 ^{c}
24 h	184.3± 18.3 ^{b}	19.05± 4.7 ^{a}
46 h	85.1± 12.8 ^{c}	14.03± 4.1 ^{b}

Table 8.2. Mean (± S.D.) concentrations and content of α-T in eggs and larvae of *P. margaritifera.* Values within the same column sharing a common superscript are not significantly different (P > 0.05).

	α-T (μg g^{-1})	α-T (pg per egg or larva)
Sample time	**Mean± SD**	**Mean± SD**
0h	162.9± 27.3 ^{b}	3.10± 0.51 ^{b}
4h	174.7± 10.0 ^{ab}	2.91± 0.45 ^{b}
8h	196.9± 7.7 ^{a}	3.88± 0.58 ^{b}
20h	72.8± 0.3 ^{c}	3.80± 0.40 ^{b}
46h	35.4± 1.0 ^{d}	7.31± 2.75 ^{a}

8.3.4 Analysis of fatty acids

Percentages of specific fatty acids and fatty acid classes showed some consistent trends during *P. margaritifera* embryo development (Table 8.3). For example, percentages of 14:0 and 16:0 reduced over time, whereas levels of 22:6(n-3) (DHA) increased. Overall, total saturated fatty acids reduced (from 42.6 to 32.4%), whereas total polyunsaturated fatty acids, and to a lesser extent mono-unsaturated fatty acids increased (from 42.7 to 50.0%, and 14.6 to 17.2%, respectively).

Concentration of total fatty acids showed a general downward trend from 142 to 8.8 mg g^{-1}, which was consistent with concomitant increases in larval dry weight over time (Fig. 8.1). The total amount of fatty acids per egg or larvae were not significantly different between the different sampling times (ANOVA; $P > 0.05$) and did not show any consistent trend, though the highest and lowest values were observed at 0 and 46 h, respectively.

8.4 Discussion

Mean dry weight of *P. margaritifera* eggs (20 ng) was similar to values reported for other bivalve species (Mann and Gallager, 1985; Whyte et al., 1991). In 46 h old *P. margaritifera*, mean concentrations of AsA (85.1 μg g^{-1}) and α-T (35.4 μg g^{-1}) were similar to vitamin concentrations reported in 48 h old scallop, *P. maximus*, larvae which ranged from approximately 80 to 120 μg g^{-1} for AsA and 30 to 80 μg g^{-1} for α-T (Seguineau et al., 1993, 1996).

Table 8.3. Percentages of major fatty acid (as % of total fatty acids) in eggs and larvae of *P. margaritifera.*

	Time post fertilisation (h)					
	0	**4**	**8**	**12**	**24**	**46**
Fatty Acid	% of Major Fatty Acids					
14:0	4.7 ± 0.2	4.6 ± 0.0	4.4 ± 0.2	4.0 ± 0.1	3.4 ± 0.1	1.8 ± 0.1
16:0	27.1 ± 1.0	25.8 ± 0.6	25.2 ± 0.8	24.4 ± 0.7	23.0 ± 0.1	18.9 ± 0.4
17:0	2.4 ± 0.1	2.6 ± 0.0	2.6 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.6 ± 0.0
18:0	7.1 ± 0.5	7.5 ± 0.0	7.3 ± 0.0	7.3 ± 0.1	7.8 ± 0.0	8.1 ± 0.3
18:1(n-9) &18:3(n-3)	11.9 ± 0.2	10.1 ± 0.2	10.4 ± 0.1	10.5 ± 0.1	9.7 ± 0.1	7.0 ± 0.1
18:1(n-7)	1.8 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.2 ± 0.0	2.2 ± 0.0	2.0 ± 0.0
18:2(n-6)	2.1 ± 0.1	1.9 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	1.9 ± 0.0	1.4 ± 0.0
18:3(n-6)	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.2 ± 0.0
18:4(n-3)	2.1 ± 0.1	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.5 ± 0.0	0.9 ± 0.0
20:4(n-6)	2.6 ± 0.1	2.8 ± 0.1	2.9 ± 0.0	3.0 ± 0.2	3.6 ± 0.1	5.0 ± 0.1
20:5(n-3)	5.8 ± 0.2	6.3 ± 0.0	6.1 ± 0.1	5.9 ± 0.2	4.9 ± 0.0	4.2 ± 0.1
22:5(n-6)	1.5 ± 0.0	1.6 ± 0.0	1.5 ± 0.2	1.6 ± 0.0	1.7 ± 0.2	3.0 ± 0.0
22:5(n-3)	1.5 ± 0.1	1.4 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.6 ± 0.0	1.5 ± 0.1
22:6(n-3)	17.2 ± 0.8	18.4 ± 0.6	19.0 ± 1.0	19.8 ± 0.5	21.1 ± 0.2	22.1 ± 0.2
22PUFA *	1.4 ± 0.3	1.6 ± 0.1	1.8 ± 0.0	1.9 ± 0.1	3.0 ± 0.1	5.3 ± 0.2
Sum saturated	42.6 ± 1.0	41.9 ± 0.7	40.8 ± 1.1	39.5 ± 1.0	37.8 ± 0.1	32.4 ± 0.6
Sum mono-unsaturated	14.6 ± 0.5	14.5 ± 0.1	14.7 ± 0.0	14.9 ± 0.1	15.5 ± 0.4	17.2 ± 0.2
Sum poly-unsaturated	42.7 ± 0.6	43.4 ± 0.6	44.3 ± 1.1	45.6 ± 0.9	46.6 ± 0.2	50.0 ± 0.5
Total fatty acids (mg g^{-1})	141 ± 24	115 ±24	117 ± 7.4	70.7 ± 8.2	25.2 ± 0.6	8.8 ± 0.3
Total ng fatty acid per egg or larva	2.86 ± 0.4	1.91 ±0.8	2.47 ±0.6	1.77 ±0.1	2.32 ±0.5	1.66 ±0.79

* Refers to non-methylene interupted dienes (NMID 22:2).

No prior study has reported on changes in AsA and α-T during embryological development of a bivalve up to first-feeding, however, similar studies with fish have shown that there is little change from egg to first feeding larvae in their total content of α-T and AsA (Ronnestad et al., 1999; M. Brown, CSIRO, pers. comm. 2004). For *P. margaritifera*, there was no consistent trend in the content of AsA during embryological development. Eggs apparently only contained trace amounts of AsA; however this level increased (though in an apparently random manner) with highest values found in 24 and 46 h larvae. This trend of increasing levels of AsA with time could indicate larval synthesis of AsA, or the liberation of AsA from a stable precursor. The latter occurs within *Artemia* cysts where ascorbyl-sulphate is converted to active (free) ascorbic acid during the hatching process (Merchie et al., 1995). However, a similar process has not been documented for any bivalve species.

The content of α-T in eggs and developing larvae showed no significant change from 0 to 20 h; but approximately doubled after 46 h. Similar to AsA, this result again may indicate that *P. margaritifera* larvae are able to produce α-T *de novo*, or convert a precursor (e.g. an α-T-ester, or related carotenoid) to free α-T; however, there is no literature evidence for this process in other marine animals during embryological development.

All fatty acids decreased on a dry weight basis (Table 8.3), due most likely to the accumulation of inorganic matter for shell production. There were no significant differences on a per egg or per larva basis. The trends in quantitative changes in total fatty acids (per dry weight and per egg or larvae) were similar to the changes in the lipid soluble vitamin, α-T (apart from the elevated 46 h α-T per larva value). On a per dry weight basis there was a rapid decrease in total fatty acids and then a levelling out between 4 h and 8 h post fertilization followed by further decreases. Rapid decline in

the total lipid content of embryos of marine bivalves has been reported to occur within 24-48 h after fertilization (Gallager et al., 1986; Whyte et al., 1991).

The nutritionally important n-3 PUFA were conserved with time, relative to most other fatty acids. There was a slight decrease in the percentage of 20:5(n-3) (EPA) with time while the percentage of 22:6(n-3) (DHA) increased. EPA is a precursor to DHA and the same observations could be a result of DHA production from chain-elongation and desaturation of EPA. It has been suggested that similar changes reported in other bivalves indicate that EPA is catabolized for energy, while DHA is conserved due to its relative importance as a structural lipid (Whyte et al., 1991; Labarta et al., 1999). The n-6 PUFA and in particular 20:4(n-6) (arachidonic acid, AA) also increased relative to the other fatty acids. AA is a common component of structural polar lipids such as phosphatidyl inositol. Also, decreases in the major saturated and monounsaturated fatty acids suggest that they may also play an important role as an energy source prior to the commencement of feeding in *P. margaritifera* larvae.

The C22 NMID (non-methylene interrupted dienes) (22 PUFA, Table 8.3) increased with time post-fertilization. These PUFA are probably synthetised from monounsaturated fatty acids and are found in a number of species, but are particularly evident in bivalves and abalone (Dunstan et al., 1993, 1996). Their function is unknown but in the oyster, *Crassostrea virginica*, they tend to be higher in the polar (structural) lipid fraction whereas the C20 NMIDs (only found in very low amounts in the current trial) are more abundant in the neutral (storage) lipid fraction (Watanabe and Ackman, 1974).

The relatively low amount of antioxidants (carotenoids, AsA and α-T) found in *P. margaritifera* eggs may also be explained by the active use of saturated fatty acids (14:0, 16:0) to obtain energy to reach first-feeding stage. These saturated fatty acids

had reduced by 25% of their initial content 46 h after fertilization while levels of mono-unsaturated and poly-unsaturated increased.

This Chapter contains new information on the use of micronutrients by pearl oyster embryos. Antioxidant (carotenoids and vitamin C and E) content would seem not to be a limiting factor during embryological development of *P. margaritifera* as they were either not present in eggs or they increased in quantity during development. However, the use of particular fatty acids during embryogenesis (previously unreported for any pearl oyster species) provides interesting new information which is likely to have relevance in providing appropriate diets for both broodstock and larvae of *P. margaritifera* and facilitate development of more effective hatchery protocols for this species.

Chapter 9

General discussion and conclusions

9.1 Introduction

Since development of the 'modern' pearl seeding technique by the Japanese in the early 1900's, there have been few changes to the original method which is still widely used. Similarly, the cultured pearl industry has been slow to adopt important advances in aquaculture and other relevant disciplines. Although reliable methods for hatchery culture of bivalves, for example, were developed in the 1960's and 1970's (Loosanof and Davis, 1963; Walne 1974) and further developed for pearl oysters in the 1980's and 1990's (Alagarswami et al., 1983, 1989; Rose et al., 1990; Southgate and Beer, 1997), pearl production from *P. margaritifera* in French Polynesia is still predominantly reliant on wild collected spat. Similarly, the Australian cultured pearl industry relies on wild collected *P. maxima* adults. This results from legislation which restricts the proportion of hatchery bred oysters that may be held on a farm. Broad industry development has been hindered by relatively low research activity, when compared to that undertaken for the other aquaculture industries. Research has commonly been conducted in-house (i.e. on a company by company basis) and results, when reported, were often presented in 'grey' (informal) literature. It is only over the past 15-20 years that significant data resulting from research in this field has been widely reported in mainstream Western literature.

The pearl oyster industry is generally considered to be conservative and uptake of new technologies and methods by the industry has been slow. Nevertheless, over recent years there has been increasing interest in research relating to general culture methods for pearl oysters (e.g. Southgate and Beer, 1997; Taylor et al., 1997), pearl

oyster genetics (particularly relating to pearl quality) (e.g. Wada and Komaru, 1996) and there has been increased reliance on hatchery production (Gervis and Sims, 1992). Further research however, is required for greater industry efficiencies. Larval survival during hatchery culture, for example, is generally low compared to other bivalve species and factors influencing this require more research.

This study addressed important issues on pearl oysters relating to broodstock selection, breeding cycle and egg quality. The major outcomes of this study are summarized in this Chapter and the importance and application of the results are summarized in Fig. 9.1 and Table 9.1. The different stages of the pearl oyster culture cycle are shown in Fig. 9.1 which also indicates on which part of the cycle the results from this study have an impact. The use of anaesthetics on pearl oysters (Chapter 3) has an impact on the donor (mantle excision) and broodstock selection processes (Chapters 5 and 6) as well as in research related to breeding cycle (Chapter 7) when the use of biopsy needles (Chapter 4) is required. Results related to the use of nutrients during breeding cycle (Chapter 7) and embryological development (Chapter 8) will help to improve these two key areas of the pearl oyster culture cycle with the aim of improving current hatchery production protocols.

9.2 Anaesthetics and saibo excision

There have been some studies aimed at improving cultured pearl quality through the use of pre-operative conditioning, use of anaesthetics and antibiotics and suture of wound after nucleus implantation. However, despite satisfactory results, many of these developments have not reached commercial application in the cultured ‘black’ pearl industry. For example, seeding technicians prefer to seed unrelaxed oysters or to use chopping blocks and other instruments made of wood instead of (preferably) plastic

and there is common use of unsterile surgical instruments. Such procedures have been shown to contribute to post-surgical infections affecting pearl quality (Scoones 1996; Norton et al., 2000). In contrast, advances such as the use of antibiotic coated nuclei and products to reduce post-operative infections and acceleration of the healing process, as well as more hygienic surgical procedures, are well established in the *P. maxima* industry.

In the past few years the international pearl market has seen an overproduction of low quality pearls, which have driven pearl prices down (Poirine 2002; Fassler, pers. comm. 2004). Pearl farms responded by increasing production even further, and in the process reducing pearl quality (Anon., 2002; Poirine, 2002; Fassler, pers. comm. 2004). Corrective measures such as reductions in pearl production and introduction of drastic pearl quality control measures, have been established in some of the main pearl producing countries to help restabilising this downward trend (Anon., 2002). Furthermore, cultured pearl industries would benefit greatly from research into production of higher quality products.

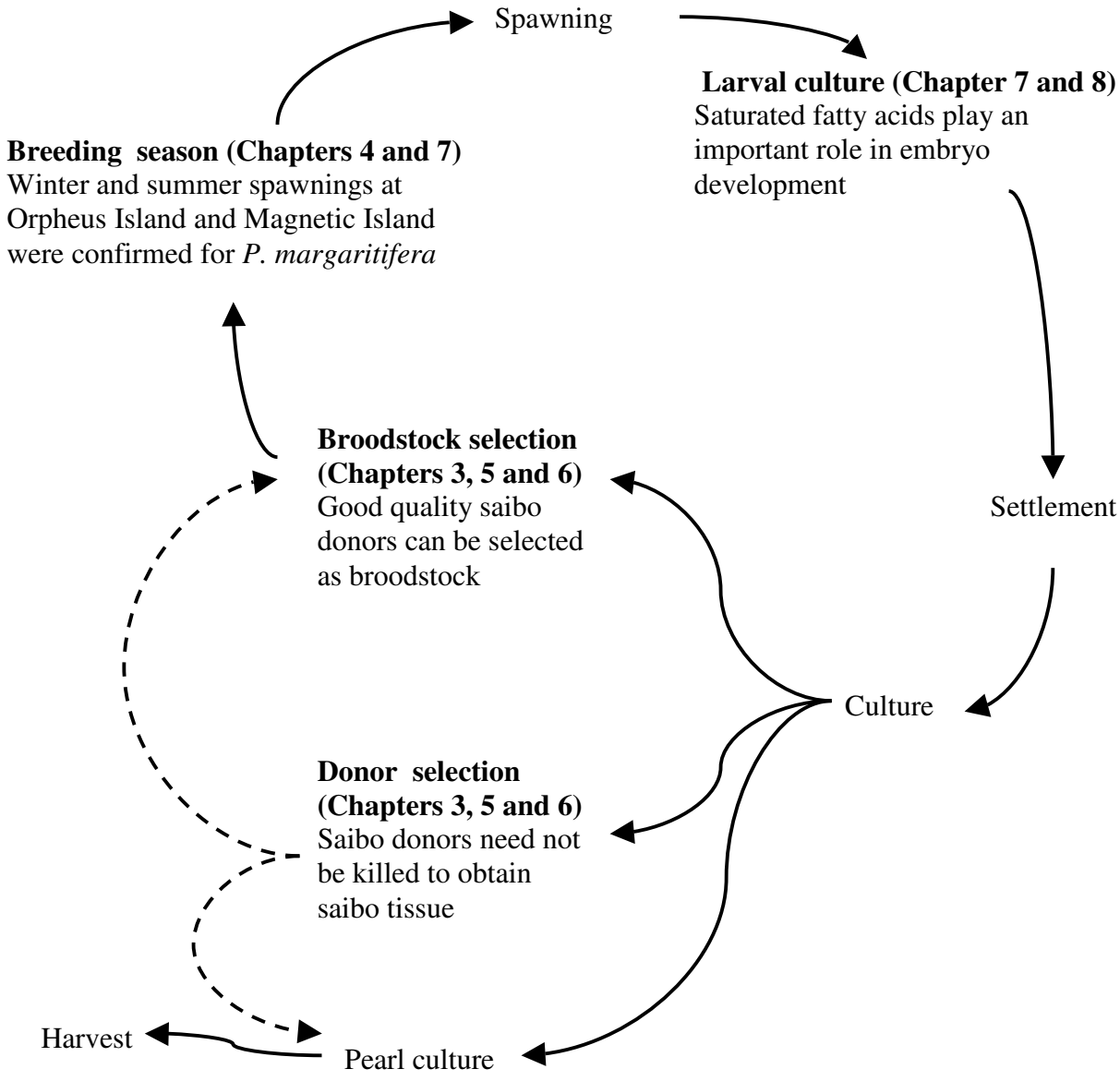

Fig. 9.1. Generalized scheme of the pearl oyster culture cycle. Important developments made in this study will allow the use of donor oysters as future broodstock, future donors or future pearl recipients (dashed line).

Table 9.1. Summary of the importance and application of the results of this thesis

Stage of culture	Main results/recommendations
Selection of saibo donors (Chapters 3 and 5)	If anaesthetised, saibo donors can be kept alive. Once oysters are relaxed, there is a minimum time of 10 minutes to obtain saibo tissue before oysters show mantle or body collapse
Use of saibo donors (Chapter 5)	Future use of saibo donors include: receptors (following seeding season); broodstock (only from specific donors) and possibly future saibo donors (further studies required)
Broodstock Selection (Chapter 5)	Apart from general fitness, nacre coloration, shape and size another selection trait can be added: quality of pearls produced by mantle of particular oysters
Breeding cycle (Chapters 3, 4 and 7)	Two spawning peaks were confirmed for *P. margaritifera* in north Queensland. Adductor muscle plays an important role in storage of protein and carbohydrate Biopsy needles can be used to identify gonad stage (males) and could be used to obtain tissue samples for biochemical analyses keeping oysters alive
Egg quality (Chapter 8)	Carotenoids were found only in trace amounts in *P. margaritifera* eggs and should not be limiting embryo development Vitamins C and E in *P. margaritifera* embryos increased in content with time and may not be limiting nutrients for embryo development. Fatty acids 14:0, 16:0 and most unsaturated C18s were highly used during *P. margaritifera* embryo development. These may be good indicators of egg quality.

As mentioned in Chapter 5, saibo donors are one of the most influential factors determining pearl quality. Anaesthetics have been studied for all commercial species of pearl oysters and techniques for anaesthesia of pearl oysters are now established. One of the first steps in improving the cultured pearl oyster industry techniques could be the widespread use of anaesthetics to obtain saibo tissue while keeping donor pearl oysters alive, even if these oysters will not be used as future broodstock.

9.3 Broodstock management and selection

Once pearl oysters reach the adult stage they are used for one of three processes: (1) the majority are used as nucleus receptors and most of them will be sacrificed to harvest pearls one or two years after seeding (a few may be seeded for a second or third time); (2) a smaller number of oysters will be sacrificed and used as mantle tissue donor, these oysters account for approximately 5% of the adult pearl oyster population under culture and the top 5% on the basis of nacre quality; mantle donors are mainly selected on the basis of their shell and nacre coloration; (3) a small proportion of cultured pearl oysters will be selected as broodstock on those pearl farms with access to a hatchery. Broodstock would be composed of small groups of up to a few hundred oysters selected for particular traits, such as growth rate, shell shape and desirable nacre colour. As well as mantle donors, broodstock represent another group of the highest quality oysters.

Broodstock are responsible for providing gametes (especially eggs) with the right amount and proportion of nutrients for the embryo to achieve successful development into first feeding larvae and beyond. Broodstock contribute directly to the genetic information of the resulting progeny. The broodstock selection process allows pearl farms to identify ‘ideal’ oysters to transmit the desirable characteristics described

above. The importance of broodstock for pearl farms is, as in any other bivalve farm, of major concern as the quality of the progeny will depend almost entirely on them. Selection of pearl oyster broodstock is normally based on similar desirable characteristics as those used for selecting saibo or mantle donors: nacre colour, shape, growth rate and fitness.

Early attempts to produce spat in hatcheries were limited to massive spawning induction of wild or cultured oysters. All gametes were used for larval production and little selection of gametes took place (Tanaka and Kumeta, 1981; Alagarswami et al., 1983, 1989). Once techniques were more established, the broodstock selection process refined. Growth rate, shell shape and nacre colour are the most important factors when selecting pearl oyster broodstock. It has been shown that the broodstock selection process improves the ability of the progeny to produce better pearls (Wada, 1986; Wada and Komaru, 1996).

Mantle donors and broodstock are selected based on very similar criteria, however mantle donors are sacrificed and broodstock are kept alive as a very valuable asset on a pearl farm. The cultured pearl industry, in contrast to other livestock industries, sacrifices the best animals instead of using them as broodstock. Such oysters are indispensable to maintain the cultured pearl industry. Mantle tissue from the best oysters has to be used to assure the production of good quality pearls and until now the traditional way to obtain mantle tissue was to sacrifice donor oysters. This conflict of interests within pearl farms makes difficult both processes, as enough oysters must be assured for each of those important stages of pearl production (reproduction and nuclei grafting). The development of the new technique for selecting donor oysters without sacrificing them described in Chapter 5 contributes significantly to the solution of this

problem by maintaining mantle donors alive and, in theory, able to be used as future broodstock to produce better quality progeny.

The research potential of this technique may create a new vision of how to manage pearl oyster broodstock and raises the possibility of selecting as broodstock only those oysters donating mantle tissue that produced the best quality pearls. Although this approach still needs to be taken up by the industry, it is supported by a strong theoretical basis.

As suggested in Chapter 5, oysters donating mantle tissue that produces high quality pearls can be used as future broodstock and all others can be used as recipient oysters. A pearl farm seeding 100,000 oysters could have an extra 5,000 oysters to seed each season if donors are not sacrificed.

The technique for broodstock selection (Chapter 5) and the biopsy technique to obtain gonad tissue (Chapter 4) have provided important tools to develop scientific research on the reproduction of pearl oysters *in vivo*. Further studies such as the testing of the broodstock selection technique, are highly recommended. Also, a study about the influence of the biopsy sample taking in the further gonad development would be of benefit for future research on pearl oyster reproduction.

9.4 Breeding cycle

The relationship between reproductive cycles and nutrient management (storage and mobilisation) is widely known for bivalve species. As a general rule, the nutritional components playing a major role in gonad development are lipid and protein.

Broodstock diets rich in medium chain fatty acids (C14, C16 and C18) may be of good nutritional value for *P. margaritifera*, as they were highly utilised during embryo development (Chapter 8); however other important long chain fatty acids must

be provided as they have been proved essential for bivalve larvae and may be of use during later stages of larval growth although this was not tested in this study.

Important components required during embryogenesis include total lipids and fatty acids, which must be provided to the broodstock conditioning diet to assure their storage on the eggs. These nutrient are used as energy reserve by the embryos to achieve successfully the first feeding D-stage larvae.

9.5 Egg quality

Chapter 8 tested the hypothesis that carotenoids and vitamins play an important antioxidant role during embryological development of *P. margaritifera*, due to the high water temperatures experienced by this tropical species and their rapid development rate. Contrary to this hypothesis, the results showed that carotenoids are present in eggs of *P. margaritifera* at unquantifiable levels.

The vitamin content of *P. margaritifera* embryos remained constant or increased with time. This may reflect a surplus of these micronutrients and perhaps these are not being limiting factors to pearl oyster embryo development. The levels of saturated fatty acids in *P. margaritifera* embryos (particularly 14:0 and 16:0) reduced considerably with time. These may be used to provide energy or they may be elongated into longer chain poly-unsaturated fatty acids (PUFAs) as has been shown in other bivalve species (Waldock and Holland, 1984; Chu and Greaves, 1991; Chu, 1996).

9.6 Future research

There is good scientific support for the use of anaesthetics during pearl seeding. Benzocaine (500 and 1200 mg L^{-1}) and propylene phenoxetol (2 mL L^{-1}) can safely be used for a number of processes such as sex determination, biopsy taking, pearl seeding

and now mantle excision. However, research to evaluate associated effects of anaesthetics on pearl quality will help to determine, for example, if there is an effect of anaesthetised mantle on pearl sac development and resulting pearl quality. Other research associated with pearl quality relates to the use of regenerated mantle on pearl sac development and pearl quality will clarify the hypothesis raised in Chapter 4 about the use of saibo donors on more than one occasion.

The early stages of mantle healing (0-3 days) deserves further study to determine aspects of the healing process in more detail and its energetic cost and other implications for the pearl oyster. On the basis of developments from this study, selected broodstock can be more accurately identified using the technique described in Chapter 4. Once broodstock are selected, genetic studies can be done to determine heritability of the pearl quality producing ability traits on the progeny. Genes particular to these oysters could be identified and perhaps the identification of these good quality saibo donors can be known from an early developmental stage by analysing small tissue samples

Broodstock diets containing medium chain fatty acids should be investigated as they were seen to be important for embryo development.

The influence of individual parents (particularly females) on pearl oyster egg quality is another important subject for research. Little is known about the effect of individual females (egg quality) on future embryo development, hatch rate and larval development. For example, how is egg quality and fecundity influenced by the energetic status and levels of stored nutrients available in female pearl oyster tissues? Furthermore, which characteristics are important in determining egg quality in *P. margaritifera*? It may be possible for future research in this field to identify a simple measurable characteristic that can be used to assess egg quality and lead to greater

hatchery efficiency. Development of better larval diets would also help increase larval survival to levels comparable to those achieved with other commercial bivalve species.

This study presents new and pertinent information relating to the breeding cycle, broodstock selection and egg quality of pearl oysters. Many of the aspects investigated in this study were conducted for the first time with pearl oysters. As well as providing information which will facilitate greater efficiencies in pearl oyster culture methods, this study describes a novel technique which, if adopted by the pearl industry, will bring about significant changes to traditional aspects of broodstock husbandry and pearl production.

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Appendix 1 Histological and histochemical methods

A. Histology

a) Mayer's Haematoxilin and Young's Eosin-Erythrosin.

1. Get sections directly from oven (60°C) and take them to water
2. Stain in Mayer's Haemalum/Haematoxilin for 5 minutes
3. Wash in tap water
4. Blue in Scott's tap water substitute for 30 seconds
5. Wash in tap water
6. Stain in Young's Eosin/Erythrosin for 2.5 minutes
7. Wash in tap water
8. Dehydrate, Clear and Mount

B. Histochemistry

a) MSB (Martius, Scarlet, Blue) trichrome stain.

Samples should be fixed in Bouin's solution. If samples were fixed in formalin, slides need to be placed in Bouin's solution or Acidified K_2CrO_7 in the oven @ 60°C for 30 minutes and then rinsed with tap water for 10 minutes between steps 1 and 2 below.

1. Get sections directly from oven (60°C) and take them to water
2. Stain in Celestine Blue for 5 minutes
3. Wash in running water
4. Stain in Mayer's Haemalum for 5 minutes
5. Wash in tap water
6. Blue in Scott's tap water substitute for 30 seconds

7. Wash in tap water
8. Rinse in 95% Alcohol
9. Stain in Martius Yellow solution for 2 minutes
10. Rinse with distilled water
11. Stain in Brilliant Crystal Scarlet for 10 minutes
12. Rinse with distilled water
13. Differentiate in 1% Phosphotungstic Acid for 2 minutes
14. Rinse with distilled water
15. Stain in Aniline Blue
16. Rinse with distilled water
17. Dehydrate, Clear and Mount

b) Masson's trichrome stain.

1. Get sections directly from oven (60°C) and take them to water
2. Stain in Celestine Blue for 5 minutes
3. Wash in running water
4. Stain in Mayer's Haemalum for 5 minutes
5. Wash in tap water
6. Blue in Scott's tap water substitute for 30 seconds
7. Rinse with distilled water
8. Stain in acid fucsin solution for 5 minutes
9. Rinse with distilled water
10. Differentiate in 1% Phosphotungstic Acid for 2 minutes
11. Rinse with distilled water
12. Stain in Aniline Blue

13. Rinse with distilled water
14. Dehydrate, Clear and Mount

c) Alcian Blue-PAS (Periodic Acid-Shiff's reagent) technique.

1. Get sections directly from oven (60°C) and take them to water
2. Stain in Alcian Blue for 5 minutes
3. Rinse with distilled water
4. Immerse sections in Periodic Acid Solution for 2 minutes
5. Rinse slides thoroughly with distilled water
6. Stain with Shiff's reagent for 8 minutes
7. Wash slides in running tap water for 10 minutes
8. Stain in Mayer's Haemalum for 5 minutes
9. Wash in tap water
10. Blue in Scott's tap water substitute for 30 seconds
11. Wash in tap water
12. Dehydrate, Clear and Mount

Appendix 2 Biochemical methods

This extraction method was modified from those described by Holland and Gabbott (1971) and Mann and Gallager (1985). Modifications to the final determination were based on those described by Dubois et al. (1956); Bradford (1976) and Barnes and Blackstock (1973) for carbohydrates, protein and lipids respectively. A LabSystems iEMS spectrophotometer equipped with a 96 well microplate reader and Genesis 3.04 software were used to read absorbances for the individual analyses.

A. Lipid assay

1. Add 300 µL homogenate to a 5 mL centrifuge tube
2. Add 100 µL H2O + 1,500 µL 1:2 $CHCl_3$:CH_3OH –1st Extraction (Bligh and Dyer, 1959)
3. Shake or sonicate
4. Stand for 10 min
5. Centrifuge at 1000 g for 10 min
6. Remove supernatant to a second centrifuge tube
7. To precipitate from 6. add 1,500 µL 2:1 $CHCl_3$:CH_3OH –2nd Extraction (Folch et al., 1957)
8. Shake or sonicate
9. Stand for 10 min
10. Centrifuge at 1000 g for 10 min
11. Remove supernatant and pool with supernatant from 6. above
12. Add 950 µL of 0.7 % NaCl to pooled supernatant –Purification of lipid extract (Folch et al., 1957)

13. Mix thoroughly, stand at 4°C for > 30 min
14. Centrifuge at 500 g for 10 min
15. Take 1,000 µL of bottom layer ($CHCl_3$) (which contain lipid) to a microtube and dry at 60°C.
16. Add 500 µL H_2SO_4
17. Shake
18. Close the microtube and heat in a water bath at 100°C for 10 minutes
19. Cool down at room temperature
20. Transfer 20 µL to a new microtube (3-5 replications)
21. Add 500 µL of the H_3PO_4-Vanillin reagent (0.5 g of Vanillin (Univar 574-100G) in 250 mL Orthophosphoric Acid [4:1 Phosphoric Acid:Water]) (Barnes and Blackstock, 1973)
22. Shake
23. Stand for 20 minutes
24. Transfer 200 µL to microplate
25. Read @ 520 nm
    a. Calibration versus cholesterol or tripalmitin dissolved in 2:1 v/v $CHCl_3$:CH_3OH at 1. above.
    b. Reagent blank: solvents only at 1. above.

B. Protein and Carbohydrate assays (modified from Bradford, 1971; Dubois et al., 1956)

1. Add 500 µL homogenate to microtube
2. Add 250 µL 15% Trichloroacetic Acid (TCA)
3. Shake

4. Stand > 10 min (preferably overnight) at 4°C
5. Centrifuge @ 1000 g for 10 min
6. Add 100 µL supernatant to a new microtube (3-5 replications)
7. Add 100 µL H_2O + 100 µL 5% Phenol
8. Mix
9. Add 500 µL H_2SO_4
10. Mix
11. Stand > 25 min
12. Transfer 200 µL to microplate
13. Read @ 490 nm
    a. Calibration versus glucose dissolved in 5% w/v TCA at 1. above.
    b. Reagent blank: solvents only at 1. above
14. To precipitate of 5. add 1 mL 0.1 N NaOH
15. Mix
16. Transfer 10 µL to microtube
17. Add 990 µL H_2O
18. Mix
19. Add 250 µL BioRad Dye reagent
20. Mix
21. Incubate for 5 min
22. Transfer 200 µL to microplate
23. Read @ 595 nm
    a. Calibration versus bovine serum albumin dissolved in distilled water at 14. above.
    b. Reagent blank: distilled water at 14. above.

Publications resulting from this thesis

Published

Acosta-Salmón, H., Southgate, P.C., 2004. Use of a biopsy technique to obtain gonad tissue from the blacklip pearl oyster *Pinctada margaritifera* (L.). **Aquaculture Research** 35, 93-96.

Acosta-Salmón, H., Martínez-Fernández, E., Southgate, P.C., 2004. A new approach to pearl oyster broodstock selection: can saibo donors be used as future broodstock? **Aquaculture** 231, 205-214.

Submitted

Acosta-Salmón, H., Southgate, P.C., Wound healing and mantle regeneration in the pearl oysters *Pinctada margaritifera* and *Pinctada fucata*.

Acosta-Salmón, H., Martínez-Fernández, E., Southgate, P.C., Use of two relaxants to obtain saibo tissue from pearl oysters.