Improving the quality of pearls from *Pinctada maxima*

Thesis submitted by

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DECLARATION

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Statement of Contribution of Others

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Abstract

The method used for cultured round pearl production was developed in the 1920's and has changed little since. It utilises mantle tissue grafts ('saibo') from donor oysters which are killed. The quality of resulting pearls is highly influenced by the nacre quality of the donor and, because of this, a pearl farm's best oysters are sacrificed for pearl production. This is potentially a major constraint to the pearling industry which, unlike most livestock industries, cannot use its highest quality animals in breeding programmes to improve the stock quality. Recent research has shown that saibo tissue can be removed from donor pearl oysters using anaesthetics, without killing the oyster, and that excised mantle tissue is regenerated within three months. Potential benefits to the cultured pearl industry from these results include the use of donor oysters producing high quality pearls as broodstock to improve farmed oyster stock, and potential multiple saibo donation by high quality donors. These benefits, however, assume that the mantle tissue of anaesthetised pearl oysters and regenerated mantle tissue perform in a similar manner to 'normal' mantle when used as saibo for cultured pearl production. Assessing this new approach and testing this assumption was the basis of this study which was conducted with the silver- or gold-lip pearl oyster *Pinctada maxima*.

The experiment reported in Chapter 2 assessed seven anaesthetics for their efficacy with *P. maxima*: 3 mL L^{-1} 2-phenoxyethanol, 500 mg L^{-1} and 1200 mg L^{-1} of benzocaine, 1.5 mL L^{-1} clove oil, 0.25 mL L^{-1} and 0.4 mL L^{-1} menthol liquid, and 2.5 mL L^{-1} propylene phenoxetol. Of the 27 oysters exposed to each treatment, the highest proportion of relaxed oysters (96.3, 88.9 and 88.9%) and the shortest exposure times required for anaesthesia (13.8 ± 6.4, 10.5 ± 7.9 and 15 ± 7.1 min), were recorded for the treatments of 3 mL L^{-1} 2-phenoxyethanol, 1200 mg L^{-1} of benzocaine, and 2.5 mL L^{-1} propylene phenoxetol,

respectively. In contrast, none of the oysters exposed into 0.25 mL L^{-1} menthol liquid became relaxed and most oysters exposed into 1.5 mL L^{-1} clove oil died during the experiment. Oysters exposed to 3 mL L^{-1} 2-phenoxyethanol and 1200 mg L^{-1} benzocaine remained relaxed for up to 30 min while the number of relaxed oysters exposed to 2.5 mL L^{-1} propylene phenoxetol decreased during that time. With the exception of oysters exposed to clove oil, all relaxed oysters recovered within 2 h of being placed back into seawater and there was close to 100% survival after one month.

The capacity for regeneration of excised mantle tissue by *P. maxima* was investigated in Chapter 3. Oysters were anaesthetised with 2.5 mL L^{-1} propylene phenoxetol prior to a piece of tissue (approximately 10 mm x 30 mm) being excised from the ventral region of the mantle. In the first experiment, 56 oysters with mean (± SD) dorso-ventral measurement (DVM) of 125.5 ± 8.9 mm had tissue excised from either the right mantle lobe, left mantle lobe or both mantle lobes. Following a further three-month period in suspended culture, oyster survival was recorded and two oysters were selected arbitrarily from each group to be sacrificed for histological examination of healed mantle. In the second experiment 36 oysters with mean (± SD) DVM of 151.6 ±13.4 mm were used for excision of the distal part of the ventral region of the left mantle lobe. Two oysters were sampled at 1, 3, 6, 12, 24, 36, 48, 72 and 120 h (5 days) after mantle excision, and then at 12, 24, 45, 72 and 90 d after mantle excision for histological and histochemical analysis of mantle regeneration. There was almost 100 percent survival in both experiments. Healing and regeneration of mantle tissue in oysters subject to excision from the left, right or both mantle lobes was evident, with regenerated mantle appearing similar to normal mantle. All external and internal components of normal mantle were present in regenerated mantle tissue. Epithelization signifying wound healing occurred within 36 to 72 hours and was characterised by a reduced wound area,

haemocyte infiltration and accumulation, and cell dedifferentiation. Within 48 hours of mantle excision, the latero-ventral edges of the wound flexed dorsally and attached to the dorsal edge of the wound reducing the wound area. Between five and twelve days after excision, the distal part of the mantle had divided into three small lobes which developed into the outer, middle and inner mantle folds two weeks later. Ninety days after excision the mantle had completely regenerated with histological observations indicating no difference in epithelial structure or in other internal mantle accessories when compared to normal mantle. Shell material began to be secreted onto the shell by regenerating mantle twelve days after excision. Initially this occurred in a position dorsal to the non-injured mantle edge. However, forty-five days after mantle excision, regenerated mantle had extended ventrally to a position similar to that of non-injured mantle. Nacre deposition by regenerated mantle had now reached the same position ventrally as that of non-injured mantle indicating full acquisition of nacre secreting abilities by regenerated mantle. Complete regeneration of the mantle had occurred 90 days after excision when no differences in epithelial structure or other internal mantle accessories were evident when regenerated and normal mantle were compared.

The results of Chapters 2 and 3 showed that appropriate anaesthetics can be used to relax *P. maxima* to allow mantle excision, and that excised mantle tissue could regenerate with secretory functions within 3 months of excision. On this basis, it would be possible to obtain saibo from living anaesthetised donor oysters and from regenerated mantle tissue. However, no prior study had investigated whether regenerated mantle had the ability to secrete the same quality nacre as normal mantle or whether anaesthetised and regenerated mantle were able to proliferate to form a functional pearl-sac when implanted into a recipient oyster. In Chapter 4, regenerated mantle from *P. maxima* was shown to produce shell material with the same structure as normal mantle. The nacre produced by both types of mantle appeared identical in

both the size and structure of nacre platelets. Regenerated mantle tissue appeared to secreted nacre at a more rapid rate than normal mantle tissue which was indicated by the greater thickness of nacre adjacent to the mantle wound site. These results confirmed that regenerated mantle has the ability to secrete nacre and the potential to secrete nacre of similar quality to normal mantle tissue. Chapter 5 investigated the ability of relaxed saibo, and saibo from regenerated mantle, to form a pearl-sac following implantation into a recipient oyster. Survival of recipient oysters implanted with relaxed, regenerated or normal saibo ranged from 90% to 100% and did not differ significantly between treatments (p-value= 0.2333). Nucleus retention was much poorer than expected with a total of only 15 oysters retaining nuclei (of 191 nucleated oysters) and showing pearl-sac development. Eight nuclei (53% of the total) were retained by oysters in the control treatment (normal saibo x normal recipient), 4 (26.7%) were retained by the anaesthetised saibo x anaesthetised recipients oysters, 2 (13.3%) by the regenerated saibo x normal recipients treatment and 1 (6.7%) by the anaesthetised saibo x normal recipients treatment. Pearl-sacs from seven of these were used for histological analysis: four from oysters in the control treatment (normal saibo x normal recipient), one from the anaesthetised saibo x anaesthetised recipient treatment, and two in the regenerated saibo x normal recipient treatment. The six-week duration of this study allowed complete pearl-sac development in oysters implanted with relaxed, regenerated or normal saibo. However, the thickness of the pearl-sac epithelium varied, indicating differences in the degree of pearl-sac maturity. Pearl-sacs in all treatments had cell accessories: epithelium and mucous cells. In the control treatment which used normal saibo, greater nacre deposition was evident compared to that produced by both relaxed and regenerated saibo. Despite variation in the thickness of the epithelium produced by each type of saibo, each pearl-sac produced approximately the same thickness of matrix or mineral deposition. This experiment confirmed the results of Chapter 4 in showing that regenerated

mantle tissue from *P. maxima* apparently regains full secretory function and showed that saibo from relaxed oysters and from regenerated mantle tissue is able to form a pearl-sac capable of mineral secretion onto an implanted nucleus.

The potential use of saibo from relaxed oysters and from regenerated mantle tissue for pearl production was investigated in Chapter 6, in an experiment conducted using 1,520 oysters at a commercial pearl farm in north Bali, Indonesia. Two pearl implanting operations were conducted three months apart. In the first, donor oysters were anaesthetised to provide saibo and then allowed to regenerate excised mantle tissue before the second operation which used regenerated mantle tissue as saibo. Pearls were harvested 24 months after the operations and graded into categories using commercial grading schemes for the following pearl quality criteria: size and nacre thickness, shape, colour, surface complexion and lustre. Pearl oyster survival varied from 90% (normal saibo) to 92% (regenerated saibo) and 95% (relaxed saibo). These values differed significantly (χ^2= 8.990048, p=0.01116441). Overall nucleus retention varied from 27% for oysters implanted with relaxed and normal saibo to 37% for those implanted with regenerated saibo. There was a very significant effect of types of saibo on nucleus retention (χ^2= 34.01114, p=0). The total number of pearls produced by oysters implanted with relaxed, regenerated and normal saibo was 240, 165 and 19, respectively, and the proportion of these that were considered to be of acceptable commercial quality was 99%, 62% and 53%, respectively. There was a highly significant difference between these values (χ^2= 112.3091, p=0). The majority of pearls were graded into the ‘round’ shape category (34.8% of a total of 425 pearls) and the majority of these were produced by oysters implanted with relaxed saibo (47% of category total). Pearls in the ‘drop’ category made up 20.2% of the total number of pearls produced and again, the majority were produced by oysters implanted with relaxed saibo (24% of category total). There was a highly significant effect of

saibo type (relaxed and regenerated) on pearl shape (χ^2= 15.32797, p=0.018). Pearls produced by relaxed saibo ranged from 3-14 mm in size with the highest proportion in the 10-11 mm category which collectively made up 46% of the total. Pearls produced by relaxed saibo attained a larger size than those resulting from both regenerated and normal saibo; those in the 12.5 mm to 14 mm size ranges made up 3% of the total number of pearls produced by relaxed saibo. Pearls produced by regenerated saibo ranged from 4 mm to 12 mm with the majority (64.2%) in the 8-9 mm size class. The largest pearls produced by regenerated saibo were in the 12 mm size class but only 3.6% of the total number of pearls fell into the 10.5 mm to 12 mm size categories. Pearl produced by normal saibo ranged from 8 mm to 11 mm and did not attain the larger sizes of pearls produced by relaxed and regenerated saibo. The majority (57.9%) of pearls produced by normal saibo were in the 8.5 mm to 9 mm size category. There was a very significant effect of type of saibo on pearl size (χ^2= 44.57578, p=0). Mean (± SE) sizes of pearls produced by relaxed, regenerated and normal saibo were 10.3 ± 0.14 mm, 8.7 ± 0.11 mm and 9.2 ± 0.33 mm, respectively. The average nacre thickness on pearls was 3.4 ± 0.12 mm for relaxed saibo, 1.8 ± 0.11 mm for regenerated saibo and 2.4 ± 0.30 mm for normal saibo. Relaxed saibo produced significant greater nacre thickness than both regenerated (p = 0.000) and normal saibo (p = 0.013), while nacre thickness of pearls produced by regenerated saibo did not differ significantly from that of normal saibo (p = 0.120). There was a weak correlation between pearl size and nucleus size (r = 0.31, n = 146) but a strong correlation between pearl size and nacre thickness (r = 0. 95, n =146). White/silver colours were dominant in pearls produced by oysters implanted with white/silver donor saibo making up 96% of total pearls produced by relaxed saibo, and 83.1% and 84.2% of the total for regenerated and normal saibo, respectively. There was much greater variability in pearl colour produced by yellow/gold oysters implanted with relaxed, regenerated and normal saibo from yellow/gold donors, when compared to pearls produced

by saibo from white/silver donors. White/silver colours shared about the same percentage in both relaxed and regenerated saibo (53.1 and 52.6%, respectively) but the proportion of pearls in the yellow/gold colour range was greater from relaxed saibo (22.5%) than from regenerated saibo (11%). There was considerable variation in the proportion of pearls in each of the major categories of surface complexion. Only 12.9% of the pearls produced in this study were graded within the A1 category characterised by no blemishes or a small blemish that can be removed by drilling. Within the A1 category, pearls produced by relaxed, regenerated and normal saibo made up 11.7%, 15.1% and 10.5% of the total, respectively. Pearls in category B1 made up 13.9% of the total pearls produced, composed of 17.1%, 8.4% and 21.1% of the pearls produced by relaxed, regenerated and normal saibo, respectively. Most of pearls produced from both relaxed (45.4%) and normal (47.4%) saibo were graded within the B2 category. There was a highly significant effect of saibo type (relaxed and regenerated saibo only) on surface complexion of pearls produced (χ^2= 26.99977, p=0). Only 10.6% of the pearls produced in this study were graded within the highest category for lustre. Pearls produced by relaxed saibo made up the majority (14.2%) of these. The majority of the pearls produced from all treatments were placed into lustre category 2 and were characterised as being bright pearls with a slightly blurred reflection. Pearls from regenerated saibo made up the majority of pearls in this category which contained 69.9% of pearls from regenerated saibo, 57.9% of pearls from normal saibo and 54.6% of those from relaxed saibo. The effect of saibo type (relaxed and regenerated only) on pearl lustre within categories 1-3 was very significant (χ^2= 10.07011, p=0.006). Based on the major criteria used to assess the performance of both oysters and pearls after implanting with relaxed, regenerated and normal mantle, the results indicate that relaxed saibo from anaesthetised pearl oysters performed better than both regenerated and normal saibo.

The final research chapter of this thesis used UV-visible (UV-Vis) spectrophotometry to analyse some of the pearls produced in Chapter 6. Of particular interest was a comparison between pearls from the same saibo donors and pearls with various colours (from white/silver to gold) and overtones. Three pearls with different colours resulting from the same gold donor showed different absorption spectra. Cream and gold coloured pearls showed a wide absorption from 320 to about 460 nm while there was just slight reflectance around 400 nm by the white pearl with a pink overtone. Cream and gold pearls reached a reflectance peak at 560 to 590 nm while the white pearl with pink overtone showed slightly wider absorption in this region. Both cream and gold pearls showed an absorption peak after the reflectance peak; at about 700 nm for the cream pearl and 750 nm for the gold pearl. Two other pearls produced by the same gold saibo donor (white with cream overtone and cream with various overtones) showed similar spectra which differed in their intensity. One of these pearls had very high lustre and its spectrum showed a much higher % reflectance than the second pearl with inferior lustre. This result may indicate that reflectance is a useful quantitative indicator of pearl lustre. The spectra of two white pearls resulting from different silver nacre donors showed a reflectance at 260 nm, followed by absorption at 280 nm and another reflectance peak at 340 nm. After this peak the spectra for these pearls remained flat until a slight absorption peak around 700 nm. Throughout the visible region, all white pearls used in this study showed similar reflectance spectra although there were differences in reflectance intensity. Unlike the spectral results from white pearls, the results from yellow and gold pearls varied according to colour saturation of the pearl. The results of this study show that similarities between absorption and reflectance spectra of cultured pearls resulting from the same saibo donor are negligible and could not be detected with UV Vis spectrophotometry. Nevertheless, this technique could have a role to play in developing less subjective methods

of assessing pearl quality and in further studies of the relationships between pearl quality and that of the donor and recipient oysters.

This study has confirmed that *Pinctada maxima*, like *P. margaritifera* and *P. fucata*, is able to be anaesthetised to allow mantle excision without mortality, and that excised mantle can regenerate within a period of 3 months. Relaxed and regenerated mantle were shown to possess secretory function, similar to normal mantle tissue, and the ability to proliferate to form a pearl-sac when used as saibo. Indeed, relaxed mantle from anaesthetised oysters was shown to produce pearls of superior quality to those produced by normal mantle tissue when used as saibo. This result has major implications for the pearling industry and indicates that minor changes to the pearl seeding process (i.e., use of relaxed mantle as saibo) could bring about improvements in pearl yield and pearl quality.

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Chapter 1

General Introduction

The pearling industry is among the most potentially profitable aquaculture businesses. For example, a hundred grams of gem pearls could be valued at more than US$ 2,600 (Anon, 2007b). This value is more than hundred times higher than that of other aquaculture commodities on a weight basis. Global cultured pearl production in 2005 was valued at around two-third of a billion US dollars from a total of more than 1,550 tonnes of pearls (Anon, 2007b) (Table 1.1). It has increased by approximately 40% compared to 2003 (Kremkow, 2005). Cultured pearl production is contributed to primarily by four leading pearl producing molluscs: (1) freshwater mussels; (2) the Akoya pearl oyster, *Pinctada fucata;* (3) the blacklip pearl oyster, *P. margaritifera*, that produces 'black' South Sea or Tahitian pearls; and (4) the silver-or gold-lip pearl oyster, *P. maxima*, that produces white or gold South Sea pearls (Southgate et al., 2008b). Cultured pearls from both *P. margaritifera* and *P. maxima* are generally referred to as "South Sea Pearls" but are differentiated on the basis of colour (Strack, 2006). For clarity in the following text, cultured pearls from *P. margaritifera* and *P. maxima* will be referred to as Tahitian and South Sea pearls, respectively (Anon, 2007a).

Freshwater cultured pearls make up 96.7% of total cultured pearl production but only 23% of total value (Anon, 2007b)(Table 1.1). Tahitian and South Sea cultured pearls contribute approximately 0.5 and 0.6% of the total volume but make up 20 and 37% of the total value, respectively. Japanese Akoya cultured pearls contribute 2.2% of the total volume, which make up 20% of total value. Therefore based on the proportion of value of pearl production South Sea cultured pearls are the predominant sector of the industry. Besides producing pearls, pearl oysters or mussels also supply valuable shells and meat. Shells are usually

supplied to various industries and needs in products including jewellery, accessories, cosmetics, health products or fertilizer (Ward, 1995); while meat - which is taken from the adductor muscle- is used for food.

Table 1.1 Production and the value of world cultured pearl production at the pearl farm level in 2005 according to Golay's estimates.

Pearl type	Production (tonnes)	Value (Million US$)	Percent of the total pearl market value
South Sea pearls	9 - 10	$236	37%
Tahitian pearls	8.1	$126	20%
Akoya pearls	34	$128	20%
Freshwater pearls	1500	$150	23%

Source: Anon (2007b)

Pinctada maxima (Jameson) is the largest species among pearl oysters (Gervis & Sims, 1992; Hynd, 1955; Strack, 2006). It produces the finest, largest and most valuable cultured pearls (Matlins, 2002; Ward, 1995) and is the target species for this study. As an introduction to the study, the remainder of this Chapter provides a general overview of the biology and taxonomy of *Pinctada maxima* (Jameson), the history of natural and cultured pearls, pearls cultivation methods, pearl formation, pearl grading and pearl quality, and a the major objectives of this study.

1.1 Pearl producing molluscs

Although logically all shell-bearing molluscs can produce pearls (Strack, 2006; Webster, 1994), they have only been recorded in several families and genera. In marine waters, pearls have been found in 17 genera of 11 families of bivalves, 11 genera of 8 families of gastropods and one genus of cephalopod (*Nautilus*) (Strack, 2006). Freshwater pearls have been found in 43 genera of two families only (Strack, 2006). Pearl producing molluscs can be divided in two groups; those producing nacreous and non-nacreous pearls.

1.1.1 Molluscs producing non-nacreous pearls

There are at least five gastropod families (Strombidae, Cassidae, Muricidae, Fasciolariidae and Volutidae) and seven bivalve families (Arcidae, Pectinidae, Spondylidae, Placunidae, Ostreaide, Tridacnidae and Veneridae) that produce non-nacreous pearls (Strack, 2006). They are taxa without nacre-lined shells. Pearls from these species are generally natural pearls which lack colour and have low value. However, some of the pearls produced by these taxa are rare, colourful and coveted; for example, those produced by several species of conch (Family Strombidae) and volutes (Family Volutidae) (Matlins, 2002; Strack, 2006).

1.1.2 Molluscs producing nacreous pearls

In freshwater, nacreous pearls are produced from bivalves only. They are distributed in two families: Margaritiferidae and Unionidae (Strack, 2006). Both are from the superfamily Unionoidea and have nacre-lined shells. Three species from the family Unionidae are commonly cultivated for pearl production: *Cristaria plicata, Hyriopsis cummingii* and *H. schlegeli* (Strack, 2006; Wang & Wu, 1994).

In marine waters, there are at least three gastropod families (Haliotidae, Trochidae and Turbinidae) and four bivalve families (Mytilidae, Malleidae, Pinnidae and Pteriidae) that produce nacreous pearls (Strack, 2006). They have nacre-lined shells (Watabe, 1988). However, only two families are cultured for pearl production: the gastropod family Haliotidae and the bivalve family Pteriidae. Two genera are cultivated for pearl production in the family Pteriidae; *Pinctada* and *Pteria* (Strack, 2006). By far the most important of these is the genus *Pinctada* (Southgate et al., 2008b). Of the taxa within the genus *Pinctada*, *Pinctada maxima* is the most important in terms of commercial cultured pearl production and as outlined above, it accounts for around 46% by value of global marine cultured pearl production (Southgate et al., 2008a). *Pinctada maxima* is the focus of this study.

1.2 *Pinctada maxima* (Jameson)

1.2.1 Taxonomic position and distribution

Pinctada maxima was recorded for the first time in northern Australian waters (Dakin, 1913). They are commonly called the silver-lipped or gold-lipped pearl oyster and produce the famous South Sea pearls (SSP)(O'Sullivan, 1992). It is the largest species in the genus *Pinctada* (Dakin, 1913; Hynd, 1955; Shirai, 1994; Xie, 1990) and it produces the largest and finest pearls (Kunz & Stevenson, 1908). Shell size may reach more than 30 centimetres and individuals may live for up to 40 years (Strack, 2006). Both *Pinctada maxima* (Jameson, 1901) and *P. margaritifera* (Linnaeus, 1758) are closely related species (He et al., 2005) and share the same position as the most primitive species within the genus *Pinctada* (Yu and Chu, 2006). *P. maxima* is distributed from the Indian Ocean to the Pacific and from the tropic of Cancer to the tropic of Capricorn (Wada & Tëmkin, 2008)(Fig. 1.1). They are found in depths of up to 90 metres although their optimal habitat is at about 35 metres (Strack, 2006).

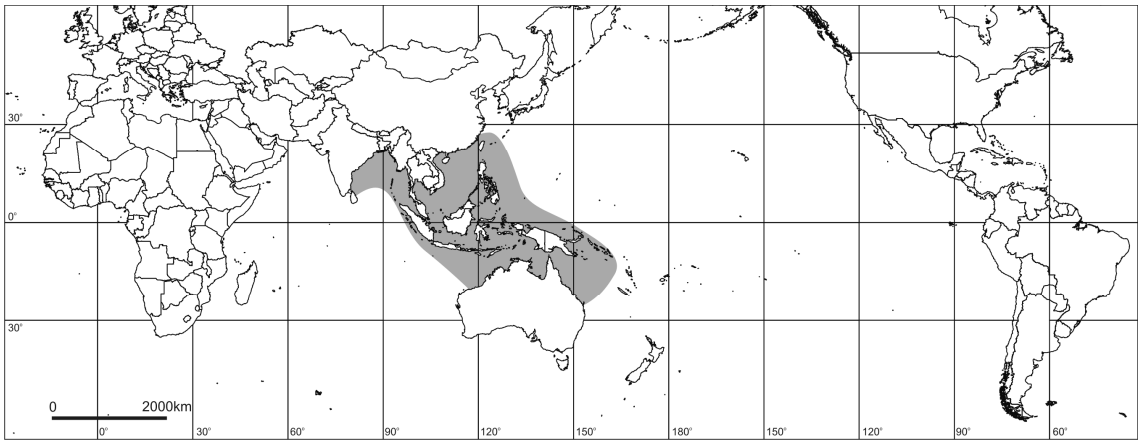

Fig 1.1. Geographical distribution of *Pinctada maxima* (Wada & Tëmkin, 2008).

The taxonomic position of *Pinctada maxima* is shown below:

Phyllum Mollusca

Class Bivalvia

Order Pterioida

Family Pteriidae

Genus *Pinctada* Röding, 1798

Species *Pinctada maxima* (Jameson, 1901)

1.2.2 Morphology and anatomy

The anatomy of *Pinctada maxima* described below relates to organs and structures that have importance to pearl production and therefore to the research conducted in this study. Like other members of genus *Pinctada*, *P. maxima* has nearly equivalve shells with less projecting posterior 'wing', compared to the genus *Pteria*, and concentric lines. Small projecting scales

may also occur on the external surfaces of shells, particularly in young individuals (Lamprell & Healy, 1997). Colour bands of gold or silver occur in distal region of the nacreous part of the inner shell (Fig. 1.2).

Fig. 1.2. The inner surface of two valves of *Pinctada maxima* from different individuals representing gold lip pearl oyster (left) and silver-lip pearl oyster (right); arrows indicate lip colour.

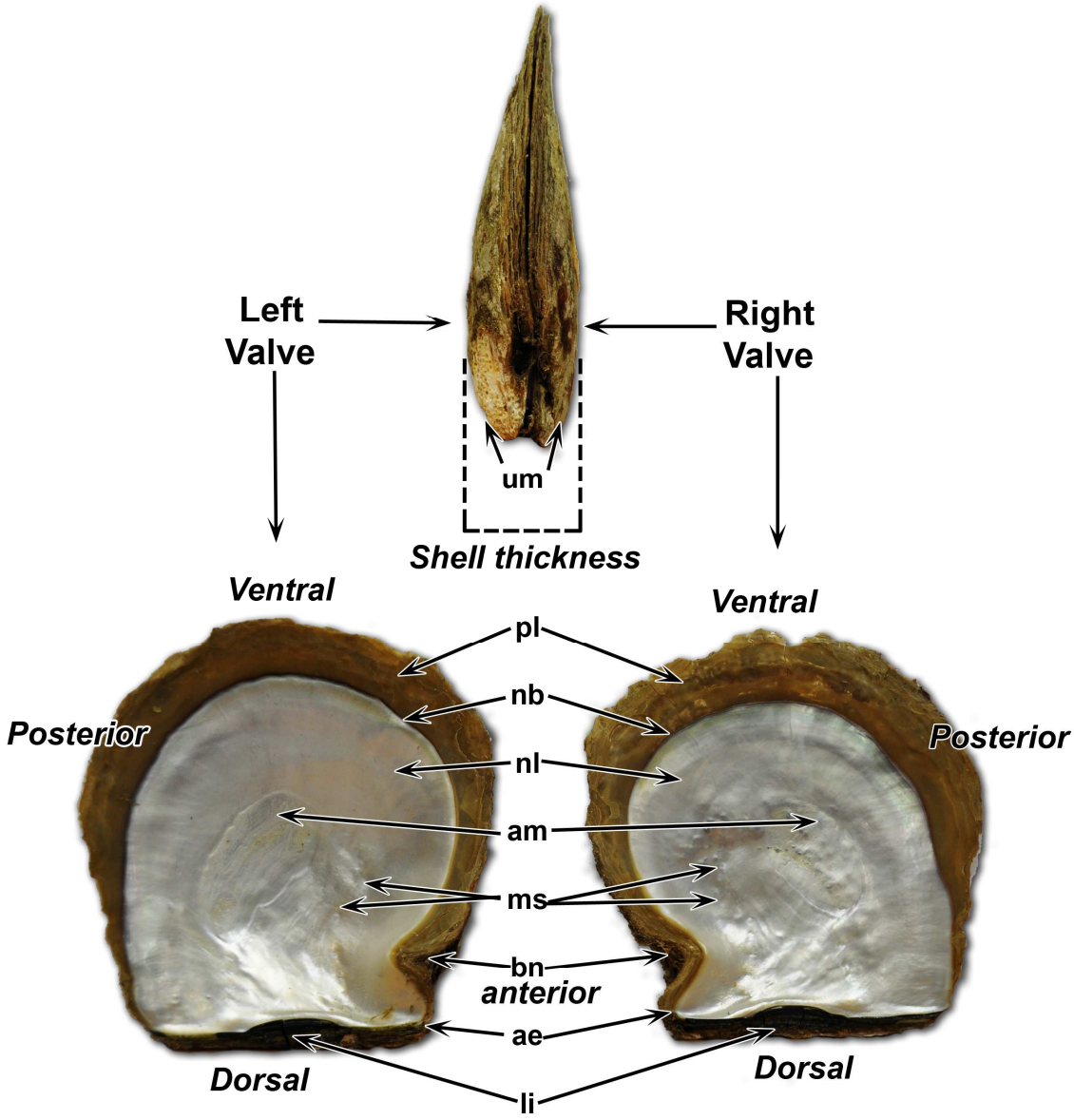

Fig. 1.3. A pair of valves of *Pinctada maxima* showing shell morphology and orientation; ae, anterior ear (auricle); am, adductor muscle scar; bn, byssal notch; li, ligament; ms, pallial muscle scar; nb, nacre border; nl, nacre layer (mother of pearl=MOP); pl, prismatic layer, and um, umbo.

1.2.2.1 The shell

Like other bivalves, *Pinctada maxima* posses a pair of valves (Fig. 1.3). Both valves are attached with a ligament in the dorsal hinge region. There are no hinge teeth (Strack, 2006). The right valve is usually flatter than the left valve. Each shell valve is composed of three layers: (1) the outer layer is the periostracum or conchiolin layer; (2) the middle layer is the ostracum or prismatic layer; and (3) the inner layer is the hypostracum or nacre (mother of pearl) layer (Fougerouse et al., 2008). The periostracum may help reduce biofouling on the outer shell surface (De Nys & Ison, 2008; Guenther et al., 2006). Unlike the periostracum which is formed mainly from proteins, the prismatic and nacreous layers are composed of different forms of calcium carbonate. The prismatic layer is composed of calcite crystals, while the nacreous layer is built from aragonite. These structures are embedded within an organic matrix framework (Addadi et al., 2006; Bedouet et al., 2001; Checa and Rodriguez-Navarro, 2005; Matsushiro and Miyashita, 2004) composed mainly of protein (Matsushiro et al., 2003).

1.2.2.2 The mantle

The molluscan mantle (Fig. 1.4) has many functions. Besides protecting internal organs, the mantle has also roles in assimilation, respiration, locomotion and reproduction (Simkiss, 1988). In relation to shell formation, the mantle is responsible for producing ions and minerals used in the biomineralisation process (Blank et al., 2003). The bivalve mantle consists of two lobes of tissue that line the inner surfaces of both shell valves. As in bivalves, each mantle lobe in *P. maxima* can be divided into three zones: the marginal, pallial and central zones (Dix, 1973; Humphrey & Norton, 2005). The central zone covers the soft tissue, the pallial zone is composed primarily of muscular threads used in mantle retraction, while

the outer marginal zone splits into three folds: the outer, middle and inner folds, each with specific roles (Fougerouse et al., 2008). Tissue from the pallial zone is used in the cultured pearl production process (Acosta-Salmón, 2004).

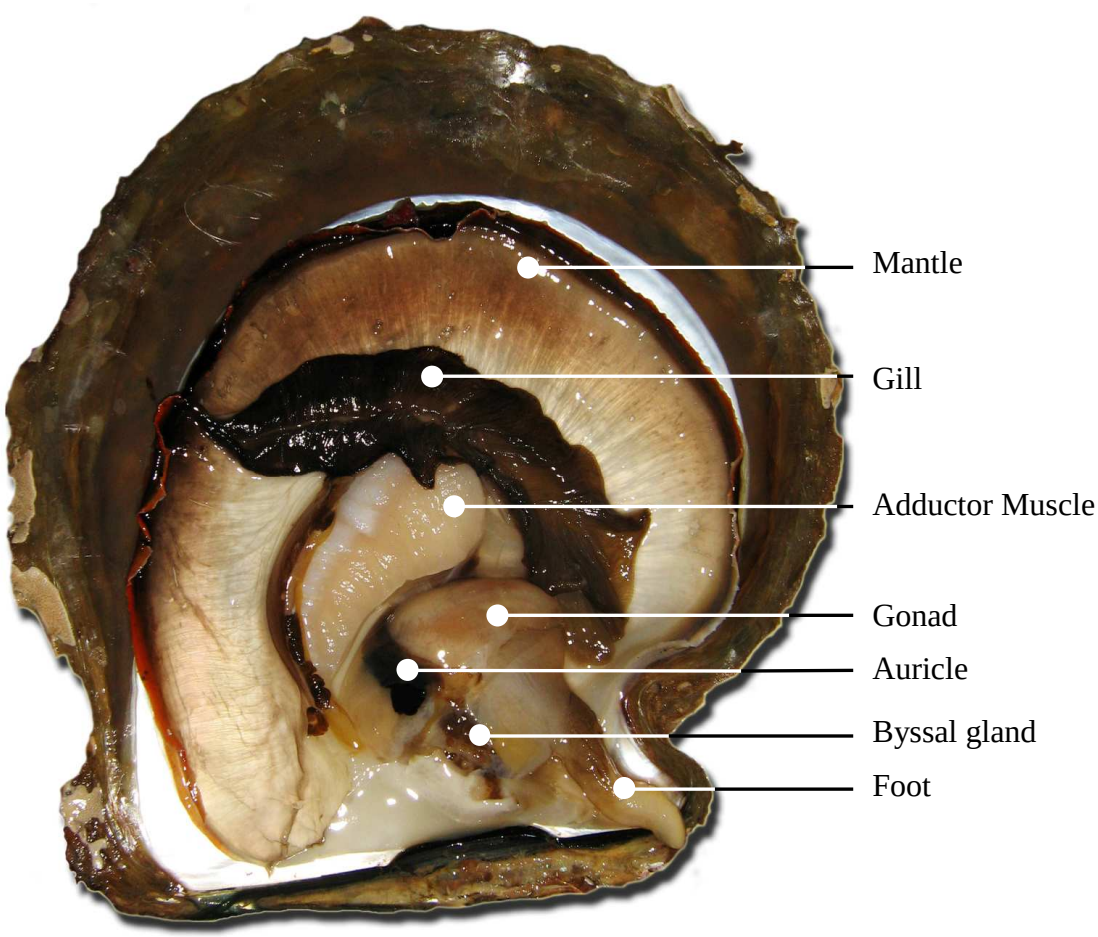

Fig.1.4. Internal anatomy of *Pinctada maxima* (Jameson)

1.2.2.3 The gonad

The gonad (Fig. 1.4) has an important role in cultured pearl production as it is used as the organ that receives the nucleus and nacre secreting tissue implant (‘saibo’) required for pearl production (Taylor & Strack, 2008). The ripe gonad of male *P. maxima* is milky white but it is creamy yellow for females. When fully ripe the gonad may occupy one-third of the internal

space of the oyster. However, pearl oysters with full gonads are not used for pearl production because space is required to house the nucleus and tissue implant. Because of this, the implantation for cultured pearl production takes place after the spawning period or following a conditioning period, which empties the gonad (see Section 1.4.2.1). Changes in water temperature are the main factor in inducing spawning of pearl oysters in nature (Behzadi et al., 1997; Hernandez-Olalde et al., 2007; Saucedo et al., 2002a; Saucedo & Southgate, 2008).

1.3 Pearl: history and origin

1.3.1 Natural pearls

Natural pearls were first discovered accidentally when human searched for food along the coastline and in lakes and rivers in prehistoric time (Dakin, 1913; Kunz & Stevenson, 1908; Strack, 2006; Ward, 1995). Pearls subsequently became parts of the rituals associated with cultural and religious activities (Strack, 2006). The shells of pearl bearing species or mother of pearl (MOP) has also been utilised for decoration throughout human history. MOP inlays were used around 4500 BC in Mesopotamia and around 4000 BC in Egypt (Strack, 2006; Ward, 1995). The use of pearls for decoration was assumed to have begun in the 5th Century BC during the Persian invasion (Kunz & Stevenson, 1908) where possibly the oldest necklace containing pearls was found in a sarcophagus at the Winter Palace of the Persian kings in Susa (Strack, 2006). During the Roman Era pearls became the most valuable gems (Ward, 1995). The 'Pearl Age' began in the 16th century. Although there was a shift from pearls to diamonds in the 18th century, pearls regained their top position in the nineteenth century when new pearl oyster beds were discovered and cultured pearl production began (Strack, 2006).

Many believe that natural pearls are formed as a reaction to an irritant in the internal part of a mollusc (Kunz & Stevenson, 1908; Strack, 2006; Streeter, 1886; Ward, 1995). The irritant may be a trapped parasites, small particles, or mantle scratches due to friction or predator damage (Strack, 2006; Ward, 1995). However, pearls will not be formed without the existence of epithelial cells from the nacre secreting mantle tissue (Simkiss & Wada, 1980). Therefore, for a pearl to form the irritant (other than mantle epithelium) must be associated with some epithelial mantle (Strack, 2006). The epithelial cells begin to proliferate and form a 'pearl-sac' to cover the irritant (Taylor & Strack, 2008). The pearl-sac then begins to deposit minerals (nacre) as a kind of internal defence mechanism (Dakin, 1913; Kunz & Stevenson, 1908). Such deposition continues and the resulting pearl grows. The shape of the irritant is usually irregular and this irregularity causes pearls to grow asymmetrically in shape (baroque type). This type of pearl is common in natural pearls (Strack, 2006).

Other types of natural pearls may also be formed on the internal surface of the shell. They are called blisters (Taylor & Strack, 2008). The formation of natural blisters results from the reaction of the host to organisms that penetrate the shells or any material trapped between mantle and the shell (Kunz & Stevenson, 1908). The penetration is mainly caused by boring sponges, *Cliona* spp. (Fromont et al., 2005), boring polychaetes, *Polydora* spp. (Alagarswami and Chellam, 1976; Okoshi and Sato-Okoshi, 1996) and several lithopagan bivalves (Doroudi, 1994; Takemura and Okutani, 1956). Borers are usually categorised as pests in the cultured pearl industry because they may kill the oysters (Che et al., 1996; Humphrey, 2008; Humphrey & Norton, 2005; Jones, 2007). In response to shell penetration, the host begins to secrete nacreous material to cover the resulting damage or irritation on the inside of the shell. This process results eventually in the production of a blister (Taylor & Strack, 2008).

Natural pearls are very rare and occur in approximately one in a thousands oysters (Haws, 2002). However, the frequency with which natural pearl occurs varies according to species and the region in which they are found. In the nineteenth century one high-valued pearl could be found at a ratio of 500:1 in the Persian Gulf, 5000:1 in the Sulu Sea, 15.000:1 in French Polynesia, and 1.000.000:1 in the Gulf of Manaar, Ceylon (Strack, 2006). Obtaining such pearls is usually costly. Pearl divers are susceptible to accidents and shark attacks (Joyce & Addison, 1992; Kunz & Stevenson, 1908). Such conditions made pearls in 19th and 20th century among the most expensive gems which were restricted to the rich and to noblemen in particular (Dakin, 1913; Ward, 1995).

Before the early 20th Century, there were several places with large beds of oysters and mussels that supported a pearl fishery. In the marine environment, large pearl oyster beds stretched from Arabian waters to the Pacific and there were smaller, patchy distributions in Central America (Kunz & Stevenson, 1908). *Pinctada radiata*[1] (synonym *P. imbricata*[1]) were abundant in the Persian Gulf and the Gulf of Manaar (Sri Lanka), *P. fucata*[1](synonym *P. martensii*[1]) in Japanese waters, *P. mazatlanica* in Pacific Central America, *P. margaritifera* in the south Pacific and *P. maxima* in the tropical central Indo-Pacific region (Strack, 2006). Particular regions like the Persian Gulf, Gulf of Manaar and northern Australia were famous for their natural pearl fisheries.

In freshwater, smaller scale pearl fisheries were mainly distributed in the northern hemisphere with *Margaritifera margaritifera* being the main species. However, nowadays freshwater pearl mussel beds in Europe have been depleted and are being conserved (Bauer,

[1] There is some confusion over the taxonomic status of these taxa (Wada and Tëmkin, 2008).

1988; Beasley & Roberts, 1996; Cosgrove et al., 2000; Cosgrove & Hastie, 2001; Young, 1991).

1.3.2 Cultured pearls

Valuable pearls are usually produced from molluscs that have a nacreous lining (MOP) to the inner surface of their shells (Webster, 1994). These molluscs are selected for pearl production. For cultured marine pearls, the commonly cultured species are from the family Pteriidae: *Pinctada maxima, P. margaritifera, P. fucata and Pteria penguin* (Gervis & Sims, 1992; Southgate et al., 2008b). Marine pearls are cultivated mainly in the Indo-Pacific region; from the Red Sea to the Pacific Ocean (Bondad-Reantaso et al., 2007; Strack, 2006). Japan is famed for Akoya cultured pearls produced by *Pinctada fucata;* Indonesia and Australia lead 'South Sea' pearl production from *P. maxima* and the Pacific island countries produce 'Tahitian' cultured pearls from *P. margaritifera* (Southgate, 2007).

The first innovation towards cultured pearl production was introduced by the Chinese in the 5th Century who produced blister pearls in the shape of Buddha (Joyce & Addison, 1992). This was carried out using freshwater mussels. More than a thousand years later in Europe, Linneaus conducted experiments by creating a hole in the shells of the river mussels, *Unio pictorum*, into which he put a limestone nucleus attached to a wire in the shell (Strack, 2006). He then left the mussels in the water for five years, however, the resulting pearls were of very poor quality (Joyce & Addison, 1992). Several attempts to produce pearls were conducted by William Saville-Kent on *Pinctada maxima* in 1890, followed by Kokichi Mikimoto three years later on hemispherical pearls (George, 1996; Simkiss & Wada, 1980). In 1914, Mikimoto applied for a patent for producing round cultured pearls (George, 1967) and he received it two years later (Ward, 1995). This modern method of culturing round pearl

production utilised a nucleus wrapped within a piece of mantle tissue, which was implanted into the gonad of a recipient oyster. This method was actually invented by Saville-Kent and adopted by Tatsuhei Mise and Tokichi Nishikawa, but Mikimoto claimed the patent (George, 1996; Matlins, 2002). The method was subsequently applied commercially in 1919 (Simkiss & Wada, 1980; Taylor & Strack, 2008).

1.4 Production of cultured pearls

Cultured pearls are divided in two types: bead nucleated and tissue nucleated pearls (also called non-nucleated pearls) (Scarratt et al., 2000). Principally, bead nucleated pearls are pearls generated from nuclei and mantle tissue while tissue nucleated pearls are generated from mantle tissue only. Bead nucleated pearls consist of blisters (mabè or half pearls), flat or coin pearls (not common) and round pearls (Fiske & Shepherd, 2007; Kennedy, 1998), while tissue nucleated pearls include several types of freshwater cultured pearls and keshi.

1.4.1 Cultured blisters or mabè

Cultured blisters or mabè are types of bead nucleated pearls. They are produced by gluing rounded or hemi-spherical nuclei (or beads) onto the inner surfaces of oyster shells (Strack, 2006). The nuclei are placed in the most lustrous area (Haws, 2002) and attached to either one or both shell valves (George, 1967). Nuclei used for mabè pearl production are manufactured from shells, plastics and paraffin (Strack, 2006). The shapes of the nuclei usually depend on operator preferences but hemispherical nuclei are commonly used.

Blisters can be produced from all molluscs with nacreous-linings to their shells but only a few of these have been commercially cultivated. For example, the abalone, *Haliotis iris*, has

been developed extensively in New Zealand for commercial blister pearl production (Strack, 2006). Most other molluscs used for culturing blister pearls are bivalves especially from the family Pteriidae. For example, the winged-pearl oyster, *Pteria penguin,* and the related *Pt. sterna* are commonly used for commercial blister pearl production (Gervis & Sims, 1992; Ruiz-Rubio et al., 2006; Southgate, 2007). *Pt. penguin* is mainly found and cultivated in the Indo-Pacific and *Pt. sterna* is in the Central America (Shirai, 1994). These types of pearl oysters are used primarily for producing mabè pearls due to their ability to produce lustrous nacre but their limited ability to be used for cultured round pearl production (George, 1967; Ruiz-Rubio et al., 2006; Shirai, 1994; Yu et al., 2004). Some mabè pearls are also developed from other members of family Pteriidae that are used primarily for round pearl production such as *Pinctada maxima* and *P. margaritifera* (Strack, 2006), however, mabè cultivation from these species is usually conducted once their use for round pearl cultivation has ceased. *Cristaria plicata* is the common species for blister production in freshwater (Webster, 1994).

1.4.2 Cultured round pearl

The second type of bead-nucleated pearls is the cultured round pearl which has greater value. Production of round pearls requires a round nucleus to be implanted with a piece of mantle (nacre secreting) tissue from a donor oyster into the gonad of a recipient oyster. This process is known as ‘pearl implantation’ or ‘grafting’ or ‘seeding’. The mantle used in this process is known as ‘saibo’ (from the Japanese meaning ‘tiny penis’). This method is commonly applied to pearl oysters and is now being applied to freshwater mussels (Fiske & Shepherd, 2007; Strack, 2006).

1.4.2.1 Pre operation phase

The oysters used for cultured pearl production are usually one to two years old (Haws, 2002). Prior to nucleus implantation, oysters selected for implantation undergo a conditioning or weakening phase for up to one month. They are usually held under crowded conditions which cause nutritional and or physiological stress that reduces their metabolic rate (Taylor & Strack, 2008; Taylor, 1999). They may also be induced to spawn or resorb material within the gonad to provide space within the gonad for nucleus implantation.

The conditioning phase may continue until 24 hours before implantation. With *Pinctada maxima*, the oysters are sometimes put into tanks overnight after the weakening phase in the sea (Taylor, 1999). Before the implantation, the water level is then lowered until the oysters are fully exposed to air. In this condition, the oysters are forced to open their shells and they are pegged open with wedges. This procedure reduces the potential for injury to the mantle (Joseph Taylor, Atlas South Sea Pearls, pers. comm).

1.4.2.2 Operation phase

In the operation phase, the pegged oysters are brought to the operator for nucleus implantation or are selected for saibo preparation (donors). This technician is skilled for pearl implantation (Haws, 1998; Tun, 1994). The donor oysters are selected on the basis of their nacre colour and lustre because these characters may contribute to the quality of the resulting pearls (Taylor, 2002). Saibo tissue is usually prepared from the central-ventral region of the mantle where the pronounced colour and lustre exist. Following excision, the mantle tissue is cleaned to remove mucus and is cut into small sections (approximately 3 x 3 mm^2) on a chopping board.

For the implantation procedure, the oyster prepared for the implantation is placed in a stand and a shell-opening tool is used to hold both valves open while the peg is pulled out. The shell opener is then turned to the posterior part of the shell to allow other operating tools to access the oyster tissue. After that, a spatula is used to move mantle and gills aside to expose the gonad. An incision is then made into the gonad near the foot, or even sometimes on the foot (Fig. 1.5). A nucleus of particular size (selected by the technician on the basis of his/her observations) is inserted into the gonad and is followed with a single piece of saibo (Fig. 1.5). The region of the mantle which secretes minerals (outer surface) is placed facing the nucleus. This procedure (Fig. 1.5) is known as pearl implantation or seeding or grafting. However, this is just one of several techniques in pearl implantation. Other technique can be started with saibo before nucleus insertion (Taylor & Strack, 2008). After the implantation, oysters are placed back into seawater for further culture. Pearl nuclei used for the implantation are traditionally manufactured from the shells of freshwater mussels belong to family Unionidae (Roberts and Rose, 1989; Sonkar, 2004; Strack, 2006; Ward, 1995; Webster, 1994).

The number of nuclei implanted into a recipient oyster varies among species. The Japanese pearl oyster, *Pinctada fucata* can be seeded with multiple nuclei in one implantation period (Alagarswami, 1976), but only one nucleus is seeded into both *Pinctada maxima* and *P. margaritifera* per implantation (Gervis & Sims, 1992; Strack, 2006). However, all species can be reseeded after pearl harvest and healthy oysters that produce good quality pearls can be used for a second (and sometimes third) implantation.

1.4.2.3 Post operation phase and culture condition

After the implantation, oysters are placed into various positions in the sea; based on farm preferences. Some farms place the oysters onto the seabed while others put them into various

types of nets or baskets that are hung from a long-line or raft. Farms that place oysters on the seabed are mostly in south Pacific countries that are surrounded with shallow atoll-reef, and several places in northern Australia. Japanese farms usually use baskets to hold oysters which are hung from rafts (Strack, 2006). The rafts are commonly set up in sheltered areas with low wind and wave actions (Southgate, 2008). However, nowadays most farms put the oysters in panel nets, which are hung from long-lines. The hanging method is an improvement of the Japanese system and makes it easier to maintain the oysters (O'Sullivan & Cropp, 1994; Ryan & O'Sullivan, 2001).

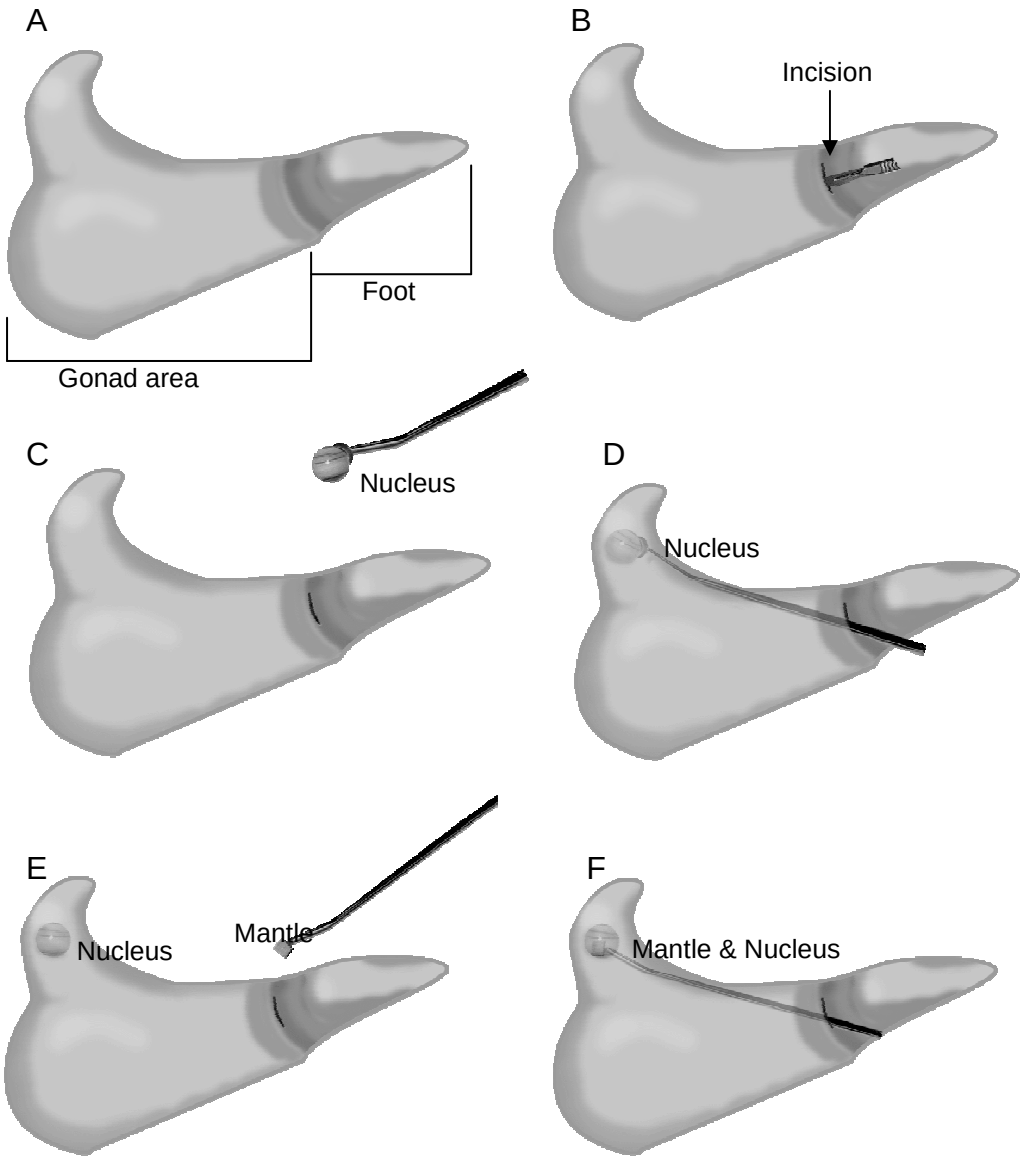

Fig. 1.5. Diagrammatic representation of the pearl implantation process: A, Foot and gonad structure of pearl oysters; B, Incision made with a blade; C-D, nucleus insertion into the gonad; E-F, mantle insertion.

Culture time for round pearl production (time between nucleus implantation and pearl harvest) varies between species. The longer the culture time the thicker the nacre coating on the nucleus will be. Akoya pearls used to be cultured for more than four years but in the mid 1990s the cultured time was reduced to 6 months only (Strack, 2006; Ward, 1995). South Sea

pearls are usually harvested between eighteen months to two years after the implantation (Fong et al., 2004; O'Sullivan & Cropp, 1994; Pouvreau & Prasil, 2001; Strack, 2006). Time for culturing freshwater pearls varies between three and five years (Fiske & Shepherd, 2007). Initially the culture time for freshwater pearls is divided into three steps: firstly, a coin bead and a piece of mantle tissue are inserted to the mantle of a recipient mussel for one year; secondly, the resulting pearl is harvested and the mussel is placed back into the water to grow a keshi for another one year; and finally, the keshi is harvested and replaced by a round bead which produces a round pearl after a further one to two years (Fiske & Shepherd, 2007). From this method the farmer may have three types of pearls within five years period: flat (coin pearls), keshi and round pearls.

1.4.3 Non-nucleated cultured pearls

A type of non-nucleated cultured pearl is called the ‘keshi’. The term keshi originated from Japanese language to describe something very small (Strack, 2006). In the pearl industry this term was adopted for small unplanned pearls that result accidentally from attempts at nucleated cultured pearl production (George, 1967; Strack, 2006). In this case, the nucleus is expelled by the recipient oyster, which retains the mantle tissue only. However, this term is also sometimes used for small pearls produced naturally by molluscs. Another type of non-nucleated cultured pearl is commonly produced from freshwater mussels when a piece of mantle tissue is the main source to produce pearl. The tissue is usually inserted into the mantle of a recipient mussel and goes on to generate a pearl. This is the traditional method for cultured freshwater pearl production which has now been modified by producing round pearls from re-operating the same recipient with nuclei (Fiske & Shepherd, 2007; Ward, 1995). Using the former method, one freshwater mussel can produce up to 50 pearls in one implantation period (Strack, 2006).

1.5 Pearl formation and pearl characteristics

Based on its composition, pearls can be categorised as nacreous and non-nacreous (Kennedy, 1998). Nacreous pearls (mostly aragonite) are produced by species with nacreous lining to the inner surfaces of their shell(s) (MOP) but non-nacreous pearls can be produced either from non-nacreous shells or from nacreous shells that may secrete pearls with less aragonite platelets. Commercially cultured pearls, however, are mostly nacreous. The following description of pearl and nacre formation is based on studies with nacreous shells or pearls.

Cultured pearl formation begins with the development of a pearl-sac that is formed from proliferation of saibo tissue (Scoones, 1996). This is the tissue responsible for nacre secretion. Along with the development of the pearl-sac, mineral deposition occurs and continues after the mantle heals or forms a sac. The process of pearl-sac formation and mineral deposition may take up to six months after the implantation in *P. margaritifera* (Haws, 2002) and the complete healing of the pearl-sac in *P. martensi* can be within two weeks only (Strack, 2006). Scoones (1996) studied pearl-sac formation and mineral/nacre deposition in *P. maxima* in detail. He reported that development of the pearl-sac took approximately 23 days from the implantation and that the first secretions from the pearl-sac onto the nucleus were evident about 30 days after the implantation.

Mineral deposition within the pearl-sac begins with the secretion of periostracum and is followed by ostracum for non-nacreous pearls. In nacreous pearl formation however, the layers of periostracum and ostracum are covered with a hypostracum (nacre) layer. The pearl formation mechanism follows the layering structure of the shell but in reversed order (Strack, 2006). In the shell, the periostracum forms the outer surface while it is the innermost layer at the interface between the nucleus and the pearl layers, in a cultured pearl. The periostracum is

a thin layer that contains mainly conchiolin. The other layers: ostracum or prismatic layer and the hypostracum or nacreous layers are two polymorphous layers of calcium carbonate. These two calcareous layers are composed of calcite (in the prismatic layer) and aragonite (in the nacreous layer). The building structure of the prismatic layer is columnar while the nacreous layer is composed of layers in a brick-mortar arrangement where the bricks are aragonite platelets and the mortar is composed with organic matrix (Addadi & Weiner, 1997; Barthelat & Espinosa, 2007; Checa & Rodriguez-Navarro, 2005; Fougerouse et al., 2008; Gre´goire, 1957; Katti & Katti, 2006; Rousseau et al., 2004) (Fig. 1.6). A study with *Pinctada maxima*, reported that the uniformity of the nacre structure may contribute to the saturation of pearl colour; the more regular the structure the more saturated the colour will be (Snow et al., 2004). However, most of the studies on pearl layers have mainly focused on shell nacre (MOP) as a parallel comparison to the pearl. There is a difference between the structure of nacre in pearls and that in shells: nacre is concentric in pearls while it is layered in MOP (Strack, 2006). Recent detailed studies on pearl structure are few.

Fig. 1.6. Schematic drawings of (a) a three dimensional view of brick-mortar arrangement of aragonite platelets and conchiolin as a coating matrix, and (b) a cross-section view of the brick mortar arrangement of nacre in a pearl (Taylor & Strack, 2008).

1.6 Pearl grading

1.6.1 Pearl quality factors

Pearls are graded using at least six main quality factors: shape, lustre, surface contour, colour, size and nacre thickness (Matlins, 2002; Strack, 2006; Taylor & Strack, 2008). The relative

importance of each depends mainly on the jeweller or pearl producer. However, nowadays all pearls are initially sorted on the basis of species origin before running through these quality factors (J. Shepherd, pers. comm., 2007); for example, South Sea pearls are from *Pinctada maxima*. This factor however is sometimes difficult to assess unless the pearl grader knows where the pearls were produced. It is even more difficult for the buyers or customers to distinguish the difference and this issue may be open for deception.

1.6.1.1 Lustre

Fig.1.7. Different types of pearl lustre of South Sea pearls from high (left) to low quality (right). Images supplied by The Autore Group.

Lustre describes the reflectance of light from the pearl surface. High lustre means high reflectivity (mirror-like reflectivity) but pearls with low lustre appear chalky or dull (Fig. 1.7). Lustre is probably the major consideration when assessing pearl quality. People tend to buy shining baroque pearls rather than chalky round pearls (Matlins, 2002). However, grading pearls based primarily on this criterion is subjective and open to wide-interpretation (Strack, 2006) due to differences in people's perception.

1.6.1.2 Shape

Fig. 1.8. The various shapes of South Sea pearls considered in pearl grading. Images supplied by The Autore Group.

Pearl shapes range from round to baroque (Fig. 1.8). The nucleus is one factor that influences the shape of the pearl. Without a nucleus, pearl shape is usually irregularly (baroque) and to create a round pearl a round nucleus is required as a basis for pearl formation although this does not always result in the formation of round pearls. On this basis, round pearls are more marketable due to their rareness (Matlins, 2002). The shape matrix for pearl grading is divided in three main categories: spherical, symmetrical and baroque (Matlins, 2002; Strack, 2006). The round category is divided in 'round' and 'nearly round', 'symmetrical in oval', 'button' and 'drop' while baroque is divided into 'semi baroque' and 'baroque' (Strack, 2006) (Fig. 1.8). In grading South Sea pearls however, a circle shape is also added into the

symmetrical category (Fig. 1.8). Tahitian pearls, for example, have a high proportion of 'circles' (concentric grooves in the surface of the pearls) (Matlins, 2002). The new pearl shapes on the market today which are based on various nuclei forms: coin pearls, bar pearls (Matlins, 2002) are excluded from this grading matrix which is only applied to round nucleus cultured pearls.

1.6.1.3 Surface complexity

'Surface complexity' assesses the degree of blemishes or flaws covering the surface of a pearl (Fig. 1.9). Blemishes may range from small spots to big chips or cracks or calcareous bumps on the pearl surface (Matlins, 2002). Generally, the fewer surface blemishes a pearl has then the higher its quality. Some types of cultured pearls have a high proportion of surface blemishes. However, in grading South Sea pearls, big ridges forming rings (usually more than three rings) in a pearl is categorised as a circled pearl (Fig. 1.8). Small spots (non calcareous) on the surface of a pearl are usually removed by polishing treatment after harvest.

Fig. 1.9. Different types of surface complexity of south sea pearls ranging from high (left) to low quality (right). Images supplied by The Autore Group.

1.6.1.4 Colour

Colours of pearls do not merely result from pigments but also from reflection and refraction (Dakin, 1913; Snow et al., 2004), and the nature of the material surrounding the nucleus (Simkiss & Wada, 1980). Thus, pearl structure is an important influence on pearl colour. The phenomenon of iridescence that shows glittering of various colours in a pearl is due to the interference and diffraction of light interacting with the specific structure of a pearl's surface (Taylor & Strack, 2008)(Fig. 1.10). Iridescence is usually considered with lustre in pearl grading. Another colour phenomenon is overtone or glow. Overtone is a translucent colour that may sometimes appear on pearls together with its original (body) colour (Strack, 2006). However, it may alter the body colour somewhat (Matlins, 2002). A pearl can be named silver-rose, which indicates that silver is the main colour and rose is the overtone (Fig. 1.11). Snow et al. (2004) studied colour in pearls from *Pinctada maxima* and found that 'edge-band structure width' (thickness of the organic matrix) controls the diffraction of lights which consequently create different colour tones; the ticker the edge-band width, the darker the colour, while the more irregular the band, the colour becomes more unsaturated. The main natural pearl colours for pearls produced by *P. maxima* are shown in Fig. 1.11.

Fig. 1.10. The interference phenomenon on a pearl surface due to the refraction and reflection of light passing through different layers of aragonite and conchiolin (Taylor & Strack, 2008).

1.6.1.5 Size

The size of pearls is usually measured in millimetres (mm). Generally, cultured pearls with size below 7 mm are categorised as small pearls, large pearls are above 7 mm (Strack, 2006). However, each type of pearl has its own categorization because larger species of pearl oysters have the capacity to produce larger pearls (Table 1.2). Large Akoya pearls from *P. fucata*, for example, are more than 7 mm while large South Sea pearls can be up to 20 mm. Large pearls are usually more valuable than the small pearls.

Fig. 1.11. Various colours of south sea pearls in pearl grading. Images supplied by The Autore Group.

Table 1.2. Size distribution of shells and pearls from the major commercially-cultured pearl oyster species.

Genera	Species	Distribution	Shell Size (DVH)	Colour	Pearl Size
Pinctada	*maxima*	Australia Indonesia Philippines	up to 300 mm	Silver Gold	10-20 mm
	margaritifera	Australia Tahiti French Polynesia Cook Island	up to 200 mm	Black Various	8-15 mm
	fucata (Akoya)	Japan China	80 mm	Silver White Pink	2-10 mm
	mazatlanica	Mexico	150 mm	Black	6-12 mm
Pteria	*penguin*	Australia Pacific	200-250 mm	Silver Gold	Various
	sterna	Mexico	<150 mm	White Silver	Various

1.6.1.6 Nacre thickness

The thickness of the nacre layers laid down on the nucleus is measured in millimetres (mm). This factor is applied mainly to cultured pearls. Nacre thickness is strongly related to the length of culture time after the implantation; the longer the culture time the thicker the nacre.

In Akoya pearls, nacre thickness below 0.15 mm is categorised as very thin while more than 0.5 mm is very thick (Matlins, 2002; Strack, 2006). South Sea cultured pearls generally have thicker nacre than other pearls and the proportion of nacre is up to 40 to 50% (Matlins, 2002). The minimum thickness of nacre for exported Tahitian pearls is 0.8 mm (Southgate et al., 2008b). Historically, nacre thickness in pearls from *P. maxima* ranged from 1.5 mm to 5 mm with an average thickness of 2 - 4 mm (Strack, 2006). However, recent years have seen a decline in the nacre thickness of South Sea pearls, particularly those produced in Indonesia (Strack, 2006).

1.6.2 Pearl grading systems

There is no universally accepted system for pearl grading (Matlins, 2002; Strack, 2006). There are currently two systems available for pearl grading: AAA-A and A-D systems. However, both of these are open to interpretation among pearl producers (Matlins, 2002); in some occasions one producer categorise a pearl as AAA grade (following the AAA-A system) while others categorise it as AA grade (Matlins, pers.comm., 2008). These systems have also never been published for scientific use and a system that could be used routinely in grading pearls produces using different experimental procedures is desperately needed. Initially, the AAA-A system was commonly applied to Akoya pearls but it could also be applied for pearls from other species. The system consists of three levels of grading i.e, AAA is the highest, AA is medium and A is the lowest quality pearls for jewellery purposes. Any characteristics below A grade is categorised as a reject pearl. On the other hand, The A-D system has mainly been applied to grading South Sea pearls. The system consists of four grading levels: A is the highest, B is medium-high, C is medium and D is the lowest. Any characteristics below D grade is categorised as a reject pearl.

The need for a universal grading system is urgent. Such grading is available for other gemstones such as diamonds. Such a system could be used with natural and cultured pearls and more importantly for the various types of cultured pearls that are common on the market. One potential problem relates to the thickness of the nacre layer of cultured pearls. The market is overwhelmed with pearls which have very thin nacre that is easy to peel off (Matlins, pers.comm., 2007). This result from a short culture time between implantation and pearl harvest and the resulting pearls have a lower price. The system could require a certain minimum nacre thickness, which would influence pearl culture practices.

1.6.3 Pearl testing

Assessing the quality of pearls involves various tools and methods. As summarized by Kennedy (1998), Strack (2006) and Webster (1994), pearl quality tests are mainly visual and often subjective. They involve the use of optical microscopes to differentiate between genuine and fake pearls. Genuine pearls can be recognised by relief lines on their surface under 20 x magnifications while a fake pearls look smooth (Strack, 2006). Natural and cultured pearls can be distinguished using a hole drilled into the pearl. Through this hole the borderline between the nucleus and the nacre can be viewed in cultured pearls. However, for pearls without holes methods using a lucidoscope or endoscope can be applied. These two apparatus need strong light gleaming through the pearls. These methods can also be used to measure the thickness of the nacre. Further methods are X-ray diffraction (lauegram method), X-radiography (skiagram method) and X-ray fluorescence. The last two methods can be applied to determine the thickness of nacre while the former is used for observing the diffraction pattern of the tested pearls. UV-Vis spectrophotometry has been used to distinguish between treated and untreated pearls (Elen, 2001) and to determine characteristic

absorption/reflectance spectra that enables identification of species-origin of cultured pearls (Elen, 2002). In conclusion, various tools are used for pearl testing but there is no single method that can be used for overall pearl grading.

1.7 Problems and potential factors for increasing pearl quality

Pearls are harvested from only about 30 to 35% of implanted oysters (Matlins, 2002). Of these, high quality pearls make up only 5 to 10% but generate around 95% of industry income (Haws, 2002). On this basis, a relatively small increase in pearl quality could bring about major benefits in industry earnings. This has driven research into methods that can be used to improve the yield of high quality pearls. They have focused on factors such as:

- reducing oyster mortality after implantation;
- increasing retention rate of grafted nuclei; and
- improving the proportion of high quality pearls (Haws, 2002).

Other factors that may have application in improving pearl quality are the use of anaesthetics, the excision of mantle from anaesthetised oysters and the use of regenerated mantle tissue from high quality donor oysters.

1.7.1 Use of anaesthetics

The pearl implantation procedure is extremely stressful for pearl oysters and may result in mortality. Some research has been conducted to determine whether the use of anaesthetics may minimise this stress and improve survival and nucleus retention. Anaesthetics have also been used to assist internal assessment of pearl oyster tissues (Acosta-Salmón, 2004; Norton et al., 1996; Strack, 2006). Several basic studies on the effectiveness of particular

anaesthetics have been conducted successfully with pearl oysters. Concentrations of propylene phenoxetol between 2 and 3 mL L^{-1} have been effectively use to relax *Pinctada albina* and *P. imbricata* (O'Connor & Lawler, 2002), *P. margaritifera* (Acosta-Salmon et al., 2005; Norton et al., 1996), and *P. maxima* (Mills et al., 1997). However, the effectiveness of various anaesthetics and the degree of success in using anaesthetics in the large-scale pearl production has not yet been determined. This may reflect satisfaction in the current implantation method and that the use of anaesthetics may increase production costs (labours and time) (Acosta-Salmón, 2004).

1.7.2 The potential of regenerated mantle

The ability to regenerate new tissue from wounds is essential for survival. This capability is a common phenomenon in metazoans (Alvarado, 2000; Bekkum, 2004), however, extensive studies on tissue regeneration in pearl oysters have only recently started. Recent studies by the Pearl Oyster Research Group, at James Cook University have shown that *Pinctada fucata* and *P. margaritifera* have the capacity to regenerate excised mantle tissues (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006; Acosta-Salmon et al., 2004). Anaesthesia of oysters is required prior to mantle excision. They found that excised mantle tissue could heal within three days and complete regeneration was evident three months after excision. Since donor oysters are generally killed to obtain donor mantle tissue used in pearl implantation (Acosta-Salmon et al., 2004; Taylor & Strack, 2008), these studies indicated that pearl oysters used to provide donor mantle tissue for pearl implantation need not necessarily be killed. This offers considerable potential benefits to the pearling industry:

- donor oysters that produces high quality pearls can be used as future parent stock to improve the quality of cultured oysters; and

- high quality oysters could potentially be used as donors on more than one occasion following mantle regeneration (Acosta-Salmon et al., 2005)

These potential benefits assume that the mantle tissue excised from anaesthetised pearl oysters or, from oysters which have regenerated previously excised mantle tissue, will perform in a similar fashion to 'normal' mantle tissue in the pearl process. This assumption has yet to be tested with any species of pearl oyster and it forms the basis of this study with *P. maxima*.

1.8 Major objectives of this study

The major objective of this study is to investigate the potential of using anaesthesia to facilitate mantle excision from *P. maxima* without mortality and to assess the use and potential anaesthetised and regenerated mantle as saibo for pearl production. It will attempt to achieve this by addressing the following questions:

- How do *P. maxima* respond to different anaesthetics and which anaesthetic is most appropriate for use with this species? (Chapter 2)

- How does *P. maxima* respond to mantle excision? Does excised mantle tissue regenerate and, if so, how quickly and are the characteristics of regenerated mantle similar to normal mantle? (Chapter 3)

- Is the composition of shell and nacre secreted by regenerated mantle the same as the normal shell? (Chapter 4)

- Can relaxed and or regenerated mantle be used for pearl implantation? (Chapter 5) If so, are pearls resulting from relaxed and or regenerated mantle similar to those produced by normal mantle? (Chapter 6)

- Do pearls from the same saibo donor have similar traits? (Chapter 7)

Several experiments were carried to address these questions and the specific aims were:

- to identify suitable relaxants for *P. maxima* and their concentrations for mantle excision (Chapter 2);

- to assess mantle healing and the possibility of regeneration following mantle excision from anaesthetised *P. maxima* (Chapter 3);

- to compare the structure and composition of shells produced by normal and regenerated mantle (Chapter 4);

- to observe the pearl-sac development between relaxed, regenerated and normal saibo (Chapter 5);

- to determine and to compare the quality pearls produced using both anaesthetised and regenerated saibo inserted into recipient pearl oysters (Chapter 6); and

- to analyse pearl quality from the same donor oyster with non-destructive method (Chapter 7).

Chapter 2

Use of anaesthetics with *Pinctada maxima*[1]

2.1 Introduction

To produce a cultured round pearl, a skilled-technician must implant a nucleus into the gonad of a recipient pearl oyster together with a piece of mantle tissue from a sacrificed donor oyster (Gervis & Sims, 1992). Subsequent proliferation of the donor mantle tissue forms the pearl-sac around the nucleus, and deposition of nacre from the pearl-sac onto the nucleus forms a cultured pearl over a period of about 2 years (Acosta-Salmon et al., 2004; Gervis & Sims, 1992). To optimise pearl quality, pearl oysters must be treated appropriately to minimise stress during and after the pearl implantation procedure which may include forced opening of their shells and incision of the gonad prior to implantation. Anaesthetics have been investigated as a means of reducing stress and mortality of pearl oysters resulting from pearl implantation (Norton et al., 2000). In a more recent development, anaesthetics were used to enable removal of mantle tissue from donor pearl oysters without killing them (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006; Acosta-Salmon et al., 2004). This potentially allows oyster donors that produce high quality pearls to be used as future broodstock. Furthermore, pearl oysters readily regenerate excised mantle tissue (Acosta-Salmon and Southgate, 2005; Acosta-Salmon and Southgate, 2006) and so donor oysters that are anaesthetised for mantle tissue removal, rather than killed, could potentially be used for pearl implantation on more than one occasion (Acosta-Salmon et al., 2004). This

[1] The data in this Chapter are published in Aquaculture (2009): Mamangkey et al., 2009. Use of anaesthetics with the silver-lip pearl oyster, *Pinctada maxima* (Jameson). Aquaculture 288, 280-284. See Appendix 1.

approach offers considerable benefits to the cultured pearl industry and justifies further investigation of the use of anaesthetics with pearl oysters.

The response of pearl oysters to anaesthetics has been shown to vary between species according to the type and concentration of the anaesthetic used. Propylene phenoxetol at a concentration of 2-3 mL L^{-1} has been used successfully with *Pinctada albina, P. imbricata, P. margaritifera* and *P. maxima* (Norton et al., 1996; O'Connor & Lawler, 2002). Benzocaine at a concentration of 1200 mg L^{-1} was similarly used to successfully induce relaxation in *P. albina, P. margaritifera* and *P. fucata* over a short period of time and with a short recovery time (Acosta-Salmon et al., 2005; Norton et al., 1996); however, this anaesthetic was less effective at a lower concentration of 500 mg L^{-1} (Acosta-Salmon et al., 2005). More natural derivatives, such as clove oil and menthol, have also shown a degree of effectiveness in inducing relaxation in pearl oysters (Norton et al., 1996).

Prolonged exposure to an anaesthetic may cause the mantle or body of pearl oysters to lose rigidity and collapse (Acosta-Salmon et al., 2005; Mills et al., 1997; Norton et al., 1996; O'Connor & Lawler, 2002). It may also result in mantle retraction and excessive mucus production (Norton et al., 1996) and render pearl oysters unsuitable for pearl implantation. Anaesthetics are difficult to manage under large-scale pearl implantation conditions and they have not yet been widely applied in the cultured pearl industry (Acosta-Salmon et al., 2005). However, donor pearl oysters are required in much smaller numbers than recipient oysters during the implantation procedure, and the use of anaesthetics with donors alone would be a more manageable proposition. Given the potential benefits of using anaesthetics with donor pearl oysters, there is a need for further research in this field with a view to overcoming some of the potential problems outlined above. With this objective, this study determined the

effectiveness of five anaesthetics, at varying concentrations, in inducing relaxation in *P. maxima*.

2.2 Material and methods

The oysters used in this study had a mean (± SD) dorso-ventral measurement (DVM) of 128.9 ± 12.5 mm and were maintained in suspended culture at James Cook University's Orpheus Island Research Station off Townsville, north Queensland, Australia. They were cleaned and maintained in a raceway prior to the experiment. Twenty-seven oysters were randomly distributed between three replicate 20 L aquaria used for each of seven anaesthetic treatments: 3 mL L^{-1} of 2-phenoxyethanol (Sigma-Aldrich, Inc.), 1.5 mL L^{-1}clove oil (Continental Flavour), 0.25 mL L^{-1} menthol liquid (Auroma Pty Ltd.), 0.4 mL L^{-1} menthol liquid, 2.5 mL L^{-1} of propylene phenoxetol (Nipa Laboratories Ltd.), 500 mg L^{-1} and 1200 mg L^{-1} of benzocaine (Sigma-Aldrich, Inc.). Thus each aquarium contained nine oysters. A further set of aquaria was used as controls and contained oysters held in 1 µm filtered sea water. The concentrations of the particular anaesthetics used in this experiment were based on those used by Norton et al. (1996).

To prepare the solutions of propylene phenoxetol, 2-phenoxyethanol, clove oil and menthol liquid, each was added to seawater in a small container and shaken vigorously before being transferred to a large container of seawater (Norton et al., 1996). The 500 mg L^{-1} solution of benzocaine was made using a preparation of 1:4 w/v benzocaine:methanol solution that was poured into hot seawater, before being transferred into a large container of seawater to reach the desired concentration (Acosta-Salmon et al., 2005). To prepare the 1200 mg L^{-1} benzocaine solution, benzocaine was dissolved in ethanol (100 g L^{-1}) and the resulting solution was mixed slowly into seawater (Acosta-Salmon et al., 2005).

Aquaria were filled with 1 µm filtered sea water and the respective anaesthetic solutions were added to give the desired final concentrations. The temperature of seawater in the aquaria was maintained at 24 ± 1ºC and pH ranged from 8.0 to 8.2. Prior to placing the oysters into the aquaria, the pH of each aquarium was recorded. Oysters used in the experiment were first placed hinge-down in a tray until their shells opened. A plastic wedge was placed between the shell valves to prevent closure and allow rapid access of anaesthetic solution to oyster tissues once they were placed into the aquaria. Oysters were placed into mesh baskets which were then suspended into the aquaria.

Once exposed to the anaesthetic solutions, oysters were observed continually. Oysters were considered to be relaxed when they no longer responded to stimulation (touching) of the mantle tissue (Norton et al., 1996). The timing of relaxation and the proportion of relaxed oyster in each aquarium were recorded for a 30 minute period which began when the first oyster in each aquarium became relaxed. Within this period oysters were also observed for 'body collapse' and 'mantle collapse' (Acosta-Salmon et al., 2005). Mantle collapse is characterised by mantle tissue that falls away from the shells, and body collapse is characterised by lack of muscular strength in all soft body parts (Acosta-Salmon et al., 2005). In both cases, they are considered unsuitable as donor oysters for pearl implantation (Acosta-Salmon et al., 2005). Relaxed oysters that did not show these characteristics were categorised as suitable donor oysters. The number of suitable donors within each anaesthetic treatment was recorded throughout the 30 min observation period.

Oysters with mantle or body collapse were removed from aquaria to a raceway containing running seawater. All other oysters were retained in treatment aquaria for the 30 min period and then transferred to running seawater where their recovery was monitored for a further 2 h. Oysters were considered to have recovered when they closed their shells in response to

touching of their mantle tissue (Norton et al., 1996). They were then placed into panel nets (Gervis & Sims, 1992) and transferred to a long-line culture system in the ocean. Oyster survival was recorded for a further month after exposure to anaesthetics.

A Kruskall-Wallis (κ) analysis was used to determine whether there was a difference between anaesthetics in terms of the time required for oysters to relax. A Pearson's Chi Square (χ^2) test was conducted to assess the effectiveness of anaesthetics based on the number of relaxed oysters and oyster survival. The Kruskall-Wallis analysis was generated by SPSS ver. 13, but the Pearson's Chi Square was by Analyse-it ver. 2.11 for Microsoft Excel 2003.

2.3 Results

The mean times required for oysters to become relaxed and to recover from exposure to anaesthetics are shown in Table 2.1. Oysters exposed to 1200 mg L^{-1} benzocaine showed the fastest time to relaxation of 10.5 (± 7.9) min while those treated with 0.4 mL L^{-1} menthol liquid required the longest time of 31.3 (± 5.2) min to reach relaxation. Oysters exposed to 3 mL L^{-1} 2-phenoxyethanol reached relaxation at 13.8 (± 6.4) min and this anaesthetic resulted in the highest proportion of relaxed oyster (96.3%) of all anaesthetics tested. Although both 2.5 mL L^{-1} propylene phenoxetol and 1200 mg L^{-1} benzocaine brought about relaxation of 88.9% of oysters, they showed differences in induction time with 2.5 mL L^{-1} propylene phenoxetol achieving this in 15 (± 7.1) min compared to 10.5 (± 7.9) min for 1200 mg L^{-1} benzocaine. The lowest proportion of relaxed oysters (51.9%) was recorded in the 500 mg L^{-1} benzocaine treatment even though the average time to relax was relatively low (17.5 ± 8.9 min). Oysters exposed to 0.25 mL L^{-1} menthol liquid did not relax. All but four of the oysters exposed to clove oil became relaxed, but all relaxed oysters in this treatment died during the

30 min observation period. Death was preceded by production of excessive mucus and mantle retraction. This condition was followed by mantle and body collapse.

Table 2.1. Mean (± SD) time required for *Pinctada maxima* to relax and the numbers of oysters that became relaxed when exposed to seven anaesthetic treatments.

Anaesthetic	**Relaxed oysters**	
	mean (± SD) time to relax (min)	**numbers relaxed**
2-Phenoxyethanol (3 mL L^{-1})	13.8 (± 6.4)	26 (96.3%)
Benzocaine (500 mg L^{-1})	17.5 (± 8.9)	14 (51.9%)
Benzocaine (1200 mg L^{-1})	10.5 (± 7.9)	24 (88.9%)
Clove oil (1.5 mL L^{-1})	10.6 (± 4.7)	23 (85.2%)*
Menthol liquid (0.25 mL L^{-1})**	-	-
Menthol liquid (0.4 mL L^{-1})	31.3 (± 5.2)	17 (63.0%)
Propylene phenoxetol (2.5 mL L^{-1})	15 (± 7.1)	24 (88.9%)

*all oysters died during the observation. **no oysters relaxed

A Kruskal-Wallis (κ) analysis conducted to include five anaesthetics (i.e. excluding the 0.25 mL L^{-1} menthol liquid and clove oil treatments) showed that the type of anaesthetic influence the time required for oysters to relax ($\kappa = 43.61$, $P < 0.01$) while Chi Square test showed a highly-significant difference in the numbers of relaxed oysters between treatments ($\chi_4^2 = 23.14$, $P < 0.01$, critical value was 0.0001).

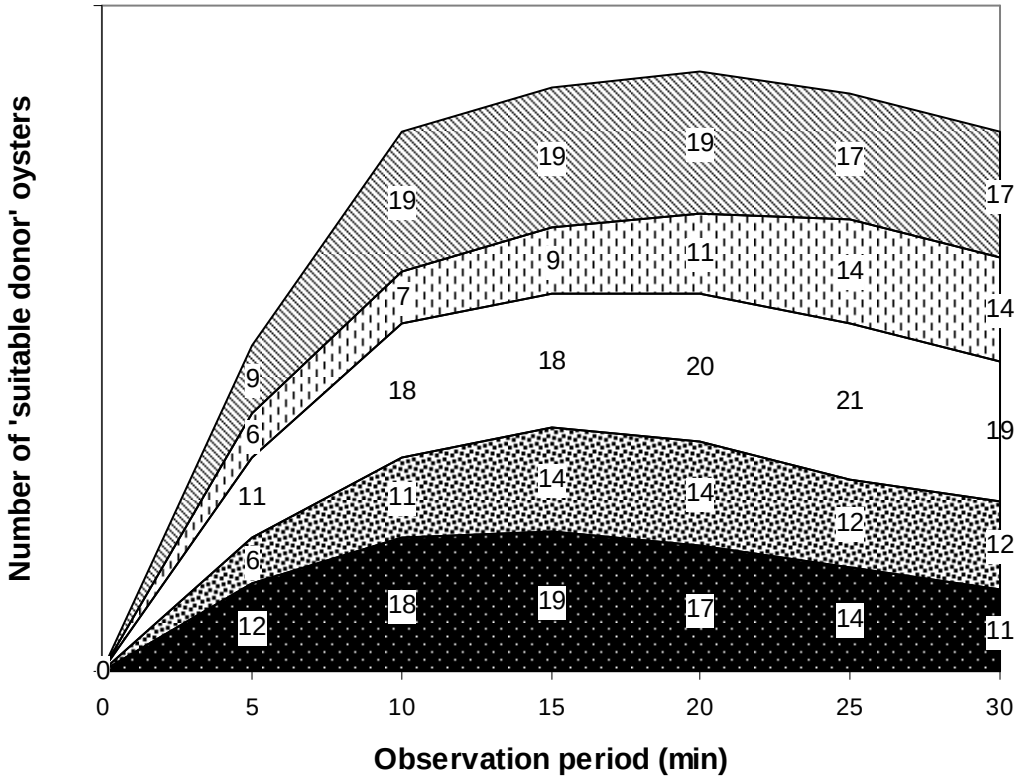

Fig. 2.1. Changes in the number of suitable *Pinctada maxima* donors within 30 minutes of exposure to the following anaesthetics: 3 mL L^{-1} 2-phenoxyethanol; 500 mg L^{-1} benzocaine; 1200 mg L^{-1} benzocaine; 0.4 mL L^{-1} menthol liquid; 2.5 mL L^{-1} propylene phenoxetol. Total number of oysters per treatment was 27.

Within the 30 min observation period following relaxation of the first oyster in each aquarium, all treatments experienced a decrease in the number of suitable donor oysters with the exception of the 500 mg L^{-1} benzocaine treatment (Fig. 2.1). The number of suitable donor oysters decreased after 20 min in both the 2-phenoxyethanol and 0.4 mL L^{-1} menthol liquid treatments, after 25 min in the 1200 mg L^{-1} benzocaine treatment, and after 15 min when exposed to propylene phenoxetol. These decreases resulted from the onset of mantle or body collapse in relaxed oysters. However, numbers of suitable donors following exposure to 2-phenoxyethanol, 1200 mg L^{-1} benzocaine and propylene phenoxetol increased rapidly to

more than half the total in each treatment (total 27 oysters) within 10 min (Fig. 2.1). The highest numbers recorded were 19 suitable donors (70.3%) for both 2-phenoxyethanol and propylene phenoxetol treatments, and 21 suitable donors (77.8%) in the 1200 mg L^{-1} benzocaine treatment (Fig. 2.1).

All oysters in all treatments, except those exposed to clove oil, reached normal condition within two hours of removal from exposure to the various anaesthetics. One month later, nearly 100% survival of oyster was observed in the treatments using 3 mL L^{-1} 2-phenoxyethanol, 500 mg L^{-1} benzocaine, 2.5 mL L^{-1} propylene phenoxetol, and the control, with only one dead oyster in each of them (Fig. 2.2). Six dead and three dead oysters resulted from exposure to 0.4 mL L^{-1} menthol liquid and 1200 mg L^{-1} benzocaine, respectively. Although all oysters that relaxed after being exposed to clove oil died shortly after exposure, the four oysters in this treatment that did not relax were still alive after one month (Fig. 2.2). With the exception of the clove oil treatment, survival of oysters from the remaining anaesthetics did not differ significantly (χ^2_6 =17.97, P > 0.05, critical value was 0.1025).

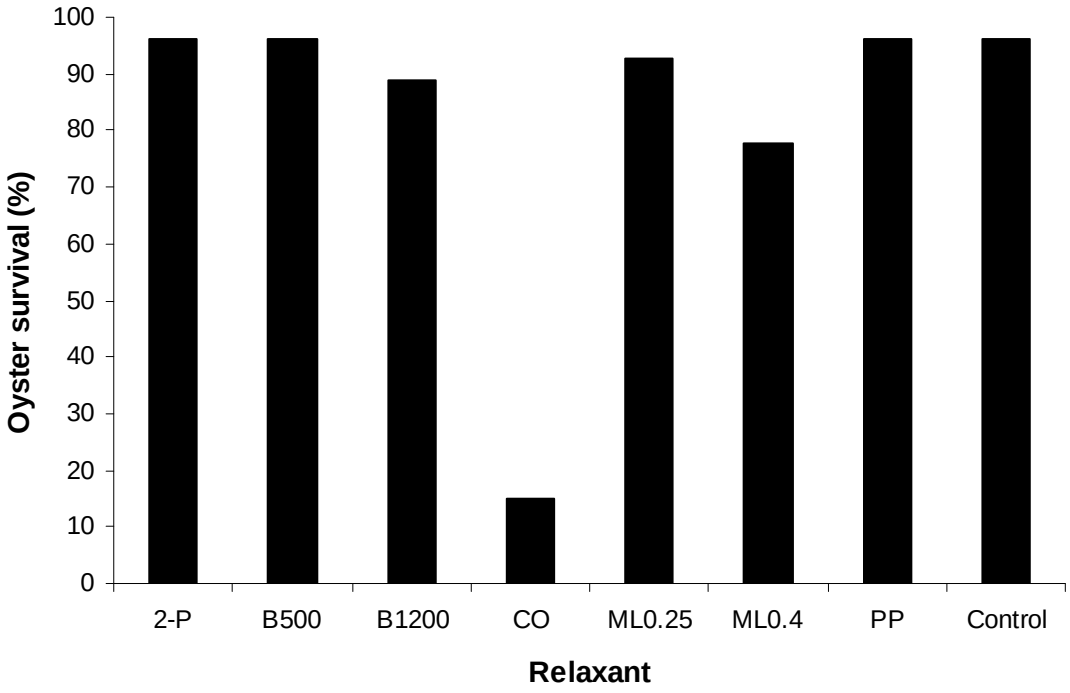

Fig. 2.2 Survival (%) of *Pinctada maxima* one month after exposure to the following anaesthetics: 2-P, 3 mL L^{-1} 2-phenoxyethanol; B500, 500 mg L^{-1} benzocaine; B1200, 1200 mg L^{-1} benzocaine; CO, 1.5 mL L^{-1} clove oil; ML0.25, 0.25 mL L^{-1} menthol liquid; ML0.4, 0.4 mL L^{-1} menthol liquid; PP, 2.5 mL L^{-1} propylene phenoxetol; and control, without anaesthetic.

2.4 Discussion

The results of this study show that benzocaine at a concentration of 1200 mg L^{-1}, 2-phenoxyethanol and propylene phenoxetol were among the better anaesthetics to use for *P. maxima*. A summary of the effectiveness and characteristics of these three anaesthetics is shown in Table 2.2. Each varied in the time required to induce relaxation, the proportion of oysters that relaxed, the time that oysters remained relaxed, the time required for relaxed oysters to recover, and oyster survival. Norton et al. (1996) suggested that anaesthetics used with pearl oysters should ideally induce relaxation in less than 15 min and allow rapid recovery of oysters following anaesthesia (< 30 min). While, benzocaine (1200 mg L^{-1}), 2-phenoxyethanol and propylene phenoxetol did induce relaxation within 15 min, none of them allowed recovery within 30 min (Table 2.2).

Different species of mollusc react differently to particular anaesthetics and concentrations (Aquilina & Roberts, 2000; Araujo et al., 1995) and a summary of a number of studies in this field is shown in Table 2.3. The induction time for *P. maxima* exposed to 1200 mg L^{-1} benzocaine in this study was similar to those observed for both *P. margaritifera* and *P. fucata* in a prior study (Acosta-Salmon et al., 2005). Furthermore, relaxation of *P. maxima* following exposure to 2.5 mL L^{-1} propylene phenoxetol was also similar to that observed for *P. margaritifera* when exposed to similar concentration of the same chemical (Norton et al., 1996). In contrast, a concentration of 2.5 mL L^{-1} propylene phenoxetol caused high mortality of the abalone, *Haliotis iris* (Aquilina & Roberts, 2000)(Table 2.3). *P. maxima* showed much slower relaxation (13.8 min) when exposed to 2-phenoxyethanol than reported for the abalone, *H. midae* (White et al., 1996). Furthermore, neither 2-phenoxyethanol or benzocaine, which were effective anaesthetics for *P. maxima* in this study, were successful in

inducing relaxation in a recent study with the queen conch, *Strombus gigas* (Acosta-Salmon & Davis, 2007)(Table 2.3).

Table 2.2. A summary of the effectiveness of three anaesthetics; 2-phenoxethanol, benzocaine and propylene phenoxetol.

Parameter	**3 mL L^{-1} 2-phenoxyethanol**	**1200 mg L^{-1} benzocaine**	**2.5 mL L^{-1} propylene phenoxetol**
Induce relaxation (less than 15 min)	Yes	Yes	Yes
Percentage of relaxed oysters	>95%	85- 90%	85-90%
Proportion of suitable donors within 30 min	Decreased after 20 min	Decreased after 25 min	Decreased after 15 min
Rapid recovery (< 30 min)	No	No	No
Proportion of recovered oysters after two hours	100%	100%	100%
Survival	>95%	85-90%	>95%
Preparation	Shake with seawater in a small container prior to application	Dilute in alcohol prior to application	Shake with seawater in a small container prior to application
Dilution	Diluted well	Crystallisation on bottom	Could create oil on surface
Toxicity	Low	Low	Low

Table 2.3. Induction time (min ± SD) for different species of molluscs when exposed to various anaesthetics.

Anaesthetic	Species	Induction time	Signs of relaxation	Mortality
3 mL L^{-1} 2-phenoxyethanol	*Pinctada maxima*[1]	13.8 ± 6.4	no response to touch	low
	Strombus gigas [2]	-	not shown	none
	Haliotis midae[3]	2.9 ± 2.3	fall off from wall	none
500 mg L^{-1} benzocaine	*P. maxima* [1]	17.5 ± 8.9	no response to touch	low
	P. margaritifera [4]	11.0 ± 7.3	no response to touch	none
	S. gigas [2]	-	not shown	none
1200 mg L^{-1} benzocaine	*P. maxima* [1]	10.5 ± 7.9	no response to touch	low
	P. margaritifera [4]	9.0 ± 4.0	no response to touch	none
	P. fucata [4]	10.27 ± 4.41	no response to touch	none
	S. gigas [2]	-	not shown	none
0.4 mL L^{-1} menthol liquid	*P. maxima* [1]	31.3 ± 5.2	no response to touch	low
	S. gigas [2]	-	not shown	none
2.5 mL L^{-1} propylene phenoxetol	*P. maxima* [1]	15 ± 7.1	no response to touch	low
	P. margaritifera [4]	9.6 ± 4.3	no response to touch	none
	Haliotis iris[5]	up to 29 min at 22 °C	fall off from wall	high
2 - 3 mL L^{-1} propylene phenoxetol	*P. albina*[6]	< 15	no response to touch	none
	P. margaritifera[6]	< 15 min	no response to touch	none

[1]This study; [2]Acosta-Salmon and Davis (2007); [3]White et al. (1996); [4]Acosta-Salmon et al. (2005); [5]Aquilina and Roberts (2000); [6]Norton et al. (1996).

In this study, the three anaesthetics that induced the highest proportions of relaxed oysters (1200 mg L^{-1} benzocaine, 2-phenoxyethanol and propylene phenoxetol) also resulted in oysters which remained in a relaxed state for longer periods (> 15 mins) before showing signs of mantle and body collapse, when compared to the other chemicals tested. This is an important factor when considering the potential use of relaxed oysters in the pearl implantation process, because it increases the period over which an oyster can be utilised as a mantle tissue donor. An inability to maintain this condition, i.e. oysters showing mantle or body collapse or even mortality, renders an oyster unsuitable as a donor (Acosta-Salmon et al., 2005).

Anaesthetics may disrupt synaptic transmission in the neural system of molluscs (Spencer et al., 1995; Woodall et al., 2003) and longer periods of exposure to anaesthetics may lead to neuro-degeneration of the important organs within animals (Woodall et al., 2003) and subsequently death. The death of a large proportion of the *P. maxima* exposed to clove oil indicates a toxic nature to this chemical. Besides changes in the morphological appearance of their soft tissues, affected oysters also produced excessive mucus. Norton et al. (1996) recorded mucus production from *P. albina* following exposure to the anaesthetic MS222, but not when exposed to clove oil at the same concentration as used in the present study. Clearly there are differences in the degree of toxicity of clove oil to various pearl oyster species. However, it is interesting to note that four of the 27 *P. maxima* exposed to clove oil in this study did not relax or have an adverse reaction to this chemical; they were still alive one month after exposure to clove oil. Mantle tissue of the *P. maxima* that died following exposure to clove oil became inflamed and more red in colour before developing lesions indicating the death of mantle epithelial cells. This epithelial irritation may have resulted from too high a concentration of clove oil and it is possible that this chemical may be

effective as an anaesthetic for *P. maxima* at a lower concentration. The death of some oysters recorded a month after exposure to menthol liquid also raises concerns about the toxicity of this chemical to *P. maxima* and its use as an anaesthetic. On the basis of our results, we do not recommend the use of clove oil or menthol liquid as anaesthetics for *P. maxima*.

Other important considerations when assessing the effectiveness of anaesthetics include their ease of use and preparation and potential toxicity to human users. All chemicals chosen in this study have low risk to human health. However, particular anaesthetics require more effort in preparation than others and may vary in their solubility in seawater. These characteristics are outlined for 2-phenoxyethanol, 1200 mg L^{-1} benzocaine and propylene phenoxetol in Table 2.2. These three chemicals were effective anaesthetics for *P. maxima* as outlined above, however, they varied in their preparation and solubility (Table 2.2). While 2-phenoxyethanol, benzocaine and propylene phenoxetol may be considered the most suitable of the anaesthetics tested for *P. maxima*, 2-phenoxyethanol and propylene phenoxetol are perhaps superior because of their ready solubility in seawater and resulting ease of preparation.

While this experiment has identified anaesthetic treatments that produce relaxation in *P. maxima* and will facilitate mantle excision, this procedure is only useful if there are no adverse effects on pearl production, when mantle tissue from anaesthetised donor oysters is used for pearl implantation. Subsequent research will address this issue (Chapter 5 and 6).

Chapter 3

Regeneration of excised mantle tissue by *Pinctada maxima*[1]

3.1 Introduction

Recent research with *Pinctada fucata* and *P. margaritifera* has shown that mantle tissue appropriate for use in pearl implantation can be removed from anaesthetised pearl oysters without mortality and that excised mantle tissue is readily regenerated within about 3 months (Acosta-Salmon & Southgate, 2005; Acosta-Salmon et al., 2004). This innovation has implications for the pearl implantation process. First of all, mantle donors do not necessarily have to be sacrificed to provide donor mantle tissue and those producing good quality pearls could later be used a parent-stock to improve the quality of pearl farm stock. Secondly, high quality oysters could potentially be used as mantle tissue donors on more than one occasion (Acosta-Salmon & Southgate, 2005; Acosta-Salmon et al., 2004).

Regeneration is a form of asexual reproduction, but the term also refers to tissue or organ repair and reconstruction to the original form (Goss, 1969). The repair of lost body parts involves at least two major steps: wound healing and regeneration (Carlson, 2007), where wound healing takes part prior to morphogenesis. Wound healing is usually characterised by a complete covering by epidermis (Carlson, 2007) before morphogenetic processes begin to build up the structure and, finally, the new body part reaches the same form as the original both anatomically and functionally (Alvarado, 2004). Although a feature of almost every phylum in the animal kingdom, complete wound healing or organ regeneration varies among

[1] The data in this Chapter are in press in Fish and Shellfish Immunology (2009), Regeneration of excised mantle tissue by the silver-lip pearl oyster, *Pinctada maxima* (Jameson). Fish & Shellfish Immunology, 27, 164-174. See Appendix 2.

taxa and is affected by factors such as wound size (DesVoigne, 1968), location of the wound (Pauley, 1966), hormones assisting the healing process (Franchini & Ottaviani, 2000), genes, age and cells complexity (Alvarado, 2000; Alvarado, 2006; Carlson, 2007; Goss, 1969). Furthermore, the capability and time required for regeneration differs between taxa (Carlson, 2007; Goss, 1969), even between closely-related species (Bely, 2006; Brockes et al., 2001; Tsonis, 2000).

The results of Chapter 2 showed that *P. maxima* are readily anaesthetised. This potentially allows mantle excision from living oysters. Although some aspects of the repair and regeneration of damaged mantle tissue has been reported for some species of pearl oyster (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006), no comparative data are available for *P. maxima*. Given the predominance of the sector of the pearling industry that relies on *P. maxima* (Southgate et al., 2008) and the potential benefits to it from retaining living donor oysters and the possible reuse of donors, this study describes the regeneration process of mantle tissue from *P. maxima*.

3.2 Material and methods

Excision of mantle tissue from live adult pearl oysters requires anaesthesia of the oysters (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006). Propylene phenoxetol at a concentration of 2.5 mL L^{-1} is an appropriate anaesthetic for *Pinctada maxima* (Chapter 2).

The *Pinctada maxima* used in this study were cultivated using a suspended culture system in Pioneer Bay at James Cook University's Orpheus Island Research Station, north Queensland, Australia (18°36'30''S, 146°29'15''E). The study was comprised of two parts: a three-month

study determining survival of oysters with excised mantle tissue and their capacity for mantle regeneration, and a more detailed study of the process of mantle regeneration.

3.2.1 Experiment 1 – Survival following mantle excision

A total of 56 oysters with mean (± SD) dorso-ventral measurement (DVM) and antero-posterior measurement (APM) of 125.5 ± 8.9 and 129.6 ± 9.1 mm, respectively, were used in this experiment. Thirty-nine of them were deployed for excision while the rest were used as controls. Following anaesthesia the oysters used for excision were divided into three groups from which tissue was excised from either the right mantle lobe, left mantle lobe or both mantle lobes. Oysters in the control group were anaesthetised but were not used for mantle excision. Excision was made at the distal part of the ventral region of the mantle (Fig. 3.1). The piece of mantle removed was approximately 30 mm long and 10 mm wide. Following mantle excision, oysters were returned to culture conditions in Pioneer Bay for three months. During this period, oysters were maintained in panel (pocket) nets (Gervis & Sims, 1992) and fouling was gently brushed from the nets at approximate monthly intervals following standard pearl oyster husbandry practices (Southgate, 2008). At the end of this three-month period, oyster survival was recorded and two oysters were selected arbitrarily from each group to be sacrificed for histological examination of healed mantle.

3.2.2 Experiment 2 – Mantle healing and regeneration following excision

The study was continued with more detailed observation of wound healing and mantle regeneration in *P. maxima*. Only the left mantle lobes were studied because the results of Experiment 1 showed that there were no differences in the healing and regeneration of mantle related to whether the left, right or both mantle lobes were excised. Thirty six oysters with

mean (± SD) DVM and APM of 151.6 ±13.4 and 143.7 ±11.7 mm, respectively were used for excision following anaesthesia using 2.5 mL L^{-1} propylene phenoxetol. Once oysters were relaxed, the distal part of the ventral region of the left mantle lobe was excised (Fig. 3.1).

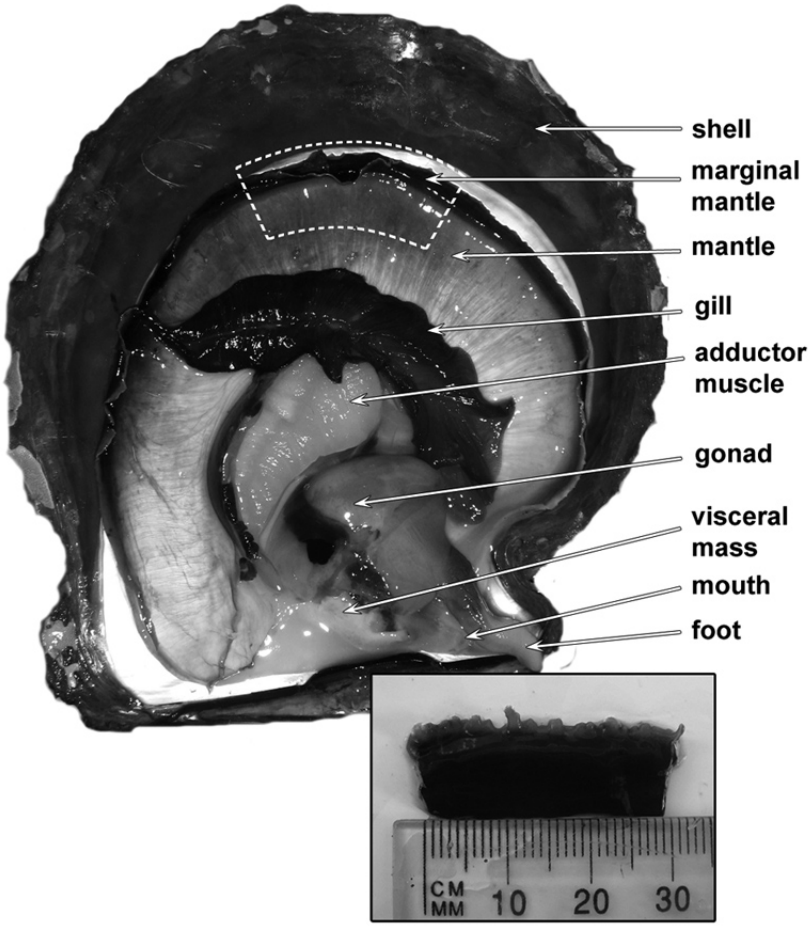

Fig. 3.1. *Pinctada maxima* with one shell valve removed showing approximate area (dotted line) of excised mantle tissue. Inset: the excised mantle.

The mean (± SD) length of mantle tissue removed was 29.7 ± 2.3 mm. After mantle excision all oysters were immediately placed into a tank with flowing seawater together with another 17 oysters that were anaesthetised without mantle excision (controls). Two of the treated oysters were sampled at 1, 3, 6, 12, 24, 36, 48, 72 and 120 h (5 days) after mantle excision.

Remaining oysters were then transferred to field culture conditions in Pioneer Bay for subsequent sampling of a further two oysters at 12, 24, 45, 72 and 90 d after mantle excision. A sample size of two oysters was influenced by the high commercial value and limited availability of *P. maxima*. Oysters sampled up to five days after mantle excision were anaesthetised and sacrificed. Oysters sampled after this time (i.e. after transfer to the field) were again anaesthetised and a further piece of mantle, that included the initial excision wound, was removed and preserved in 10% formaldehyde for histological analysis; they were then held under field culture conditions and their survival was monitored for a further three months.

3.2.3 Histological analysis of mantle tissue

Excised mantle tissue from oysters in Experiment 1 and Experiment 2 was subject to histological analysis using Mayer's Haematoxylin and Young's Eosin-Erythrosin, MSB (Martius Scarlet Blue) trichrome and Alcian Blue-PAS stains (Culling et al., 1985) following sectioning to 5 μm. The morphological structure of regenerated mantle tissue was compared with non-regenerated mantle tissue excised from an uninjured part of the mantle adjacent to the wound site. This comparison also involved reference to descriptions of 'normal' mantle morphology for *P. maxima* (Dix, 1973; Humphrey & Norton, 2005) and other pearl oysters (Garcia-Gasca et al., 1994).

3.3 Results

3.3.1 Experiment 1 – Survival following mantle excision

Only one oyster death was recorded within the three months following mantle excision. Healing and regeneration of mantle tissue in oysters subject to excision from the left, right or both mantle lobes was evident, with regenerated mantle appearing similar to normal mantle (Fig. 3.2). All external and internal components of normal mantle were present in regenerated mantle tissue.

Histological examinations showed that the inner fold of regenerated mantle tissue grew bigger than the other folds, and consisted of a large amount of muscle fibres (Fig. 3.2 A-D). In the area between the outer and middle folds, known as the periostracal grove, a conchiolin layer was secreted from the base of the regenerated mantle (Fig. 3.2 A-B). Epithelial cells had completely covered all folds and pigmentation was particularly evident around the inner fold and the inner surface of middle fold. (Fig. 3.2 B,D). Along the cells and sub-epithelial cells areas there were irregular distributions of both eosinophilic and basophilic cells (Fig. 3.3).

Three-month after mantle excision, approximately one in four oysters had developed an unusual stricture in the proximal area of the regenerated inner fold of the mantle (Fig. 3.4). Pigmentation in the marginal zone was clear but the pallial zone seemed to be more transparent than in normal mantle. New nacre secreted on the inner surface of the shell adjacent to the wound area was thin and appeared different to nacre at other point in the shell.

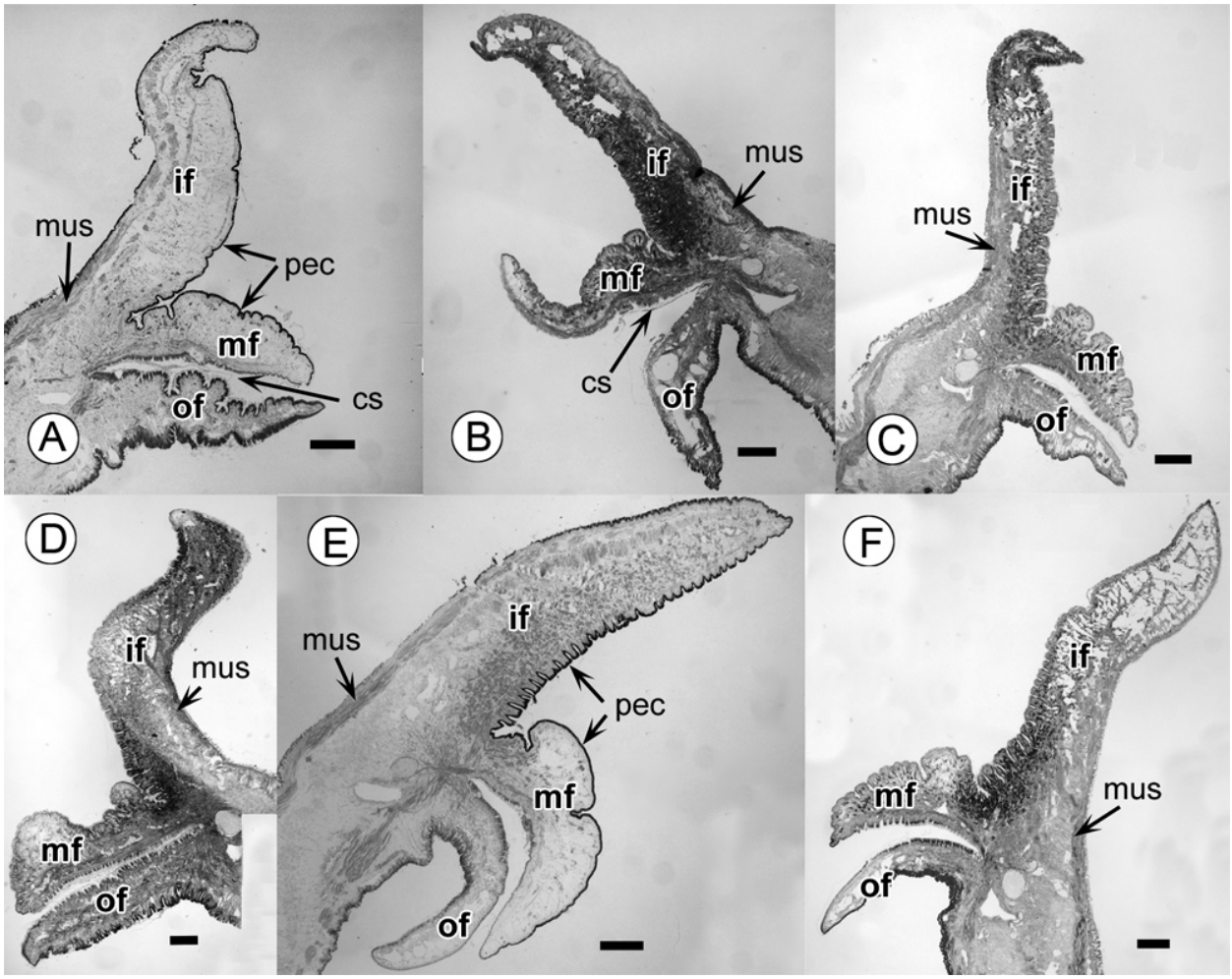

Fig. 3.2. Histological view of regenerated mantle tissue, three months after excision (A-D) and normal mantle tissue (E, F) of *Pinctada maxima*. A. regenerated left mantle following excision from both mantle lobes; B. regenerated right mantle following excision from both mantle lobes; C. regenerated mantle following excision of left mantle lobe; D. regenerated mantle following excision of right mantle lobe; E. normal left mantle; and F. normal right mantle (if: inner fold, mf: middle fold, of: outer fold, cs: conchiolin secretion, mus: muscle, and pec: pigmented epithelium cells. A, E stained with Haematoxilin eosin– erythrosin technique, and B, C, D, F with MSB trichrome technique. Bar scale 200 µm).

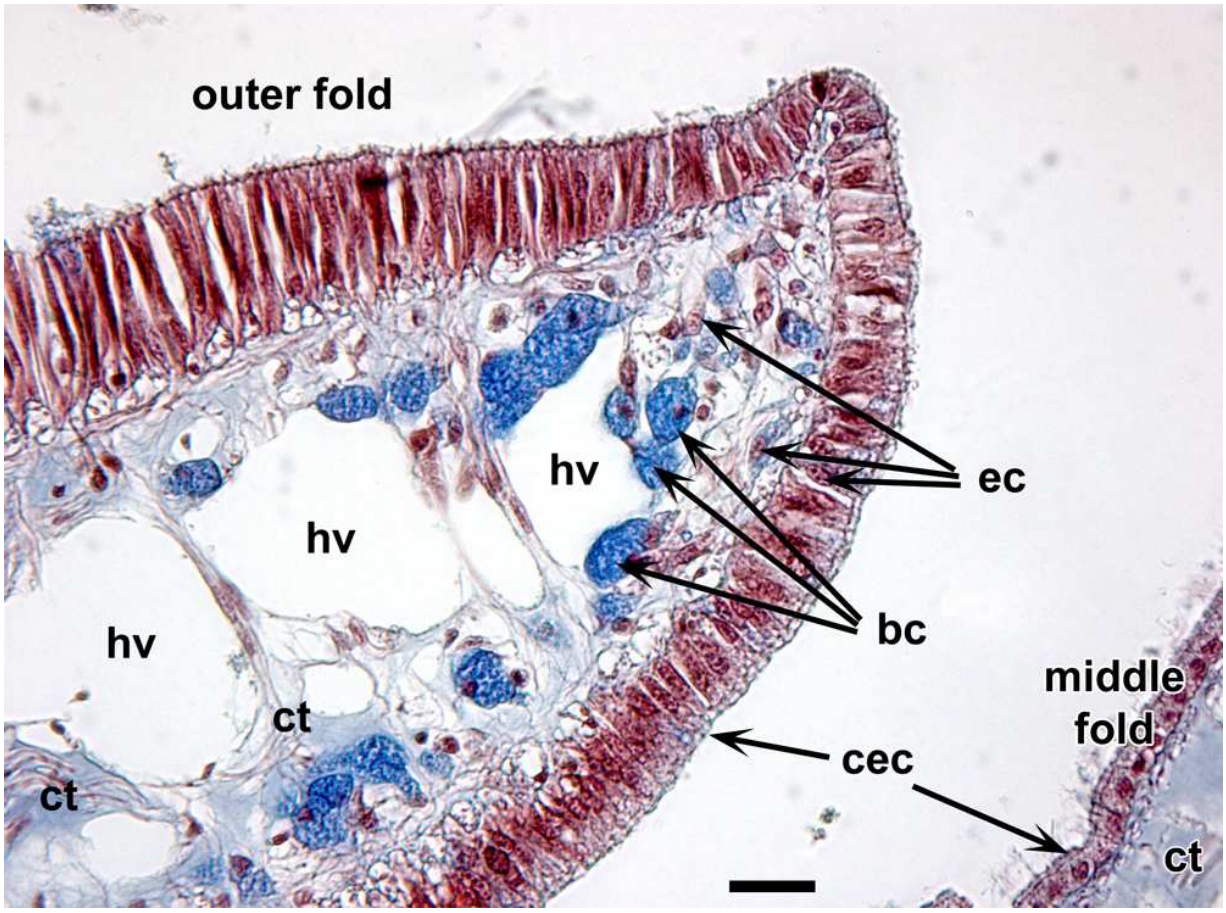

Fig. 3.3. Photomicrograph of the distal part of the outer fold and part of middle fold of regenerated mantle three months after mantle excision showing haemolymph vessels (hv), connective tissue (ct) and both basophilic (bc) and eosinophilic cells (ec) distributed along the epithelium and at the subepithelial region of the folds. Columnar epithelial cells (cec) are the main cell type of the epithelium. Stained with MSB trichrome. Bar scale 20 µm.

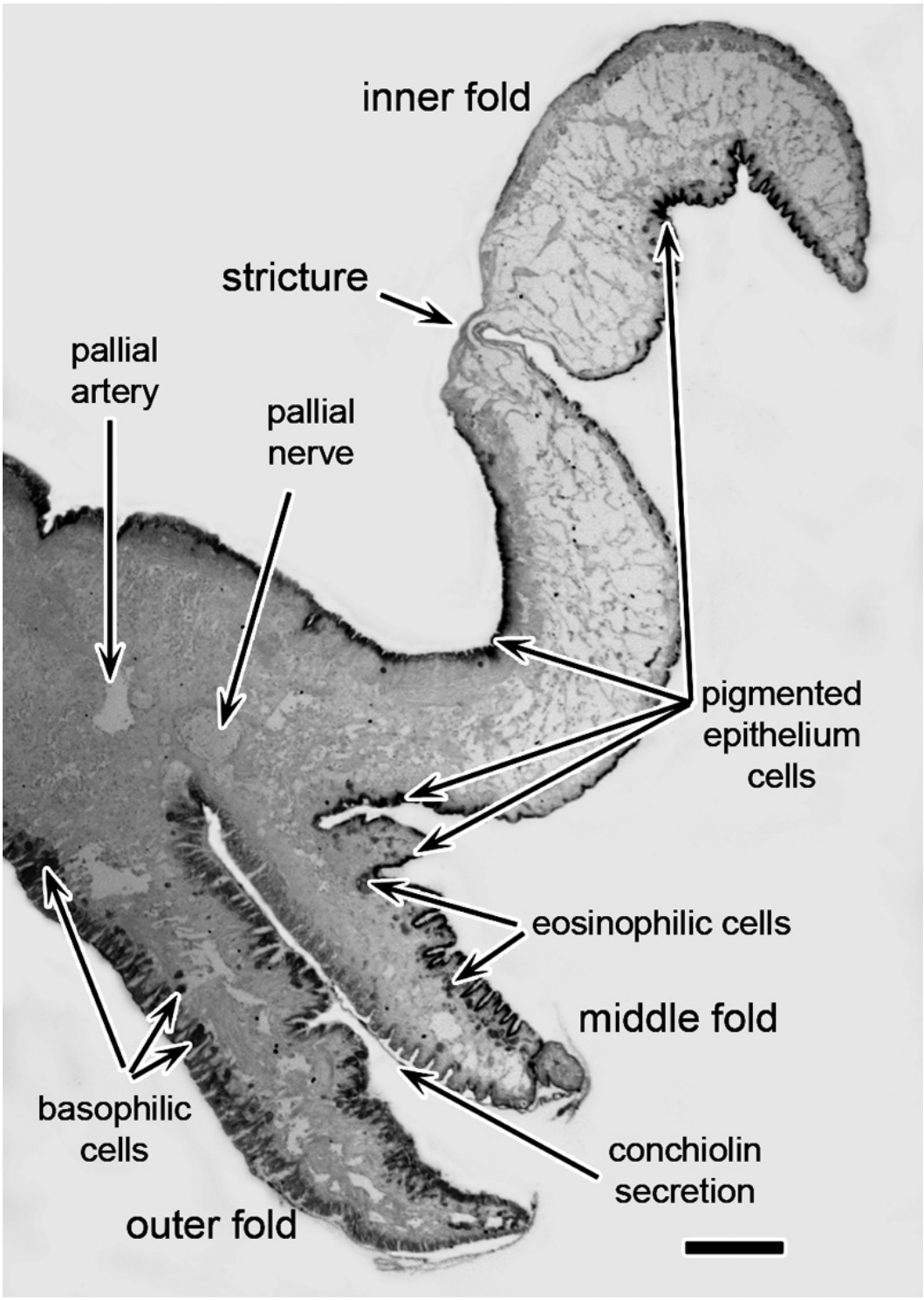

Fig. 3.4. Stricture on the inner fold of the regenerated mantle tissue of *Pinctada maxima*; three months after excision. Bar scale 200 µm.

3.3.2 Experiment 2 – Mantle healing and regeneration following excision

Regular observation allowed wound healing and regeneration to be described in a sequential manner. Initially, this process was characterised by an invagination of both edges of the wound within 48 hours of excision (Fig. 3.5). The latero-ventral edges of the wound flexed dorsally and attached to the dorsal edge of the wound thus reducing the size of the exposed area of the wound (Fig. 3.5B). This process was not obvious in the first day after excision (Fig. 3.5A). The flexed mantle edges retained their form until 5 days after excision (Fig. 3.5C). Newly regenerated mantle extended from the dorsal edge of the wound and material was deposited onto the shell 12 days after excision (Fig. 3.6A). As the mantle regenerated and grew in size, this deposition continued (Fig. 3.6B), and a thin layer of nacre has been developed by 45 days after excision when the distal part of the new mantle had reached the same position ventrally as the normal, non-injured, mantle (Fig. 3.6C).

Histological analysis showed that the first reaction of the mantle one hour after excision was movement of muscle to minimise the area of the wound on both sides of the epithelium (Fig. 3.7A). This was more evident within three hours (Fig. 3.7B), where the contraction of subepithelial muscle brought about constriction of the wound site transversally. Six hours after mantle excision the constriction loosened and haemocyte assemblages were evident at the wound site (Fig. 3.7C), while the epithelial cells around the wound flattened and became smaller. Six hours later, haemocytes had completely plugged the wound site (Fig. 3.7D). Squamous-like epithelial cells covered the wound area within 24 hours of excision, and extended from both the outer and inner epithelia; however, there was still an opening in the middle of the wound (Fig. 3.8). Connective tissue, with the appearance of muscle fibres, and their differentiation, characterized the 36 hour post-excision sample (Fig. 3.9A, B). Although

the gap became smaller, the wound site between 36 and 72 hours after mantle excision was composed of haemocytes and the epithelium had covered the entire wound site (Fig. 3.9C).

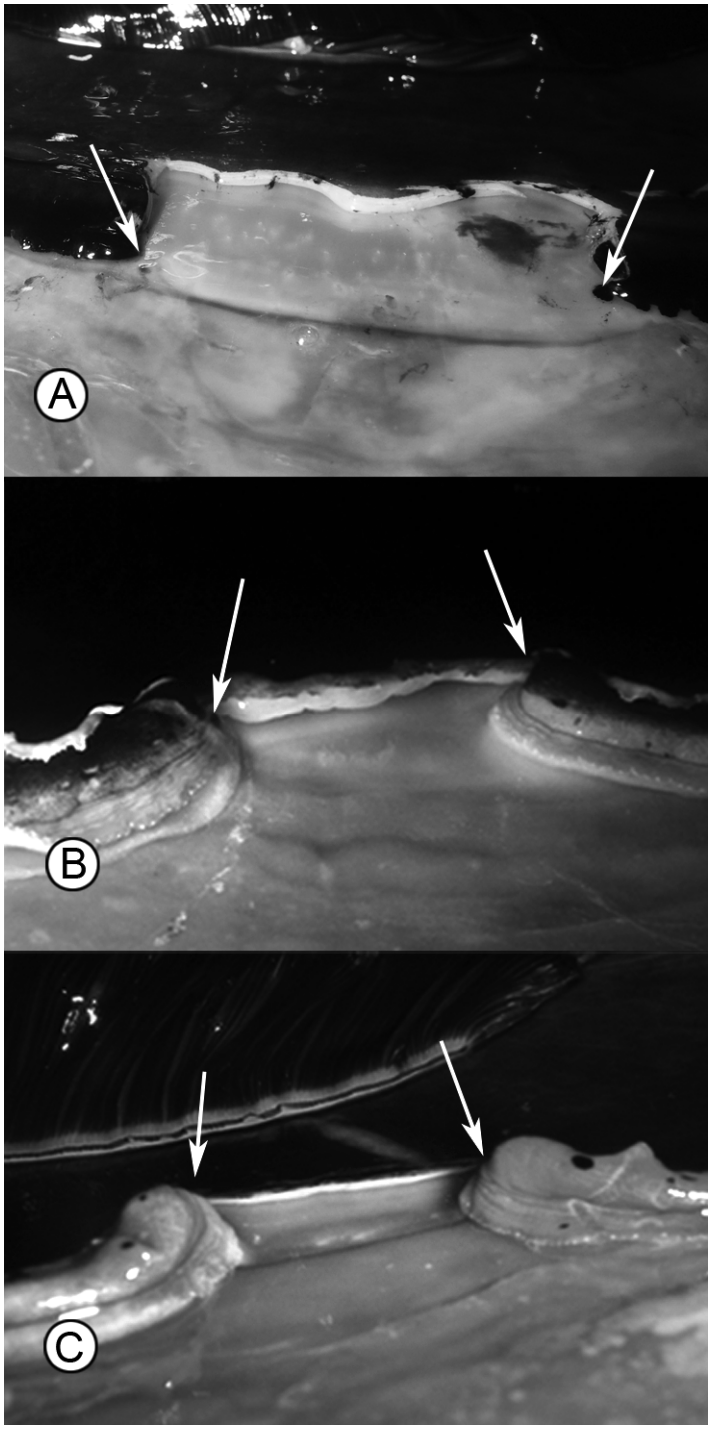

Fig. 3.5. Early wound healing following mantle excision in *Pinctada maxima*: A. 3 hour, B. 2 days, and C. 5 days after excision. Note the invagination of both latero-ventral edges of the wound area (arrows) in B and C.

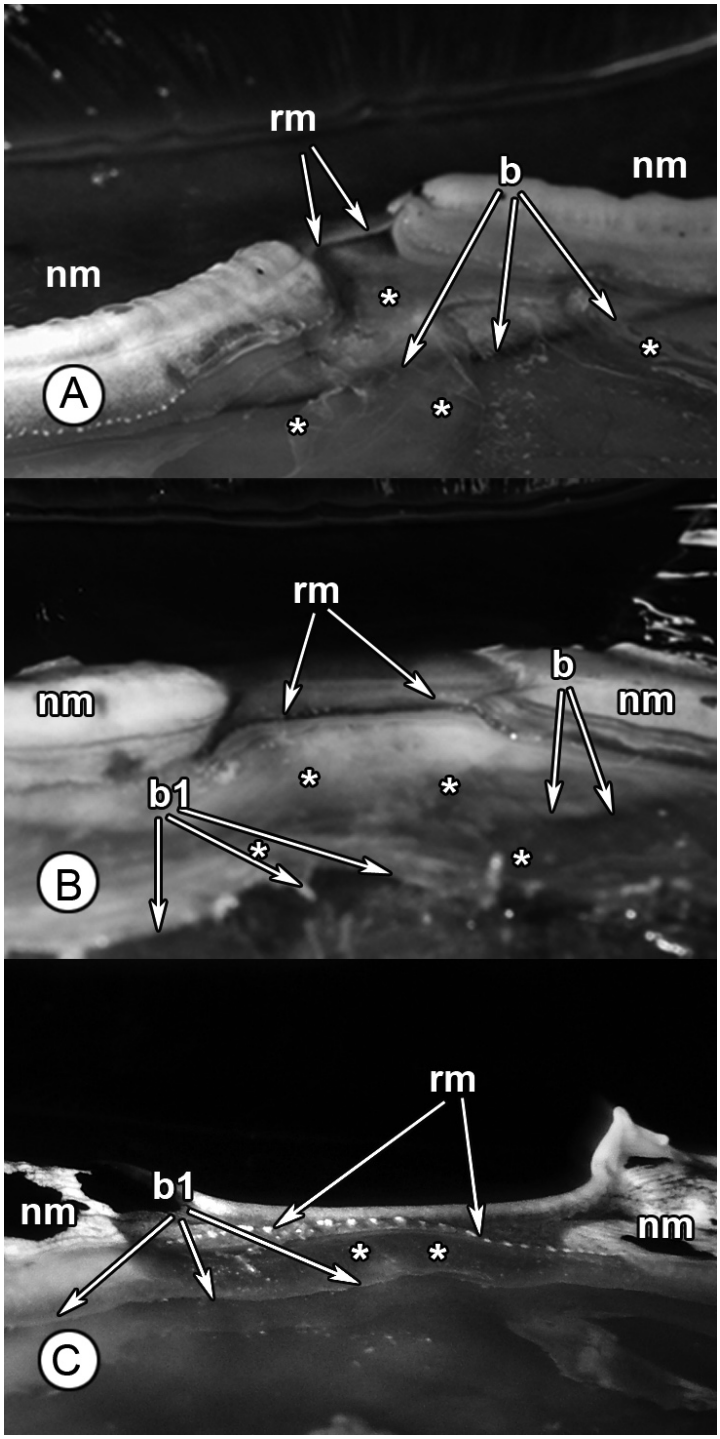

Fig. 3.6. Development of regenerated mantle (rm) and shell material deposition (*) on A. 12 days, B. 24 days and C. 45 days after excision of mantle in *Pinctada maxima*. Notice the border between nacreous secretion and the periostracum (b) is reduced from A to B due to material deposition (*), and finally in (C) the establishment of a new area of material deposition with a new border (b1); the area is characterised by darker background due to a thin cover of the deposition. Normally developed mantle (nm) is on the left and right of the picture.

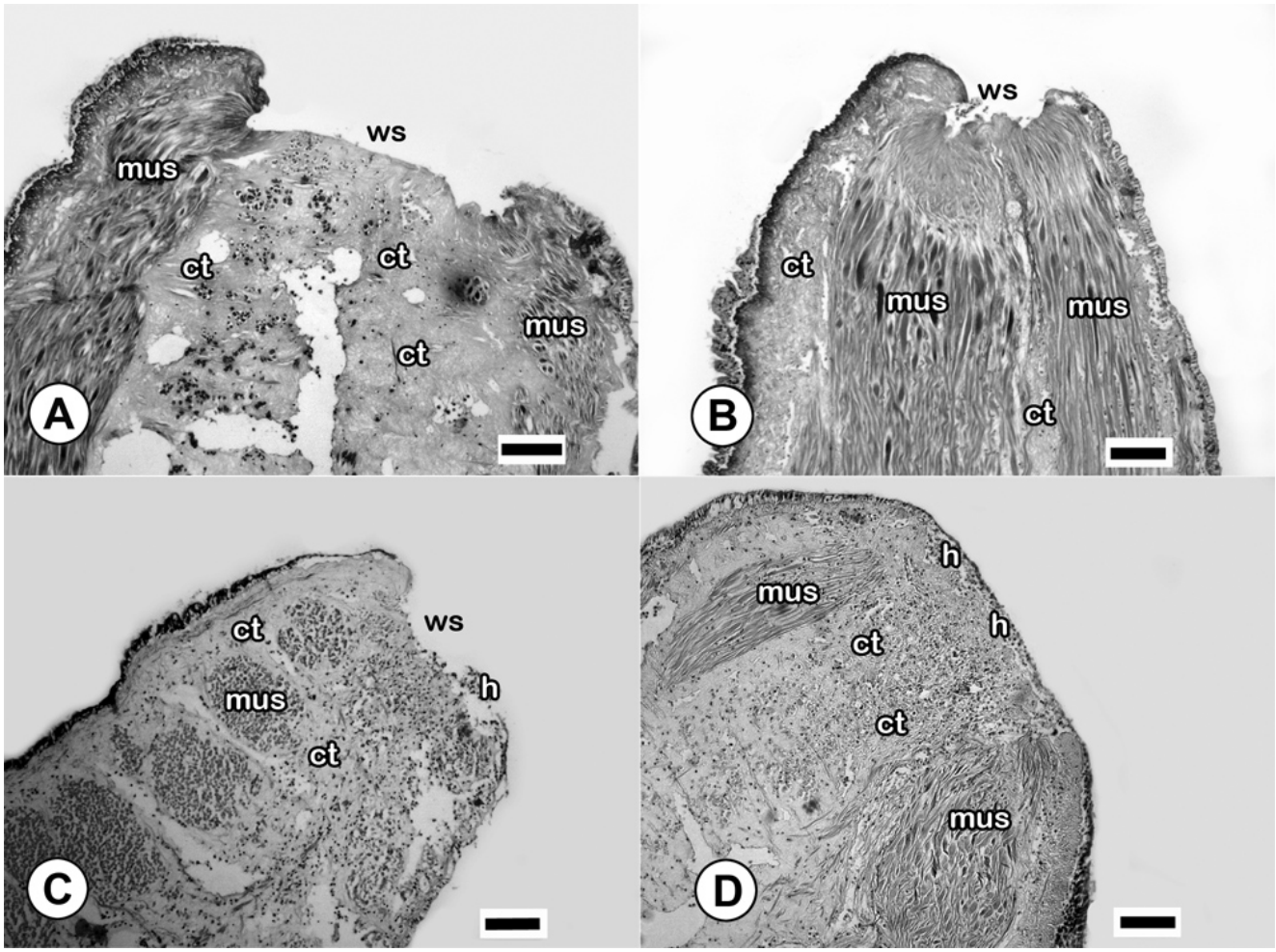

Fig. 3.7. Transverse view of the mantle wound site of *Pinctada maxima,* A: 1 hour, B: 3 hours, C: 6 hours, and D: 12 hours after excision. Scale bars 100 µm. Notice the contraction of muscle (mus) that squeeze the connective tissue (ct) in B and accumulation of haemocytes (h) at the wound site (ws) and finally the haemocytes seal the wound after 12 hours (D).

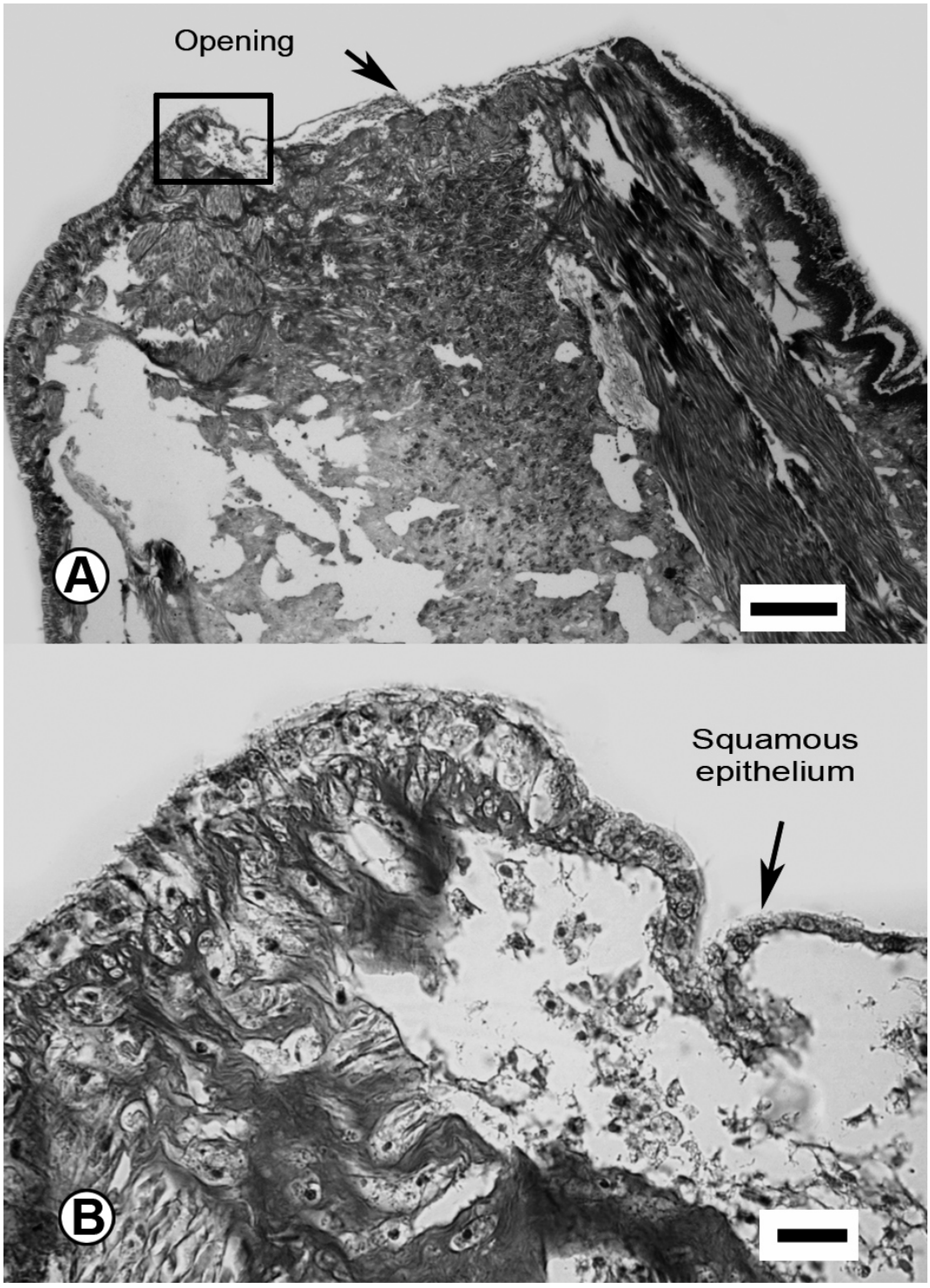

Fig. 3.8. Photomicrographs of sections of the mantle tissue of *Pinctada maxima*; 24 hours after excision. A. Formation of the wounded area of the mantle tissue (Scale bar 200 µm) B. Detailed view of the inset at A (Scale bar 20µm).

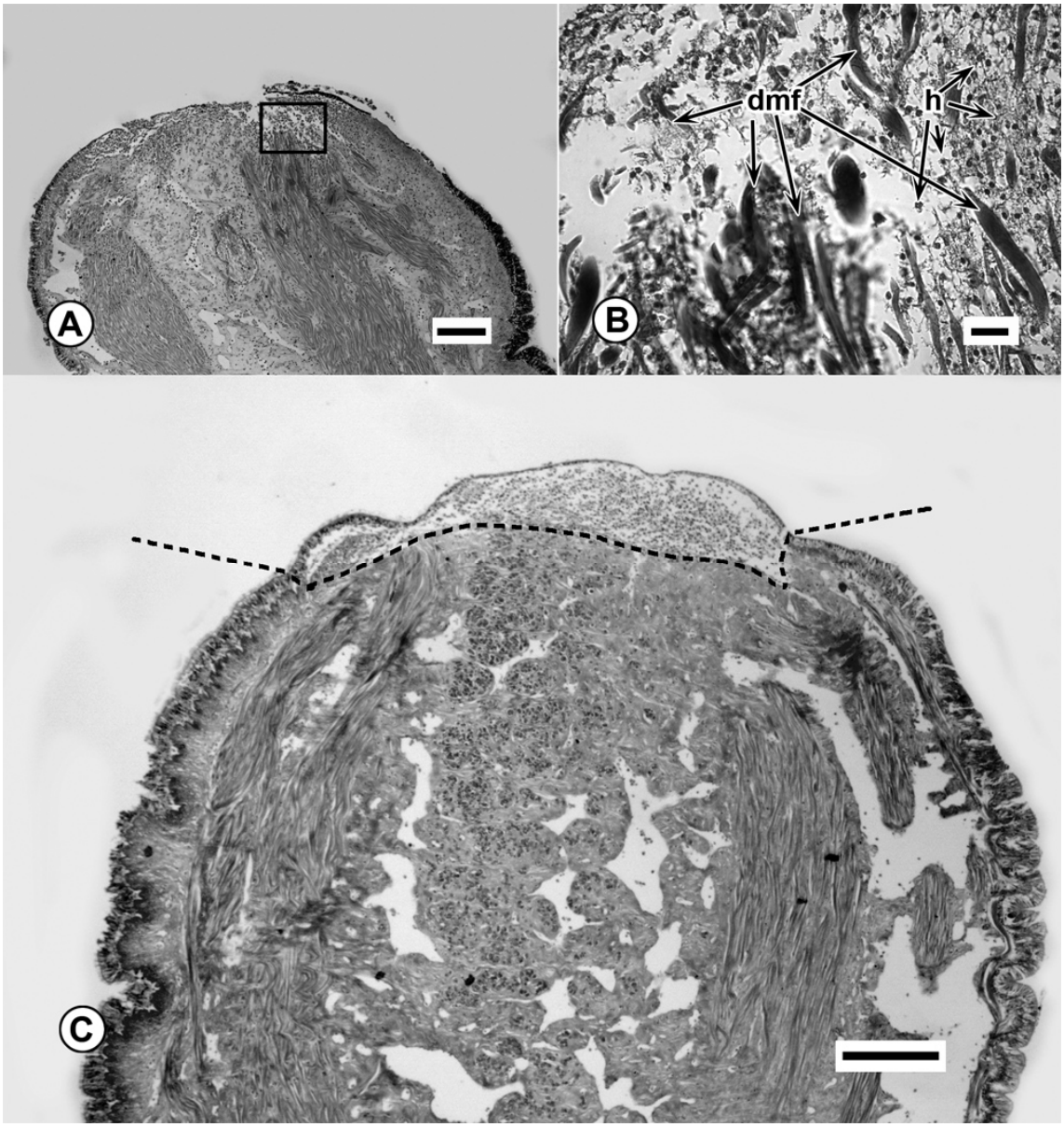

Fig. 3.9. Sections of the mantle tissue of *Pinctada maxima* at 36 hours (A, B) and 72 hours after excision (C). B is the enlargement of the inset in A, showing dedifferentiating muscle fibres (dmf) among hemocytes (h). A, C Scale bars 200 µm, B Scale bar 20 µm. The dashed line in C indicates the initial wound site.

At three to five days after excision, the healing distal edge of the mantle developed an invagination and the epithelial cells elongated distally to form columnar cells (Fig. 3.10A). Between five and twelve days after mantle excision, the distal part of the mantle had divided into three small lobes (Fig. 3.10B) which had apparently developed into the outer, middle and inner mantle folds two weeks later (Fig. 3.10C). Conchiolin secretion between the outer and

middle fold illustrated regeneration and functioning of the periostracal groove (Fig. 3.10C). However, both the middle and inner folds were smaller than the outer fold, and muscle fibres in the former were not as obvious as in the outer fold. Moreover, cuboidal and squamous forms of epithelial cells were common in these small folds while columnar cells composed the epithelial part of the outer fold (Fig. 3.10C). At this stage, the pallial nerve was clear, in the subepithelial region of the periostracal groove.

At 45 days after excision, the regenerated mantle tissue had developed pigmentation, especially in the marginal zone (Fig. 3.11). The outer fold was still much bigger than the other folds. Both basophilic and eosinophilic mucous cells were spread along the epithelium of the outer fold but they were common in the subepithelial region (Fig. 3.11). Several goblet-shaped basophilic mucous cells were evident around the basal region of the inner surface of the inner fold or at all the epithelial surfaces of the outer fold (Fig. 3.11B). However, in the middle and inner folds, eosinophilic mucous cells (goblet and granular forms) were more common (Fig. 3.11C). Muscle fibres and small haemolymph vessels were developed in all folds, but the remaining distal regions of the folds were composed of collagen only. The distal regions of each fold were covered with cuboidal epithelium while the proximal areas of all folds were columnar (Fig. 3.11A, B, C). At 72 days after mantle excision, the folds were either equal in length or the inner fold became bigger and longer than the other folds (Fig. 3.12). Muscle fibres became denser radially and longitudinally and several threads of nerves appeared around the pallial nerve.

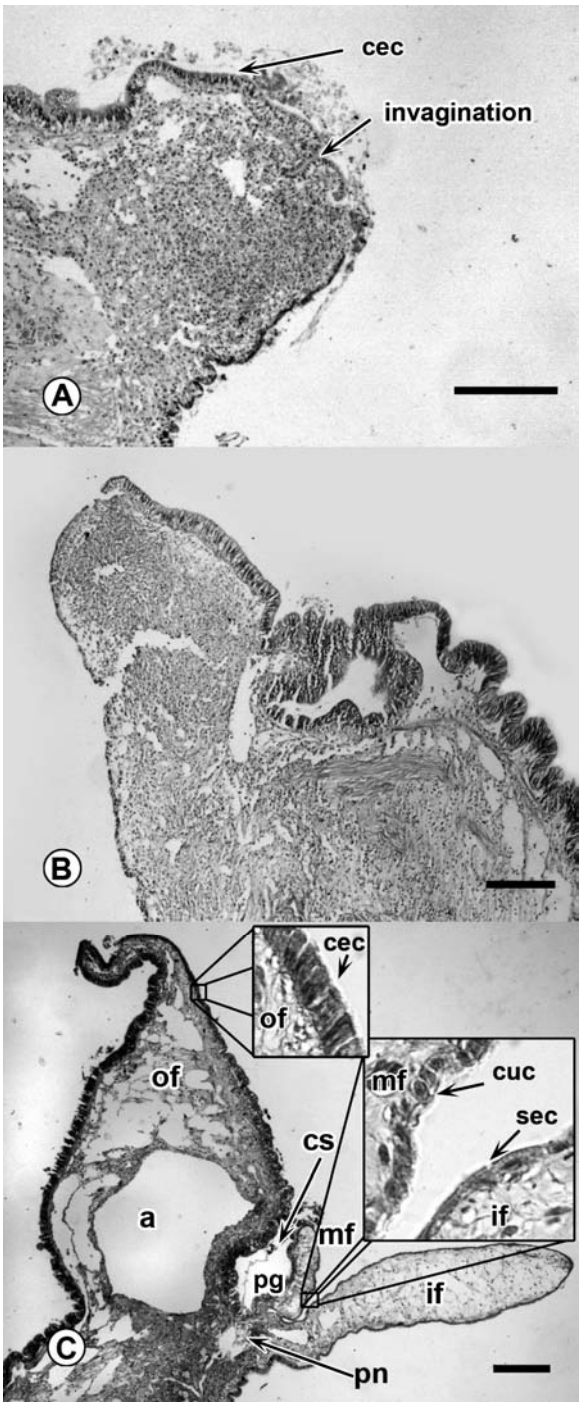

Fig. 3.10. Histological sections showing formation of regenerated mantle folds in *Pinctada maxima* at A: four days, B: twelve days and C: twenty-four days after mantle excision. Scale bars 200 µm. a: artery, cec: columnar epithelium cells, cuc: cuboidal epithelium cells, cs: conchiolin secretion, if: inner fold, mf: middle fold, of: outer fold, pg: periostracal groove, pn: pallial nerve, and sec: squamous epithelium cells.

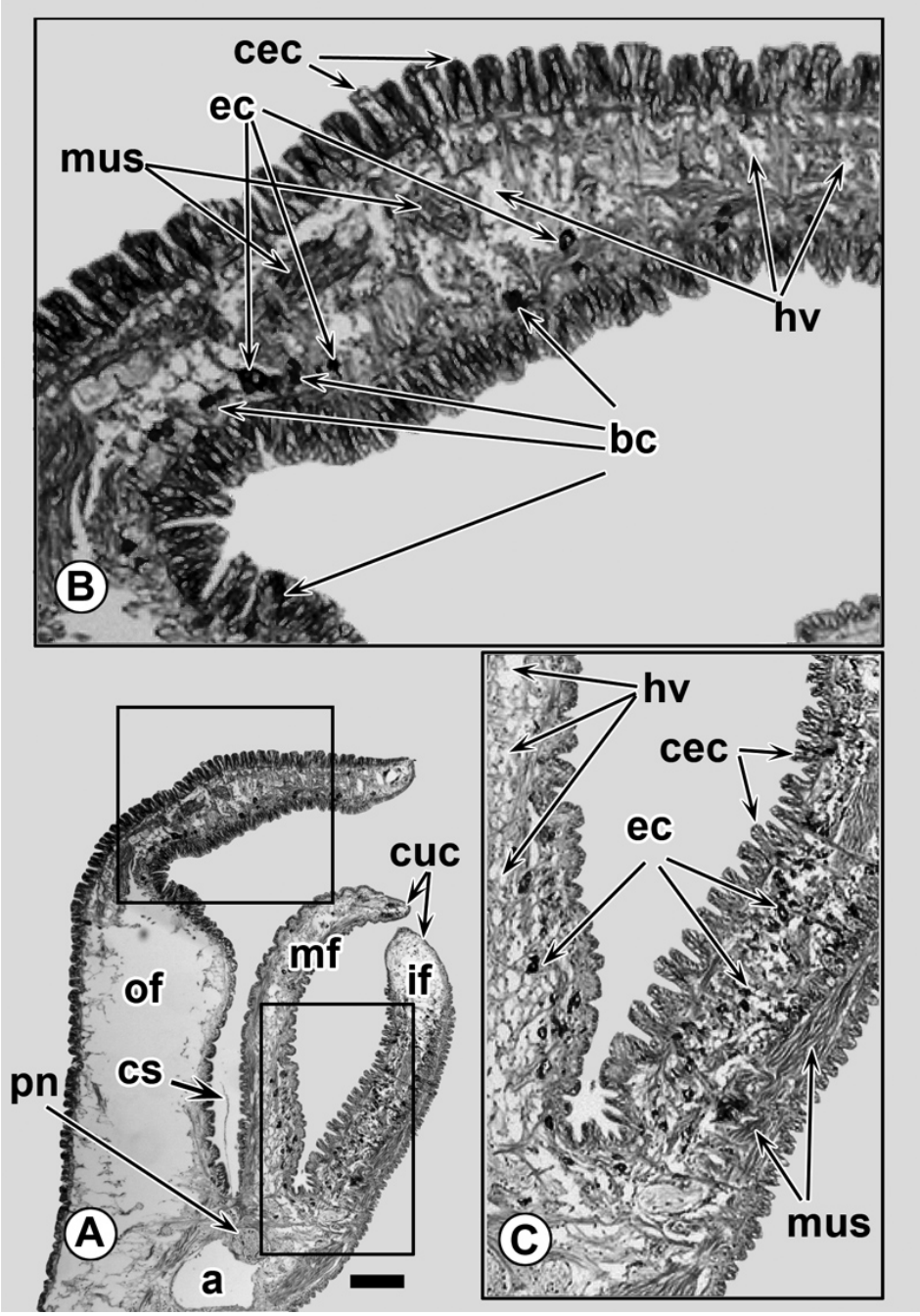

Fig. 3.11. Sections of regenerated mantle tissue of *Pinctada maxima* 45 days after excision, A: overall structure of mantle development with two insets, characterised by: outer fold (of), middle fold (mf), inner fold (if), conchiolin secretion (cs), pallial nerve (pn) and artery (a); B: enlargement of the inset covering part of outer fold area in A, and C: enlargement of the inset covering part of middle and inner folds area in A. Note the distribution of muscle (mus), haemolymph vessels (hv), basophilic (bc) and eosinophilic (ec) cells as well as columnar epithelium cells (cec) and cuboidal epithelium cells (cuc). Scale bar 200 µm.

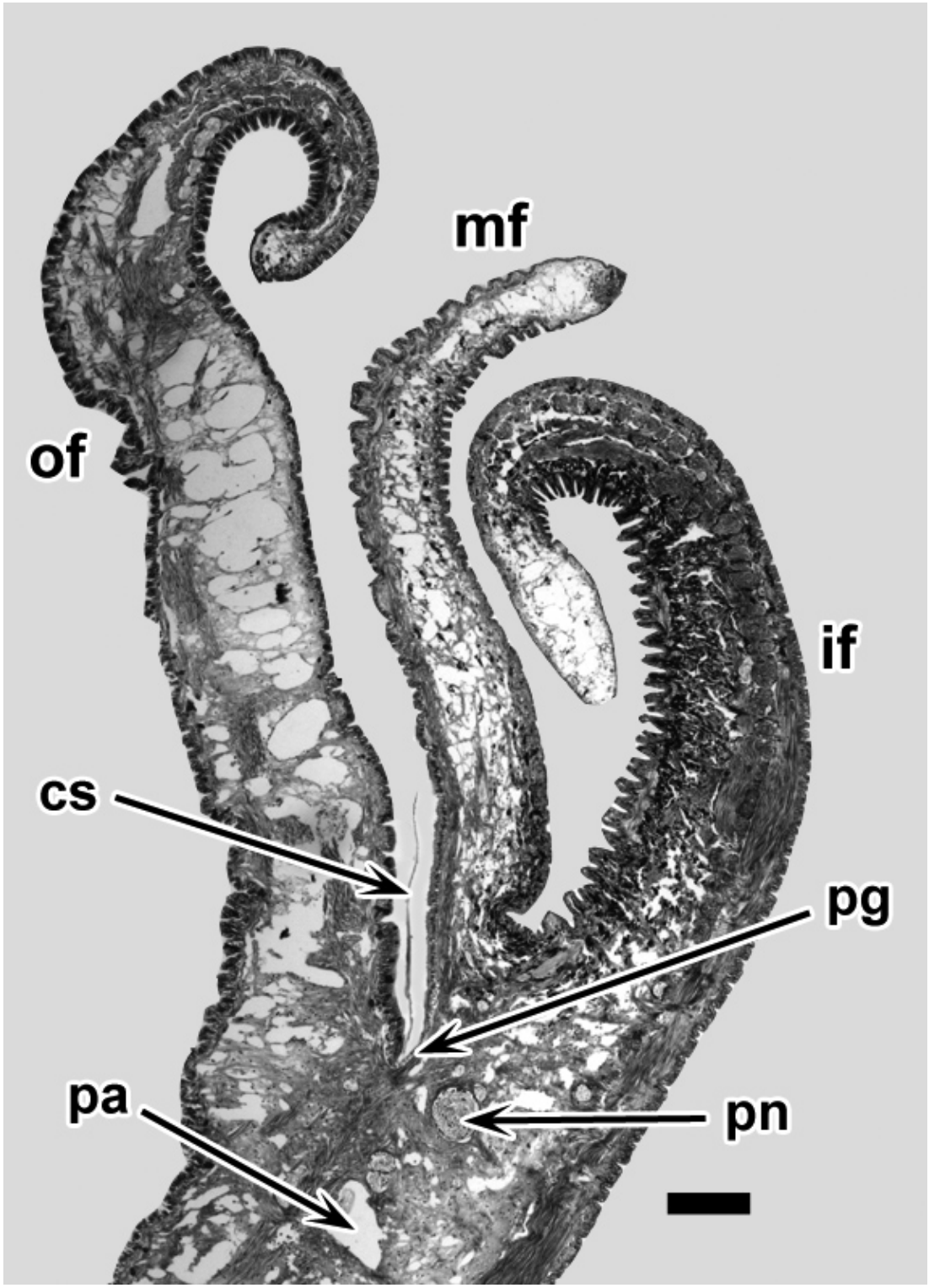

Fig. 3.12. Development of the mantle folds of *Pinctada maxima*; 72 days after excision, cs: conchiolin secretion, if: inner fold, mf: middle fold, of: outer fold, pa: pallial artery, pg: periostracal groove, pn: pallial nerve. Scale bar 200 µm.

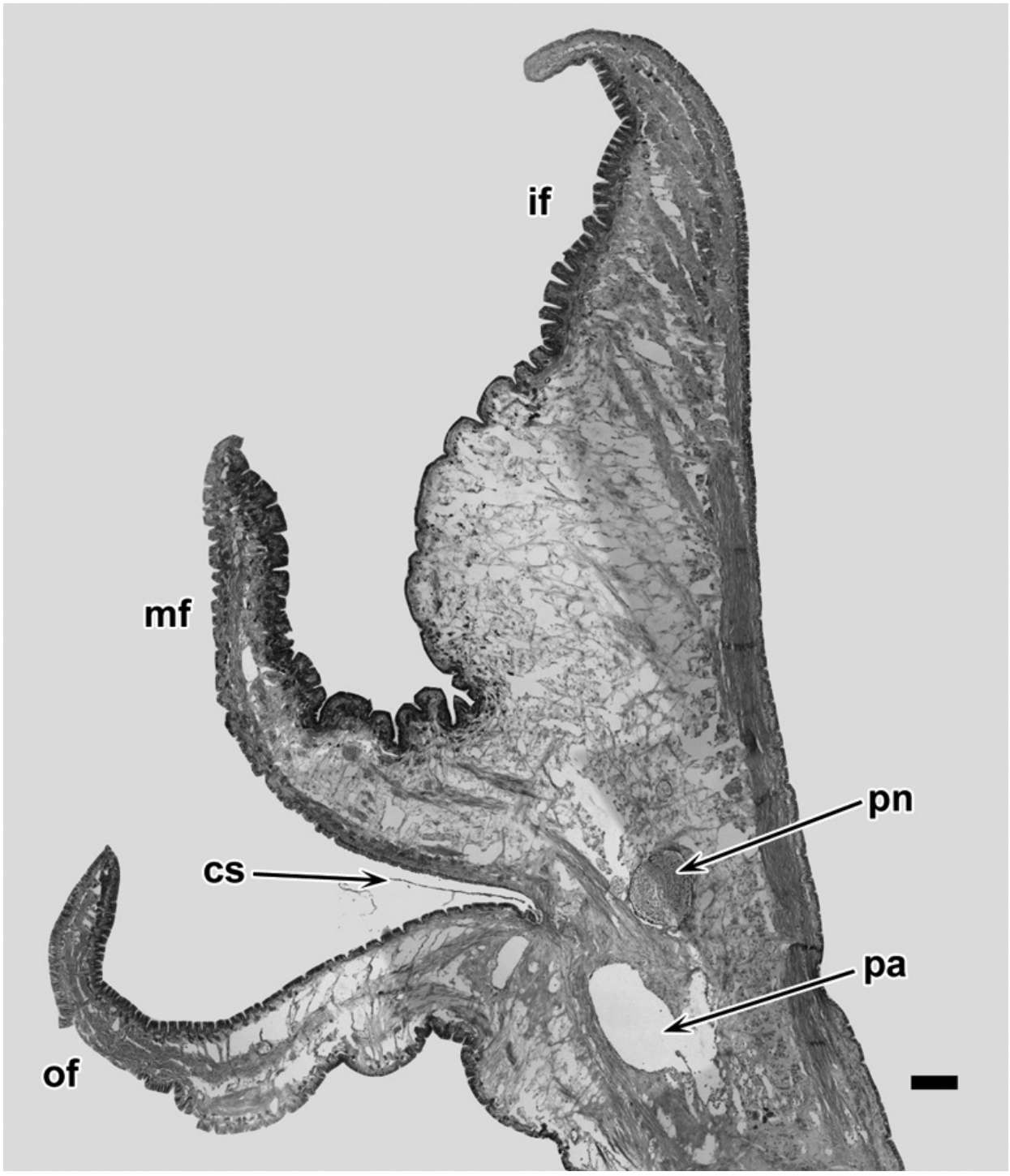

Fig. 3.13. Structure of the mantle folds of *Pinctada maxima*; 90 days after excision, cs: conchiolin secretion, if: inner fold, mf: middle fold, of: outer fold, pa: pallial artery, pn: pallial nerve. Scale bar 200 µm.

Ninety days (three months) after excision, the mantle had completely regenerated (Fig. 3.13).

The inner fold was bigger than other folds and the epithelial cells in marginal zones were

developed commonly in columnar or low-columnar types as in normal mantle tissue. Visually, pigments of regenerated mantle in the marginal zone showed the same colour tones as the adjacent mantle but the pallial zone was not as strongly pigmented as its adjoining normal mantle. However, histological observations showed no difference in epithelial structure or in other internal mantle accessories when regenerated mantle was compared with 'normal' or non-regenerated mantle. One hundred percent survival of oysters was recorded from this second observation.

3.4 Discussion

High survival following first and second mantle excisions in *Pinctada maxima* indicates that this species has the ability to survive from haemorrhage resulting from significant wounding of the mantle. Closure of the wound immediately after excision may also prevent infection. This ability was also assumed in *P. margaritifera* and *P. fucata* following similar trauma (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006). Another important factor influencing the health status of an oyster following mantle excision is the capacity to deposit shell material in the area adjacent to the wound site. This restricts the establishment of fouling organisms which could prevent shell closure and increase susceptibility to predation.

This study showed morphological similarity between regenerated left lobe and right lobe mantle tissue in *P. maxima,* and between these and normal mantle tissue. Regeneration appeared complete within three months of mantle excision as indicated by tissue structure (e.g. distribution of muscle fibres, haemolymph, secretory cells and the formation of three mantle folds that were similar in size to those of normal mantle tissue adjacent to the regenerated mantle area) and demonstrated secretory activities (e.g. production of nacre and

periostracum). Similar mantle regeneration was observed in the two different age groups used in this study; both regenerated apparently fully-functional mantle within three months of excision.

Although complete regeneration was achieved within three months of mantle excision, the cause of some anatomical deformities of the regenerated inner fold is unknown. Similar abnormalities were not reported during mantle regenerated by other species of pearl oysters (*P. margaritifera* and *P. fucata*) (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006). However, similar abnormal tissue development was reported during the regeneration of boring organs of several muricid gastropods (Carriker & Zandt, 1972). Carlson (2007) categorised this type of incomplete restoration as 'hypomorphic regeneration' possibly caused by nutritional or other types of deficiency (Carlson, 2007).

This study confirms that there are four stages in the complete covering of a wound by the epithelium through the process of epithelization: muscular contraction to reduce the wound area, haemocytes infiltration, inflammation (Bubel, 1984) and cellular dedifferentiation (Alvarado, 2000; Bekkum, 2004; Carlson, 2007). Epithelization, which characterizes many epimorphic systems (Carlson, 2007), is probably an important step prior to restoration of the original form of the mantle. Epithelium isolates and protects the lesion from further infection and this barrier provides protection against osmotic imbalance for aquatic animals (Carlson, 2007). This process was completed within 72 hours by *P. maxima*, but was completed within 48 hours by the related Akoya pearl oyster *P. fucata* (Acosta-Salmon & Southgate, 2006), and between 52 and 96 hours was required for epithelization of wounds to the siphons of *Scrobicularia plana* (Hodgson, 1982). Epithelization in the gastropod mollusc *Haliotis cracherodii* was reported within 4 days for mantle tissue and within 48 hours for the foot (Armstrong, 1971).

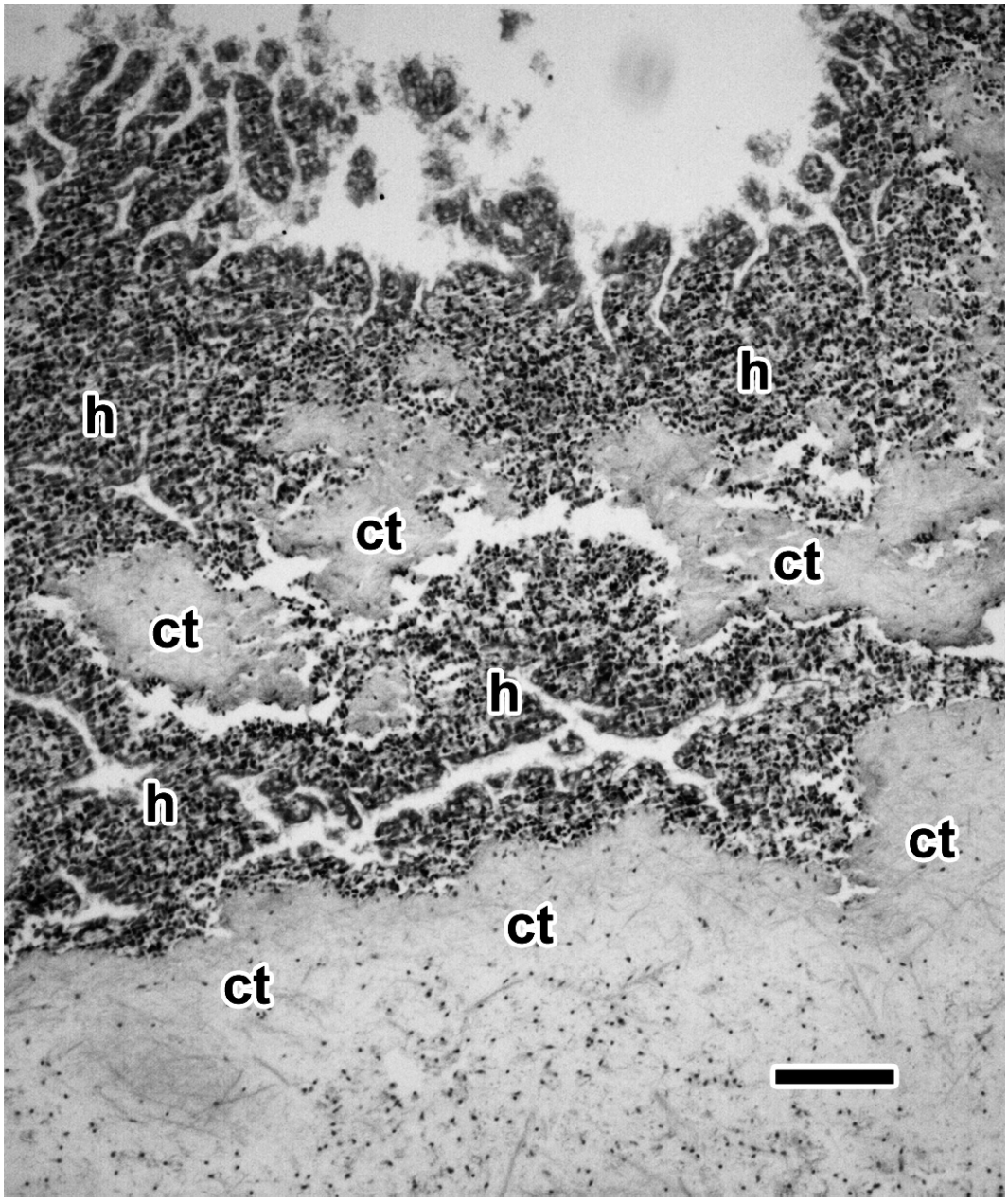

Fig. 3.14. A parallel section of mantle three days after excision showing a wound site covered with haemocytes (h) and an early development of connective tissue (ct). Scale bar 100 µm.

During the epithelization process other cellular reconstruction takes place internally. However, it is unclear whether the new cells developed at the wound site or whether they migrated from the surrounding tissues. This study recorded the development of connective tissue among haemocytes (Fig. 3.14) and the apparent dedifferentiation of muscle fibres. As muscle fibres might also increase the chance of the development of blastemal cells through

the conversion of dedifferentiated cells (Carlson, 2007), it is possible that part of this development was conducted by cells around the wound. The healing process at the epithelial level may result from migration or duplication of epithelial cells from both sides of the mantle wound. The mantle of pearl oysters has proliferative capacity although this varies between the different zones of the mantle (Fang et al., 2008). This proliferative capacity is the basis for cultured pearl production (Taylor & Strack, 2008) which requires a piece of mantle tissue to be grafted with an inert nucleus into the gonad of a recipient oyster. Subsequent proliferation of the mantle tissue graft envelopes the nucleus to form the 'pearl-sac' and secretion of nacre from pearl-sac onto the nucleus eventually forms a cultured pearl (Awaji & Suzuki, 1995; Fang et al., 2008; Haws, 1998; Taylor & Strack, 2008). The area of mantle removed from *P. maxima* in this study is the same as that preferred for donor mantle tissue used in cultured pearl production. On this basis, it is likely that the cell proliferation observed in this study, in response to mantle wounding, was stimulated primarily by cells adjacent to the wound.

Once the epithelium completely covers the wound, morphogenesis occurs and restores the original shape of the mantle (Carlson, 2007). In this study, restoration was observed when the three mantle folds formed; the outer fold emerged first, presumably because of the need for shell deposition adjacent to the would site. The functions of the outer fold include periostracum production (Hillman, 1961; Pan & Watabe, 1989), regulation for biomineralization (Chen et al., 2004) and even ingestion of particulate matters (Bevelander & Nakahara, 1966). Secretion of nacre onto the shell adjacent to the wound site indicates full acquisition of nacre secreting abilities by regenerated mantle in *P. maxima*. This study confirms that secreting ability emerges during mantle restoration (morphogenesis) as mineralisation was evident on the 12^{th} day after mantle excision. Initially this secretion seems

to be conducted by new mantle tissue protruding from the proximal part of the wound in a position dorsal to the non-injured mantle edge. Similarly, Acosta-Salmon and Southgate (2005) recorded nacre secretion from regenerating mantle tissue in *P. fucata* and *P. margaritifera* 15 days after mantle excision. Forty-five days after mantle excision, regenerated mantle in *P. maxima* had extended ventrally to a position similar to that of non-injured mantle. By this stage nacre deposition by regenerated mantle had reached the same position ventrally as that of non-injured mantle. It is reasonable to assume that after this point is reached regenerated mantle would continue to thicken the nacre layers of the shell.

Further morphogenetic development resulted in complete regeneration of the three folds of the mantle of *P. maxima*. Complete regeneration requires all cellular functions to be in place; this process takes much longer than wound healing (Carlson, 2007). In this study, wound healing was completed with epithelization within three to four days of mantle excision while regeneration, characterised by morphogenesis, required approximately three months. The results of this study and that of Acosta-Salmon and Southgate (2005) indicate that three months is required for mantle regeneration for at least three species of pearl oysters used for cultured pearl production; *Pinctada maxima, P. margaritifera* and *P. fucata*.

This study makes a significant contribution to our knowledge of tissue regeneration in pearl oysters. In commercial pearl farms it is usual practice to obtain mantle tissue used for cultured pearl production from sacrificed donor oysters. Our results show that the mantle tissue required for this purpose can be removed from *P. maxima* without mortality. Furthermore, excised mantle tissue can be completely regenerated with all internal structures within three months of excision, and this may provide the basis for high quality oysters to be used as mantle donors on more than one occasion. These results provide the basis for significant potential benefits to the cultured pearl industry. Prior studies have recognised however, that the rate of wound healing

in pearl oysters is likely to be influenced by their physiological state (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006), and by seasonal patterns of nutrient storage and utilisation that have been described for marine bivalves including pearl oysters (Saucedo et al., 2002). These processes are likely to provide energy sources and nutrients required for mantle healing in pearl oysters, and the rate of the healing process may be influence by seasonal fluctuations in nutrient availability (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006).

The results of this Chapter confirm that, like *P. margaritifera* and *P. fucata*, *P. maxima* can regenerate excised mantle tissue with apparently normal morphological and functional characteristics. There is then potential for regenerated mantle to be used as saibo for cultured pearl production. However, this potential relies on the ability of regenerated mantle to function identically to normal mantle with regard to nacre secretion and proliferation to form a pearl-sac. These aspects are investigated in Chapter 4 and Chapter 5, respectively.

Chapter 4

Shell secretion by regenerated mantle of *Pinctada maxima*

4.1 Introduction

During 'normal' shell development in pearl oysters, the shell is constructed from three layers: the periostracum on the outer shell surface, the prismatic layer is in the middle, and the nacreous layer which lines the interior of the shell (Lowenstam & Weiner, 1989; Simkiss & Wilbur, 1989). Shell development and structure in molluscs can be obstructed by damage caused by predators, infestations of marine borers or disease (Fleury et al., 2008; Mount et al., 2004). Shell material secreted to repair such damage is generally similar in structure to 'normal' shell (Fleury et al., 2008; Lin et al., 2008; Reed-Miller, 1983), however, in pearl oysters such repairs may result in changes in the shape or structure of the shell. For example, a common response of pearl oysters to shell boring organisms is to increase nacre secretion on the inner shell surface which often forms protrusions or blisters (section 1.3.1; (Doroudi, 1994; Mao Che et al., 1996; Taylor & Strack, 2008).

Recent studies have reported on the regeneration of excised mantle tissue in *P. margaritifera* and *P. fucata* (Acosta-Salmon & Southgate, 2005; Acosta-Salmon & Southgate, 2006; Acosta-Salmon et al., 2004) and this ability was confirmed for *P. maxima* in Chapter 3. All these studies have reported that regenerated mantle apparently regains complete secretory functions indicated by the formation of shell material adjacent to the regenerated mantle. Complete regeneration of excised mantle tissue required around 3 months when cell accessories supporting shell formation such as epithelium and mucous cells also appeared in

the regenerated mantle of the tested species (Chapter 3; Acosta-Salmon & Southgate, 2005). The results of Chapter 3 of this study also showed the formation of mineral deposition following extension of the new regenerated mantle. However, there is no further information available about the layer structure of the shell material deposited by regenerated mantle. Is regenerated mantle capable of producing shell material with the same structure as normal mantle? This question has important implications for the potential use of regenerated mantle as saibo for pearl grafting. This Chapter investigate the structure of shell material secreted by regenerated mantle from *P. maxima* and compares it to shell secreted by normal mantle tissue.

4.2 Material and methods

The shells used in this study were collected two years after mantle excision and regeneration. A piece of mantle (approximately 10 mm x 30 mm) was excised from the distal part of the ventral mantle following anaesthesia (Chapter 2; see Fig. 3.1) and excised mantle tissue regenerated over a period of approximately 3 months (Chapter 3). Following mantle excision, oysters were maintained in suspended culture on a longline in Pioneer Bay, Orpheus Island. Five oysters were collected for shell analysis two years after excision. The soft parts were removed to allow shell sectioning, and two adjacent areas of the same shell valve, representing shell secreted by regenerated and normal mantle were sampled (Fig 4.1). Each of these two areas of shell was cut from the complete shell valve using a Dremel™ tool before being crushed mechanically to expose a transverse section. The shell pieces were glued onto SEM stubs with plastic conductive carbon cement (Proscitech, Thurringowa), gold coated in a JEOL JUC-5000 Magnetron Sputtering Device and viewed at 10000 kV in a JEOL JSM-541OLV SEM. Images from SEM were taken from the section area as well as from the inner (nacreous) surface of the shell.

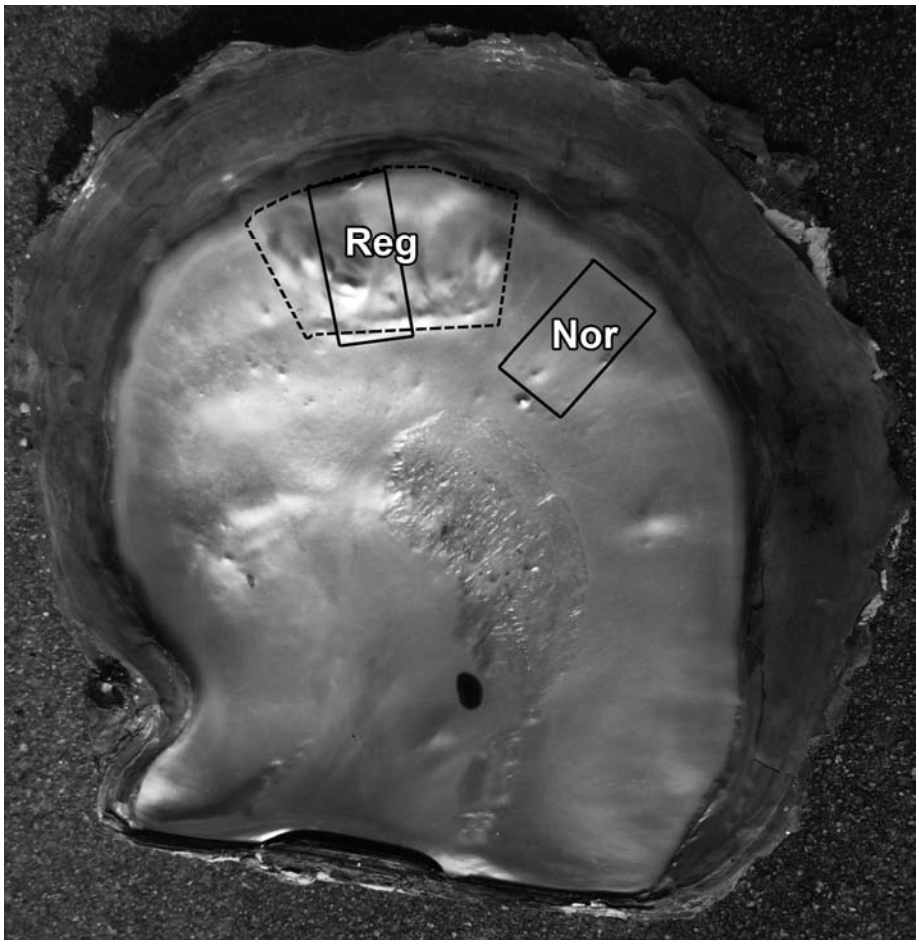

Fig. 4.1. Two areas of shell sections (solid line) representing regenerated (Reg) and normal (Nor) shell. The dashed line indicates the area of shell where nacre was secreted by regenerated mantle.

4.3 Results

Macroscopic observations of shell material deposited by regenerated mantle showed a bio-fouling assemblage sandwiched between two layers of mineral depositions (Fig. 4.2A, B); this differed greatly from normal shell formation (Fig. 4.2C) and was seen in four of the five oysters analysed. In these oysters, shell formed by regenerated mantle established a new layer of both prismatic and nacreous material over the top of this bio-fouling assemblage (Fig. 4.2A,B). A clear border was obvious where the new deposition layers juxtaposed with the old

layer and the border ended proximally. Eroded nacre, characterised by its chalky, flakey and brittle nature, was recorded in all shells with bio-fouling assemblages (Fig. 4.2A,B). The thickness of the new nacreous and prismatic layers was similar or greater in the median part of the new deposition layers (secreted by regenerated mantle) compared to the original deposition layers. The thickness of the nacreous layer then reduced distally while the prismatic layer increased in thickness at the last point of attachment with the nacreous layer, and reduced after this point.

A similar structure in the thickness of the prismatic and nacreous layers was also seen in normal shell (Fig 4.2C). The thickness of the nacreous layer reduced until it reached the point of separation with the prismatic layer, but the prismatic layer continued to increase in thickness then decreased at the end growth (Fig. 4.2C). However, the total thickness of shell secreted by regenerated mantle (minus the bio-fouling cavity) was nearly double than of the normal shell from the same oyster/shell.

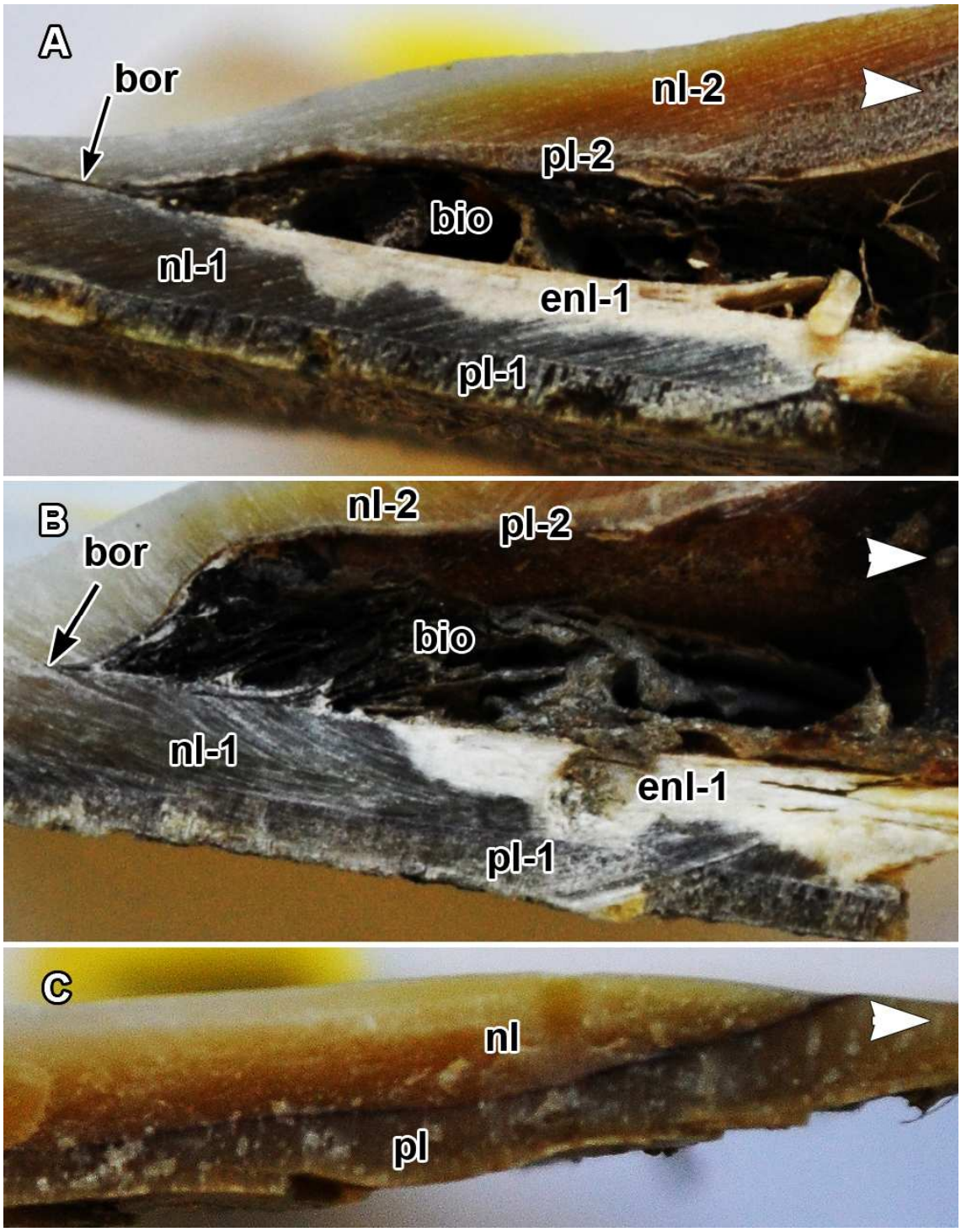

Fig. 4.2. Photographs of two sections of a shell of *Pinctada maxima* showing: A,B, the development of new layer of mineral depositions from regenerated mantle over an assemblage of biofouling and C, the development of normal shell. bio, biofouling; bor, border between first and second deposition; enl-1, eroded nacreous layer; nl, nacreous layer; nl-1, nacreous layer of first deposition; nl-2 nacreous layer of second deposition by regenerated mantle; pl, prismatic layer; pl-1, prismatic layer of first deposition; and pl-2, prismatic layer of second deposition by regenerated mantle. Arrow heads indicate direction of growth.

The formation of shell by regenerated mantle (regenerated shell) was characterised by more uniform development of the prismatic layer (Fig. 4.3A) when compared to the prismatic layer of the 'normal' part of the same shell (Fig. 4.3B). The thickness of the aragonite platelets of the nacreous layer of both areas of the same shell was similar. However, the transition area of the regenerated section was narrower than in the normal section of the shell (Fig. 4.3). The section of regenerated area of other shell (Fig. 4.4A) showed a loose transition from prismatic to nacreous layer (Fig. 4.4C) although a typical formation of prismatic edge was the same as in the normal shell (Fig. 4.4B). The transition area was characterised with a spherulitic formation of calcareous (Fig. 4.4D). On the surface of the inner shell (the interface between the mantle and the shell), the formation of aragonite platelets of both regenerated and normal development of the shell was similar. The platelets were half developed in both regenerated and normal shell development (Fig. 4.5) and a fully development recorded in another shell from both regenerated and normal area (Fig. 4.6). The average thickness of platelets in both regenerated and normal shell was the same: 0.78 ± 0.16 and 0.78 ± 0.13 µm (mean ± SD, n = 28), respectively (Fig. 4.7). A t-test showed that these means were not significantly different ($t = 0.0629$, $df = 26$, p-value = 0.95).

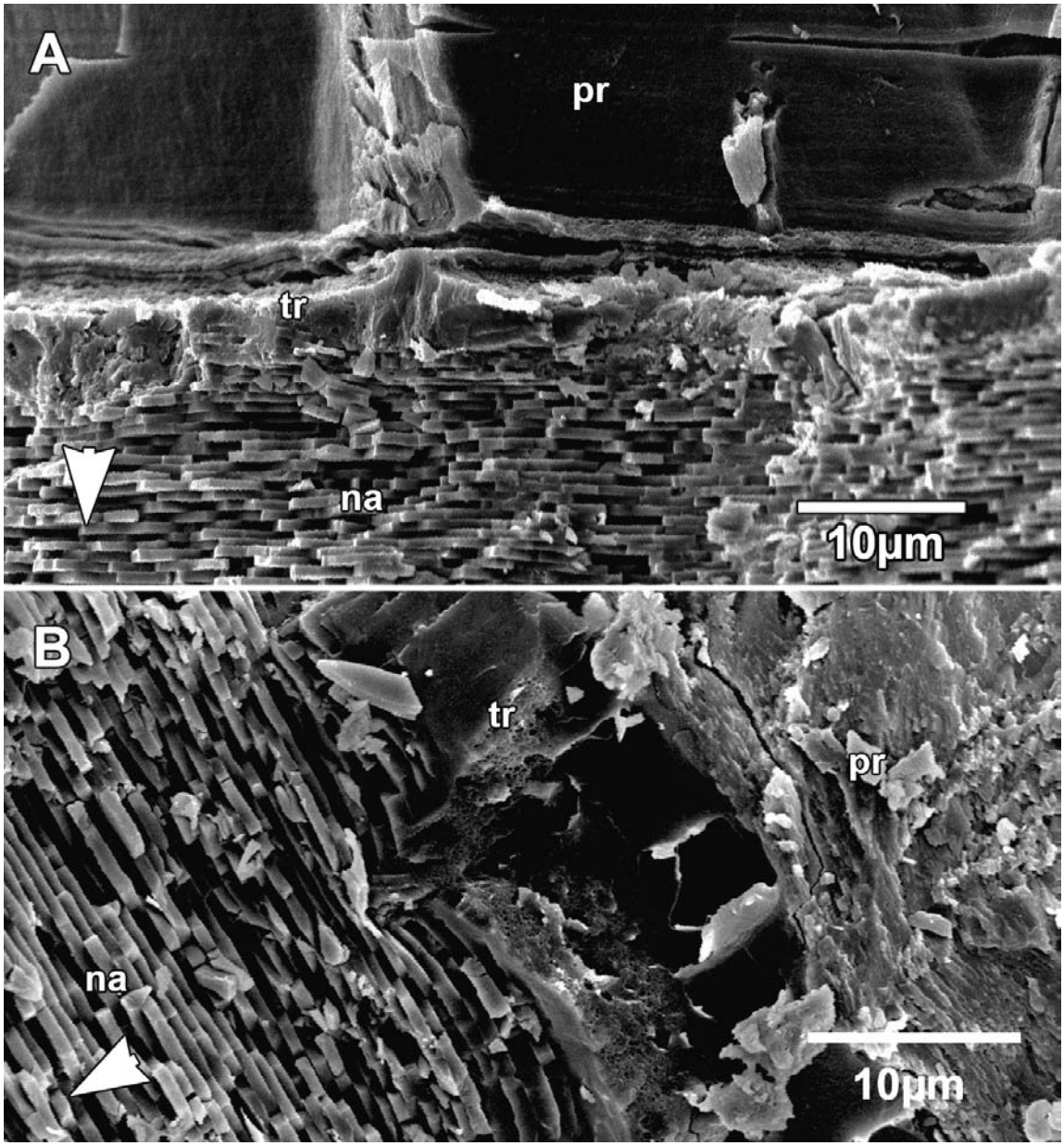

Fig. 4.3. The SEM structure of a shell of *Pinctada maxima* showing: A. shell formed by regenerated mantle; and B. by normal mantle of the same oyster; na, nacreous layer; pr, prismatic layer, and tr, transition area; while arrow heads indicate the direction towards the internal surface of the shell.

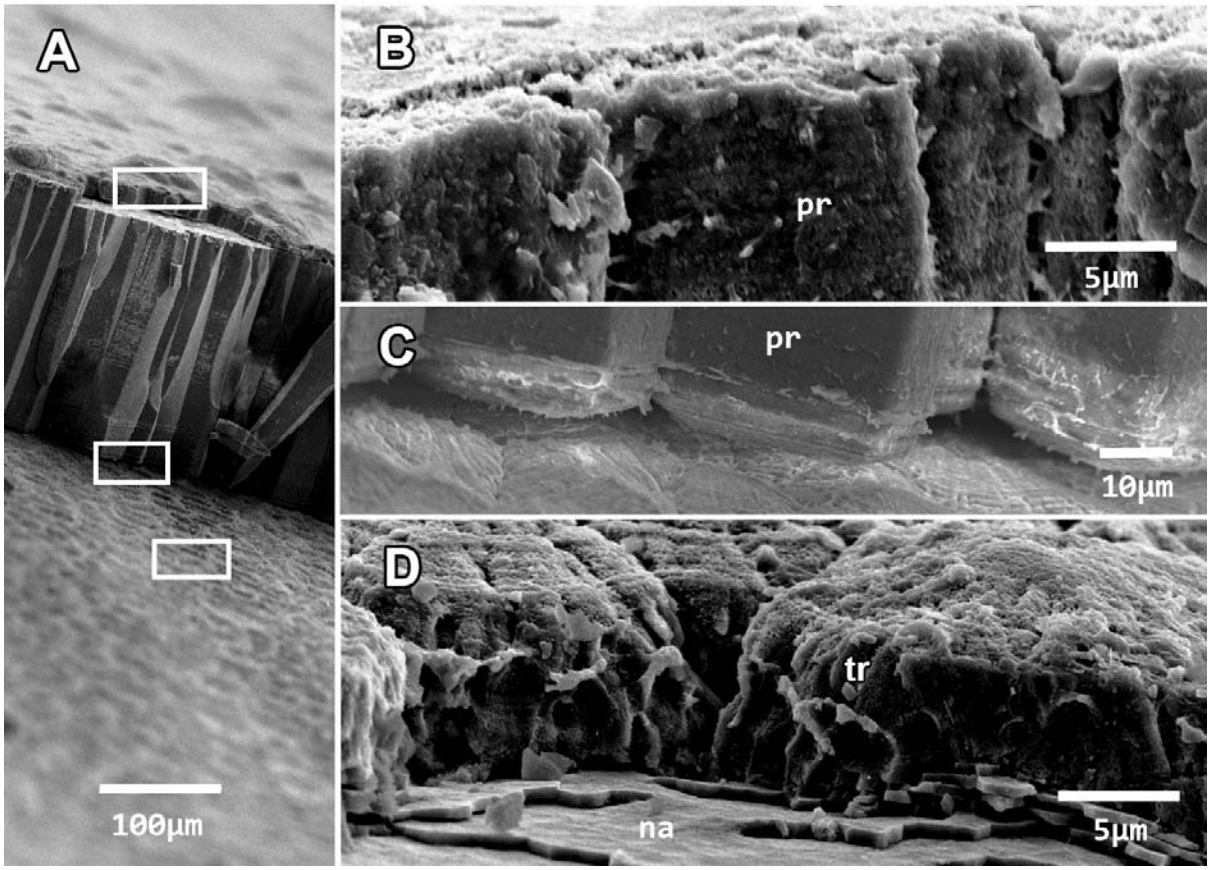

Fig.4.4. SEM of regenerated shell of *Pinctada maxima* showing the whole structure representing prismatic (pr) and nacreous (na) layers of the shell: B, the edge of the prismatic layer from the top inset of A; C, the bottom part of the prismatic layer of the medium inset of A showing a loose attachment of the prismatic and nacreous layers; and D, the transition area (tr) of prismatic and nacreous layers of the bottom inset of A.

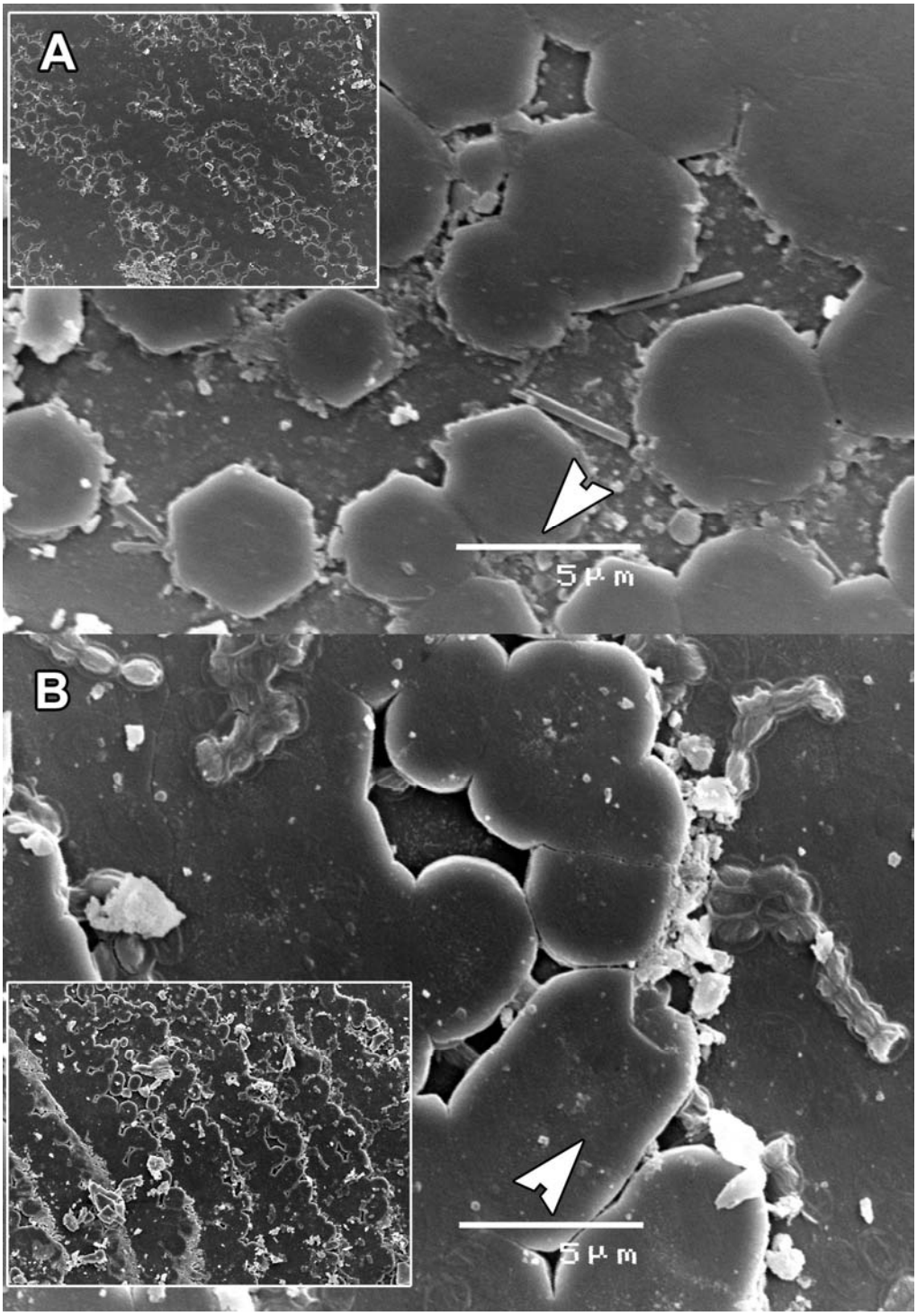

Fig. 4.5. An intermediate development of nacre platelets of the inner surface of the shell of *Pinctada maxima* showing: A, nacreous layer constructed by the regenerated mantle; and B, by the normal nacre of the same animal. Insets show the wide view of the surface and arrowheads indicate the directions of growth.

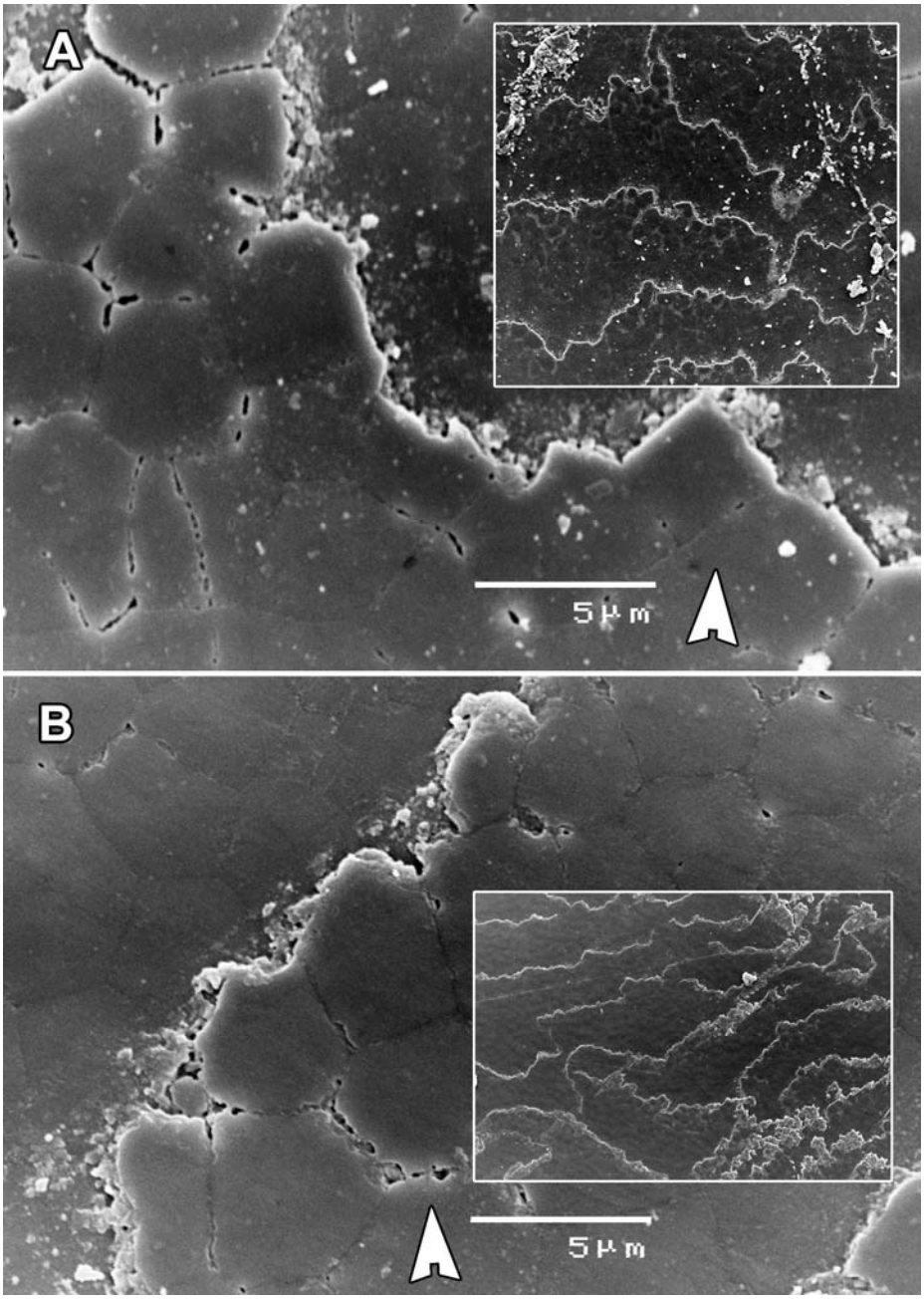

Fig. 4.6. A full development of nacre platelets of the inner surface of the shell of *Pinctada maxima* showing: A, nacreous layer constructed by the regenerated mantle; and B, by the normal nacre of the same animal. Insets show the wide view of the surface and arrowheads indicate the directions of growth.

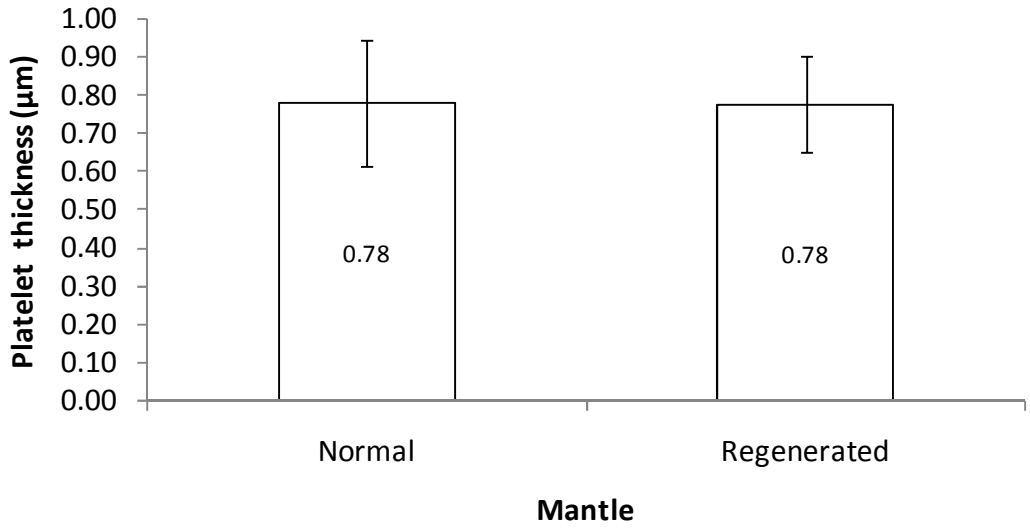

Fig. 4.7. Mean (± SD, n = 28) thickness of nacre platelets from *Pinctada maxima* shell secreted by normal and regenerated mantle.

4.4 Discussion

The results of this study confirm that regenerated mantle tissue in *P. maxima* is capable of secreting shell material and on this basis has the potential to be used as saibo for cultured pearl production. Four of the five oysters used in this study had an accumulation of biofouling on the original inner shell surface and new shell material had been laid over the top of this by the regenerated mantle. This form of shell structure is commonly known as 'double-back' and is generally associated with mantle retraction indicating disease or trauma in pearl oysters (Humphrey, 2008). In these particular cases, however, biofouling was able to colonise the ventral inner surface of the shells as a result of mantle excision. Given that full

regeneration of excised mantle requires approximately 90 days (Chapter 3), this period of time is clearly sufficient for biofouling to become well established and firmly attached to the shell surface with the result that it cannot be pushed out or removed from the shell surface once the mantle tissue has regenerated.

These results also show that the total thickness of regenerated shell was greater than that of normal shell. This may be a response to the intrusion of biofouling. Pearl oysters commonly increase the rate of nacre secretion in response to irritation or intrusion by foreign objects or boring organisms (Taylor & Strack, 2008). This process commonly results in the occurence of 'blisters' or protuberances on the inside of the shell adjacent to the site of irritation (Taylor & Strack, 2008; section 1.3.1). The resulting blisters may be very large. The results of this study indicate that when such intrusions occur closer to the shell margin, the oyster similarly responds by increasing the rate of nacre secretion and this process may result in the formation of a new ventral shell margin, oblique to the original plane of the shell valve. While it is likely that the increase in the rate of nacre secretion by regenerated mantle is a response to the presence of foreign material on the inner shell surface, another possibility is that regenerated mantle tissue has a higher rate of nacre secretion than normal mantle tissue. This possibility may have significant relevance to the use of regenerated mantle tissue as saibo for cultured pearl production.

New shell material initially secreted by regenerated mantle to cover biofouling was composed of prismatic layer and this was the overlaid by nacre. The results of Chapter 3 showed that during regeneration of excised mantle tissue in *P. maxima*, the outer fold of the marginal zone emerged first. The outer fold is responsible for secreting the periostracum and the prismatic layers of the shell of pearl oysters (Fougerouse et al., 2008) and regulation for biomineralization (Chen et al., 2004). Its regeneration prior to the inner and middle folds

presumably indicates the importance of shell deposition adjacent to the wound site to minimise biofouling and potential predation. Nacre is secreted on top of the new prismatic layer, presumably once the regenerated mantle is fully extended so that the nacre secreting pallial zone of the mantle covers the regenerated prismatic material.

The structure of the shell secreted by regenerated mantle was similar to that produced by normal mantle. This study confirms the similar structure of the prismatic layer, transition area and the nacreous layer of shell material secreted by regenerated mantle and normal mantle tissue. The transition area is the region where the prismatic and nacreous layers meet. This area is usually characterised by the development of spherulitic forms of calcareous structure from both prismatic and nacreous layers (Falini et al., 1996) which have also been recorded in shell regeneration in other molluscs (Fleury et al., 2008; Lin et al., 2008; Reed-Miller, 1983; Ubukata, 1994). This is the most common form of shell repair in molluscs (Simkiss & Wilbur, 1989).

The formation of aragonite platelets by both regenerated and normal mantle of *P. maxima* was similar. There was no significant difference in the mean thickness of platelets within the nacre indicating similarity in the structure of nacre secreted by regenerated mantle tissue to that of normal mantle. This indicates that regenerated mantle has similar potential to normal mantle tissue to be used a saibo for cultured pear production.

In summary, this experiment has shown that regenerated mantle from *P. maxima* is capable of producing shell material with the same structure as normal mantle. There appeared to be no difference in the size or structure of nacre platelets produced by the two type of mantle, and regenerated mantle secreted nacre at a more rapid rate than normal mantle tissue. The results do not indicate any constraints relating to the use of regenerated mantle as potential saibo.

Chapter 5

Pearl-sac development in *Pinctada maxima* using regenerated mantle as saibo

5.1 Introduction

As outlined in Chapter 1, the method used for cultured pearl production in pearl oysters requires a skilled-technician who implants a nucleus into the gonad of a recipient pearl oyster together with a piece of mantle tissue from a sacrificed donor oyster during the seeding or grafting procedure (Gervis & Sims, 1992). Subsequent proliferation of the donor mantle tissue forms the pearl-sac around the nucleus. Scoones (1996) provided a detailed description of pearl-sac development in *Pinctada maxima*. He reported that pearl-sac development around the nucleus is completed around 30 days after the grafting operation and that nacre secretion begins very soon after the pearl-sac is complete. Further deposition of nacre from the pearl-sac onto the nucleus forms a cultured pearl over a period of about 2 years (Acosta-Salmon et al., 2004; Gervis & Sims, 1992).

Pinctada maxima recovers from anaesthesia in less than two hours (Chapter 2), and can regenerate excised mantle with all internal structures within three months while showing high survival (Chapter 3). Furthermore, Chapter 4 showed that regenerated mantle has full secretory function that can produce nacre which is indistinguishable from that secreted by normal mantle. These results indicate clearly that there is potential for using regenerated mantle tissue as saibo for pearl production.

Following nucleus implantation, the first major step towards cultured pearl production is successful formation of the pearl-sac from proliferation of implanted saibo (Scoones, 1996; Simkiss & Wada, 1980; Taylor & Strack, 2008). This step is, therefore, a key determinant of the potential of saibo from anaesthetised oysters or regenerated mantle in pearl seeding operations. This study investigated pearl-sac formation in *P. maxima* following implantation with saibo from anaesthetised donor oysters and from oysters in which mantle tissue had been regenerated following prior mantle excision.

5.2 Material and methods

A total of 191 oysters, with dorso-ventral measurements (DVM) of between 12 and 17 cm, were used in this experiment. They were maintained in suspended culture on a long-line in Pioneer Bay, Orpheus Island (18°36’30’’S, 146°29’15’’E) prior to the experiment, and were cleaned before nucleus implantation.

This study used oysters that had been treated in three different ways to determine the effects of these treatments on pearl-sac development: (1) anaesthetised oysters (Chapter 2); (2) oysters that have previously had mantle tissue excised which had subsequently regenerated; and (3) ‘normal’ non-anaesthetised oysters with normal non-regenerated mantle. These oysters were used as both donor oysters providing saibo and as nucleus recipients in various combinations (Table 5.1). All oysters were operated by the same professional pearl seeding technician.

Table 5.1. Four implantation combinations conducted in this study.

Implantation			No. Oysters
Donor	**-**	**Recipient**	
Anaesthetised	-	Anaesthetised	24
Anaesthetised	-	Normal	49
Regenerated	-	Normal	48
Normal (Control)	-	Normal (Control)	70

Oysters were anaesthetised as outlined in Chapter 2. Once relaxed, the recipient and donor oysters were set aside by the technician who firstly prepared relaxed oysters to be used as donors. Tissue used as saibo was removed from the mantle, trimmed and chopped into small pieces (3 x 3 mm)(Acosta-Salmon et al., 2004). Each piece of saibo was inserted with a nucleus into the gonad of both relaxed and normal recipient oysters (Table 5.1).

Oysters with regenerated mantle were also prepared for the operation. These oysters experienced original mantle excision more than three months prior to the experiment. Excision followed the method described in Chapter 3 where a piece of mantle approximately 10 mm x 30 mm was cut from the distal part of the ventral region of the mantle. The mantle regenerated to its original extent and original form within 3 months (Chapter 3). In this experiment, these oysters were used as saibo donors only. The mantle taken from these oysters was cut from regenerated mantle within the original wound area and chopped into smaller pieces (3 x 3 mm) required for implantation. Regenerated mantle tissue was used to implant normal oysters only (Table 5.1). Finally, as a control, normal non-regenerated mantle was excised from non-anaesthetised oyster and used as saibo to implant normal non-anaesthetised oysters.

The nuclei used in these operations varied in size from 2.3 to 2.5 and 2.6 bu (1 bu equals to 3.03 mm) and the particular size used was chosen by the technician following inspection of the gonad of the recipient oyster. The grafting operations were conducted by a professional commercial pearl seeding technician. Implanted oysters were placed within a fine mesh bag (used to determine nucleus rejection). They were then held in a raceway with flowing sea water for two days prior to being placed into a panel net and returned to suspended culture conditions on the longline in Pioneer Bay.

After six weeks, the experiment was terminated because of high numbers of expelled nuclei. Oysters that retained the nucleus were killed and the gonad removed into 70% ethanol for 24 hours before being transferred into 4% formalin-seawater for few days. In the laboratory, pearl nuclei were removed and the pearl-sac was filled with gelatine to help maintain its shape; it was then put back into 4% formalin solution for a further night prior to preparation for histological analysis. Sections of 5-µm were cut from preserved pearl-sacs after embedding with paraffin following the methods of Acosta Salmon and Southgate (2005; 2006) and those described in Chapter 3. Mayer's Haematoxilin and Young's Eosin-Erythrosin, MSB (Martius Scarlet Blue) trichrome and Alcian Blue-PAS were used to stain.

Statistical analysis of oyster survival data was conducted with S-Plus ® Ver. 8 for Windows.

5.3 Results

5.3.1 Survival

Survival of oysters in all treatments was high and ranged from 90% for 'normal' oysters implanted with 'normal' saibo, to 100% for anaesthetised oysters implanted with saibo from

anaesthetised donors (Fig. 5.1). Survival did not differ significantly between treatments (χ^2=12.0, p-value= 0.2333).

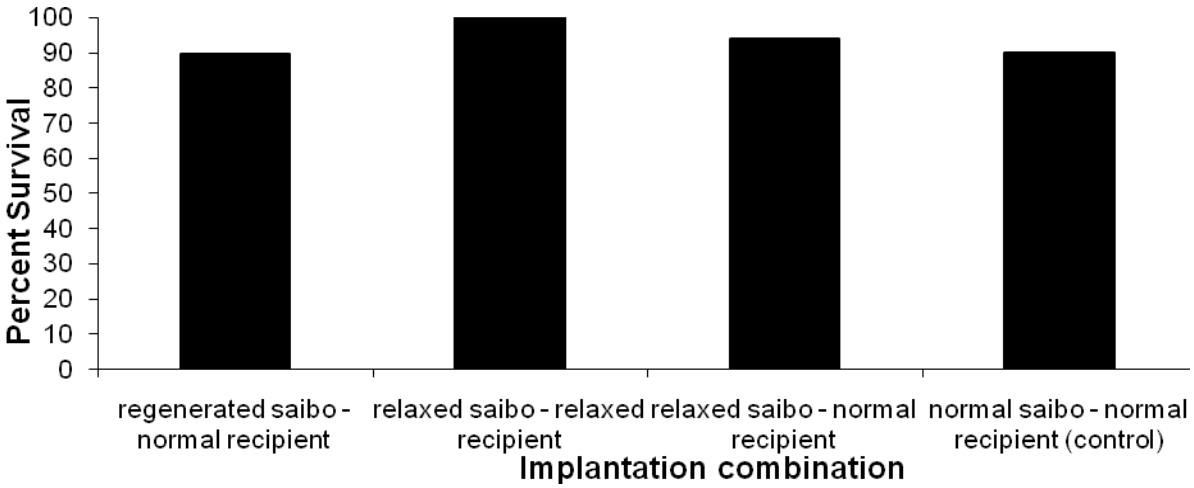

Fig. 5.1. Survival of *Pincatda maxima* following seeding for pearl production using different combinations of donor and recipient oysters.

5.3.2 Nucleus retention

Nucleus retention was much poorer than expected with a total of only 15 oysters retaining their nuclei and showing pearl-sac development. Eight nuclei (53% of the total) were retained by oysters in the control treatment (normal saibo x normal recipients), 4 (26.7%) were retained by the anaesthetised saibo x anaesthetised recipient oysters, 2 (13.3%) by the regenerated saibo x normal recipients treatment and 1 (6.7%) by the anaesthetised saibo x normal recipients treatment.

5.3.3 Histological analysis

The pearl-sacs produced in this study were extremely delicate and difficulties were encountered in their processing and histological preparation. This often resulted in damage

which provided difficulties in interpreting histological slides. Because of this, pearl-sacs from only seven of the 15 gonads that retained a nucleus were used for histological analysis: four from oysters in the control treatment (normal saibo x normal recipient), one from the anaesthetised saibo x anaesthetised recipient treatment, and two in the regenerated saibo x normal recipient treatment.

The six-week duration of this study was sufficient for complete pearl-sac development in oysters seeded with the three types of saibo (relaxed, regenerated and normal saibo) used in this study (Fig 5.2) with pearl-sac tissue completely enveloping the nucleus. However, the thickness of the pearl-sac epithelium varied indicating differences in the degree of pearl-sac maturity (Fig. 5.2). Oysters from all treatments showed evidence of tissue continuity between the saibo and recipient tissue indicating break down of saibo tissue and fusion with recipient tissue on the side distal to the nucleus. Pearl-sacs in all treatments had cell accessories: epithelium and mucous cells. In the control treatment which used normal saibo, greater development of nacre deposition was evident compared to that resulting from the use of both relaxed and regenerated saibo (Fig. 5.2C right). In pearl-sacs produced by relaxed and regenerated saibo, nacre deposition was not obvious but the deposition of an organic matrix covering the whole surface of the nucleus was clear (Fig. 5.2A-B). Despite variations in the thickness of the epithelium produced by each type of saibo, each pearl-sac produced approximately the same thickness of matrix or mineral deposition (Fig. 5.2).

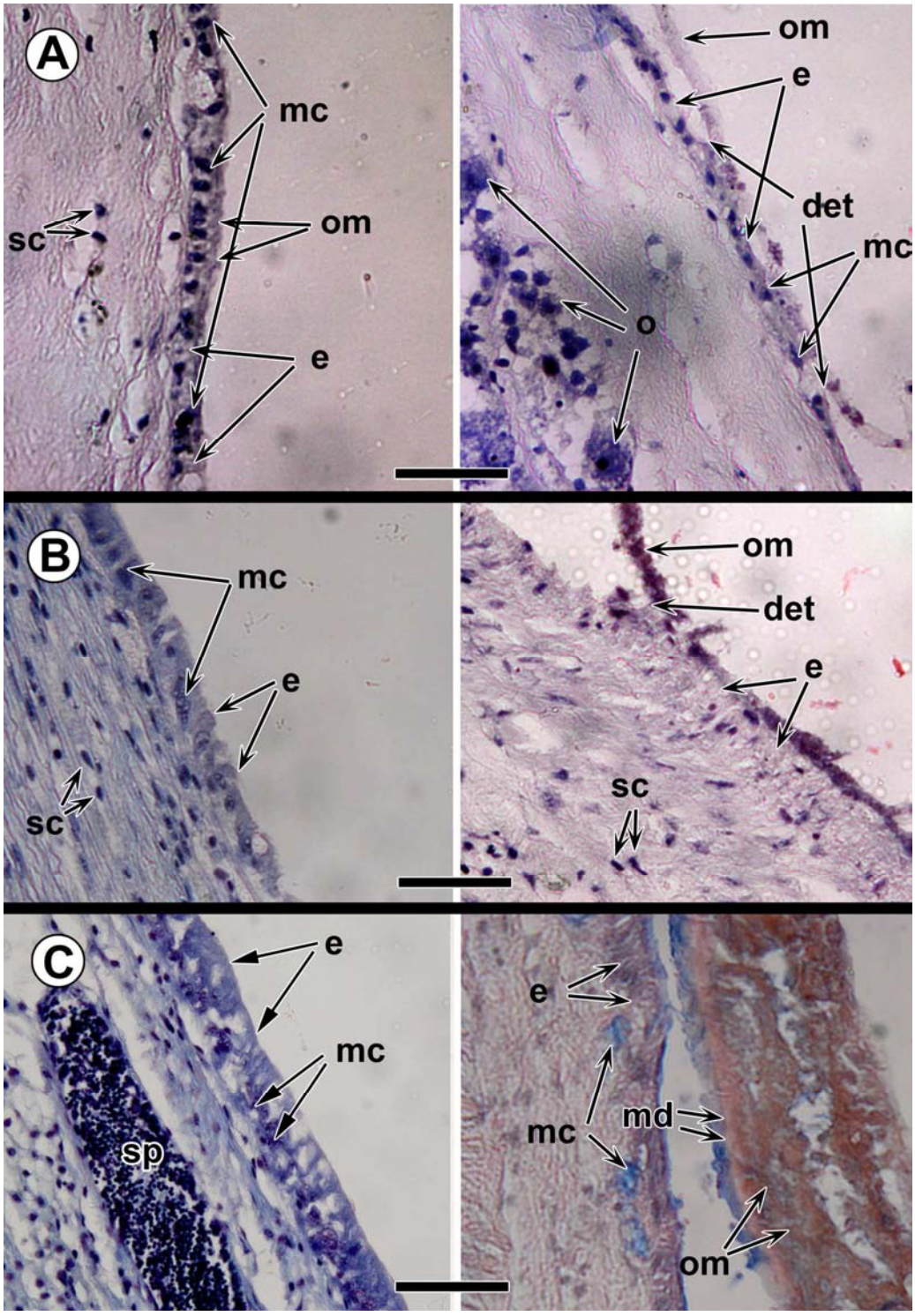

Fig. 5.2 Two areas (left and right) of epithelial regions of the pearl-sac of A. regenerated saibo, B. relaxed saibo and C. normal saibo from *Pinctada maxima* six weeks after pearl implantation. det, detachment; e, epithelium; ms, mucous cells; md, mineral deposition; o, oocytes; om, organic matrix; sc, stem cells; and, sp, spermatozoa. Scale bars 20 µm. All was stained with MSB trichrome.

5.4 Discussion

Scoones (1996) reported on pearl-sac development in *Pinctada maxima* every two days for a period of 50 days after seeding. Like this study, he reported that a significant number of samples could not be used in his analysis because of difficulties in processing them (i.e. incorrect orientation and damage during processing and sectioning). Nevertheless he provided the most detailed study so far on pearl-sac development in *P. maxima* which is summarise in Table 5.2.

Table 5.2. Pearl-sac development in *Pinctada maxima* (Scoones, 1996).

Days after operation	Key developmental features
2	No continuity evidence between the implant and the recipient nor implant tissue breakdown
4	A record of continuity (attachment) between the recipient and the implant
6	Continuity continues, but no evidence of epithelial development
8	Evidence of epithelial development as well as inflammation of haemocytes
10	Epithelial development continues
16	Record of well developed epithelial layers
30	Record of a complete epithelial cover
34	Thin organic layer appears
38	Thin nacre layers on nucleus
50	Thickening mineral deposition

Implanted saibo developed continuity with recipient tissue within 4 days of grafting and development of the pearl-sac epithelium became evident after a further 4 days (Table 5.2). The resulting pearl-sac completely covered the nucleus approximately 30 days after saibo implantation which was followed shortly after by the first evidence of nacre deposition onto the nucleus (Scoones, 1996), with a thickened layer of mineral appeared on day 50 (Table 5.2). The samples used to assess pearl-sac development in the current study were taken six-week after saibo implantation. The results confirm that this duration is sufficient for complete pearl-sac development in *P. maxima* and for initial deposition of material from the pearl-sac onto the nucleus. The appearance of mineral deposition after 50 days reported by Scoones (1996) is similar to the results of this study, particularly for depositions from the pearl-sacs produced from normal mantle.

The pearl-sac of *P. maxima* is surrounded by a thin epithelium layer composed of cuboidal or even squamous cells (Scoones, 1996). These types of epithelial cells were similarly recorded in this study, and in the results of Chapter 3, and they are assumed to have specific functions according to type. Squamous and cuboidal cells are thought to be related to nacre deposition, while columnar cells are associated with the organic matrix (Dix, 1973). These assumptions are based on the prevalence of these various types of epithelial cells in the different zones of the pearl oyster mantle. Squamous and cuboidal cells are common in the pallial to central zones of the mantle where nacre is routinely secreted (Garcia-Gasca et al., 1994; Fougerouse et al., 2008) and columnar cells are common in the marginal zone where the periostracum is formed (Scoones, 1996).

Despite unexpectedly low nucleus retention, the results of this study confirm that regenerated and relaxed saibo are able to generate complete pearl-sacs of similar structure and at a similar rate to normal saibo. However, the results indicate that there may have been a slower rate of mineral deposition from pearl-sacs that developed from both relaxed and regenerated mantle when compared to that from 'normal' pearl-sacs. This aspect required further investigation but may reflect low sample size and variability within samples. Marked variability in the rate of pearl-sac development in *P. maxima* between individual oysters was pointed out by Scoones (1996), who reported much thicker nacre deposition in some oysters 42 days after saibo implantation. He hypothesized that this resulted from variation in the time required for pearl-sac completion.

Low retention of implanted nuclei was recorded for all treatments in this study and may have resulted from sub-optimal conditions following saibo implantation. It is normal practice for pearl-oysters to be transferred back to the sea as soon as possible after operation (Taylor & Strack, 2008) where they are preferably maintained in calm conditions with minimal current and wave activity to minimise stress (Southgate, 2008). In this study, poor weather conditions prevented newly operated oyster from being placed back into ocean-based culture systems soon after the operation. They were instead retained in land-based raceways for two days before transfer to the ocean. The same weather conditions resulted in rough seas which again may have influenced nucleus retention by oysters once they were return to the sea. A nucleus retention rate of 70-90% is normally achieved for *P. maxima* (Taylor & Strack, 2008).

This experiment has confirmed the results of Chapter 4 in showing that regenerated mantle tissue from *P. maxima* apparently regains full secretory function. The results show that saibo

from relaxed oysters and saibo from regenerated mantle tissue is able to form a pearl-sac capable of mineral secretion onto an implanted nucleus, in a similar manner to ‘normal’ saibo. These findings indicate that both saibo from relaxed oysters and from regenerated mantle tissue has similar potential to normal mantle in its use for cultured pearl production. This hypothesis is investigated in Chapter 6.

Chapter 6

Cultured pearl production from *Pinctada maxima* (Jameson) using relaxed and regenerated mantle tissue

6.1 Introduction

The method used for cultured pearl production in pearl oysters has changed a little since it was developed a century ago (Taylor & Strack, 2008). Briefly, a skilled-technician must implant a nucleus into the gonad of a recipient pearl oyster together with a piece of mantle tissue from a sacrificed donor oyster (Gervis & Sims, 1992), a process known as grafting or seeding. Subsequent proliferation of the donor mantle tissue forms the 'pearl-sac' around the nucleus, and deposition of nacre from the pearl-sac onto the nucleus forms a cultured pearl over a period of about 2 years (Acosta-Salmon et al., 2004; Gervis & Sims, 1992). Donor oysters are selected primarily on the basis of their nacre quality which greatly influenced by of resulting pearls (Taylor, 2002). For example, *P. maxima* may have nacre which is predominantly white-silver or gold in colour (Shirai, 1994) and the colour of resulting pearls is greatly influenced by that of the nacre of the donor oyster (Taylor, 2002). Furthermore, very recent research using xenotransplantation of saibo between *P. maxima* and *P. margaritifera* showed that pearl colour and complexion was strongly influenced by the donor oyster species (McGinty et al., 2010). In cultured pearl production therefore the best individuals are sacrificed in the hope of producing the highest quality pearls. This approach differs considerably from most livestock industries where higher quality individuals are used

in breeding programmes to improve the quality of culture stock. This aspect of pearl culture provides a potential bottleneck in the cultured pearl industry.

Recent research however has shown that it is possible to excise mantle tissue from anaesthetised donor pearl oysters without killing them (Acosta-Salmon & Southgate, 2005; Chapter 3). Furthermore, *P. maxima*, like other species of pearl oyster, is able to regenerate excised mantle tissue with apparent full functionality within three months while showing high survival (Chapter 3). These findings potentially provide significant benefits to the *P. maxima* cultured pearl industry. For example, as outlined in section 1.7.2, donor oysters whose mantle tissue produces high quality pearls could be kept alive and potentially used as future broodstock to improve the quality of culture stock and pearl production. Furthermore, high quality oysters could be used as mantle donors on more than one occasion. However, these potential benefits rely on two major assumptions: (1) there are no adverse effects on pearl production, when mantle tissue from anaesthetised donor oysters is used for pearl implantation; and (2) there are no adverse effects on pearl quality when regenerated mantle tissue is used for pearl grafting.

The results of Chapter 4 and Chapter 5 showed that regenerated mantle tissue is able to secrete nacre with similar structure to 'normal' nacre, and that both relaxed and regenerated mantle tissue are capable of forming a pearl-sac following implantation. They indicate that relaxed and regenerated mantle tissue from *P. maxima* may be able to generate pearls when used for cultured pearl implantation. Testing this assumption is the basis of the experiment described in this Chapter, which assesses the yield and quality of pearls produced from *P. maxima* grafted with mantle tissue from anaesthetised donor oysters, regenerated mantle tissue and 'normal' mantle tissue obtained from sacrificed donor oysters.

6.2 Material and methods

This study was conducted at a commercial pearl oyster farm at Penyabangan, North Bali, Indonesia (8º 09' S and 114º 44' E). Oysters used in the study were hatchery produced. Three groups of oysters were used which came from three separate hatchery cohorts: two resulted from spawning in March 2004 (MS1 and MS2) and in April 2004 (AS). Oysters were divided into two groups: donors and recipients (Table 6.1). Furthermore, donor oysters were grouped according to their nacre colours: silver (for silver donor) and gold (for gold donor). Silver donor oysters were from the MS1 cohort while gold donors were from the AS cohort. Recipient oysters were from both AS and MS2 cohorts (Table 6.1).

6.2.1 Experimental design

Table 6.1. Oysters used in the first and second operations in this study.

1st Operation (30-31July 2006)			**2nd Operation (9-11 November 2006)**		
Donor Oysters		Recipient Oysters	Donor Oysters		Recipient Oysters
Silver	Gold		Silver	Gold	
MS1	**AS**	**AS**	**MS1***	**AS**	**MS2**

* The same oysters used in the 1st operation
MS1: oysters from the first spawning in March 2004
MS2: oysters from the second spawning in March 2004
AS: oysters from a spawning in April 2004

Pearl grafting operations took place over two time periods. The first operation was conducted about three months earlier than the second (Table 6.1). The first operation was conducted

using relaxed mantle excised from anaesthetised donor oysters. The second operation was conducted with regenerated mantle excised from anaesthetised donor oysters (Fig. 6.1). Regenerated mantle was excised 3 months after initial excision because prior research in our laboratory has shown that completed regeneration of excised mantle is achieved within three months of excision. To prepare the strips of mantle tissue (saibo) used in the operations, donor oysters were excised after exposure to anaesthetic solution of 2.5 mL/L propylene phenoxetol (Chapter 2).

Recipient oysters used for the first operation were from a 110 mm size group of AS, while for the second operation they were from a 100 mm size group of MS2. A total of 794 oysters were used for the first operation: 220 oysters were implanted with relaxed saibo from anaesthetised silver donors and 574 were implanted with relaxed saibo from anaesthetised gold donors. For the second operation 623 oysters were used: 200 oysters were implanted with relaxed regenerated saibo from anaesthetised silver donors and 423 oysters were implanted with relaxed regenerated saibo from anaesthetised gold donors. A control group was also established in the second operation composed of 103 oysters with using standard silver saibo from non- anaesthetised oysters. Thus a total of 1,520 oysters were implanted in this study.

Prior to operation, oysters underwent a weakening or conditioning period for two weeks following standard farm practice. Nets containing the oysters were covered in a material with small pores to reduce water flow and held in deeper water. This process stimulates oysters to spawn and will allow technicians to operate in gonads with adequate space for nucleus implantation (Taylor & Strack, 2008). One day before the operation, the oysters were cleaned and placed them into tanks containing filtered seawater until being prepared for the operation.

6.2.2 First operation

Water in tanks containing the oysters was lowered to stimulate shell opening and once open, a plastic wedge was inserted between the shell valves of each oyster. Pegged oysters to be used as recipients were collected in baskets and placed besides the technicians who were to conduct the implanting operation. Five technicians were used to implant the oysters used in this study. Donor oysters were selected on the basis of their nacre colour and were sorted into silver/white and gold/yellow colour groups. They were anaesthetised with 2.5mL/L propylene phenoxetol (Chapter 3) and, once relaxed, a strip of mantle was cut from the centre of the ventral marginal region (Chapter 4). This strip was long enough to provide approximately eight pieces of mantle (each 3 x 3 mm), allowing each donor oyster to supply saibo for at most eight recipient oysters. After mantle excision, donor oysters were put into a recovery tank before transportation back to field-based culture conditions where they remained until they were used again in the second operation (Fig. 6.1).

During the operation, a piece of saibo was implanted together with a nucleus into the gonad of each recipient oyster following the method used commercially (Taylor & Strack, 2008). The nuclei used were yellow bio-coated nuclei (Orca Marine Supplies, Australia). Nucleus size selection was conducted arbitrarily by each technician based on their experience. The range of nucleus sizes used was 1.9 to 2.8 bu (1 bu equals to 3.03 mm). After the operation, recipient oysters were put into eight-pocket panel nets and hung under a fine mist sea water spray before being returned to field culture conditions. Each panel net contained six to eight oysters implanted from the same saibo donor.

6.2.3 Second operation

The procedure used for the second operation was the same as that used for the first with the exception that saibo was taken from regenerated mantle of the donor oysters used in the first operation (Fig. 6.1). Saibo was excised from the ex-wound area of the donor oysters. Before excision, the donor oysters were again anaesthetised as detailed for the first operation. After mantle excision, donor oysters were placed into a recovery tank and then transported back to field-based culture conditions.

The size of the nucleus used, the colour of donor nacre and the identity of the technician was recorded for each recipient oyster during both operations. Pearls resulting from first operation were harvested at the end of July 2008 but the second operation was on the first week of November 2009; both were harvested 24 months after nucleus implantation.

6.2.4 Data analysis

Survival of implanted oysters was recorded three months before pearls were harvested. A Chi Square Test for Independence was conducted using S-Plus ® Ver. 8 for Windows to determine whether oyster survival differed between the five technicians. The same test was then used to detect any differences in survival of oysters implanted with the different types of saibo (relaxed, relaxed-regenerated and normal). Nucleus retention was determined on the basis of the numbers of nucleated pearls retained in surviving oysters at harvest. Implanted oysters may reject the nucleus but retain saibo tissue in the gonad resulting in the formation of a non-nucleated pearl or ‘keshi’. Only nucleated pearls were included in this analysis. Nucleus retention was analysed using a Chi Square Test for Independence using S-Plus ® Ver. 8 for Windows.

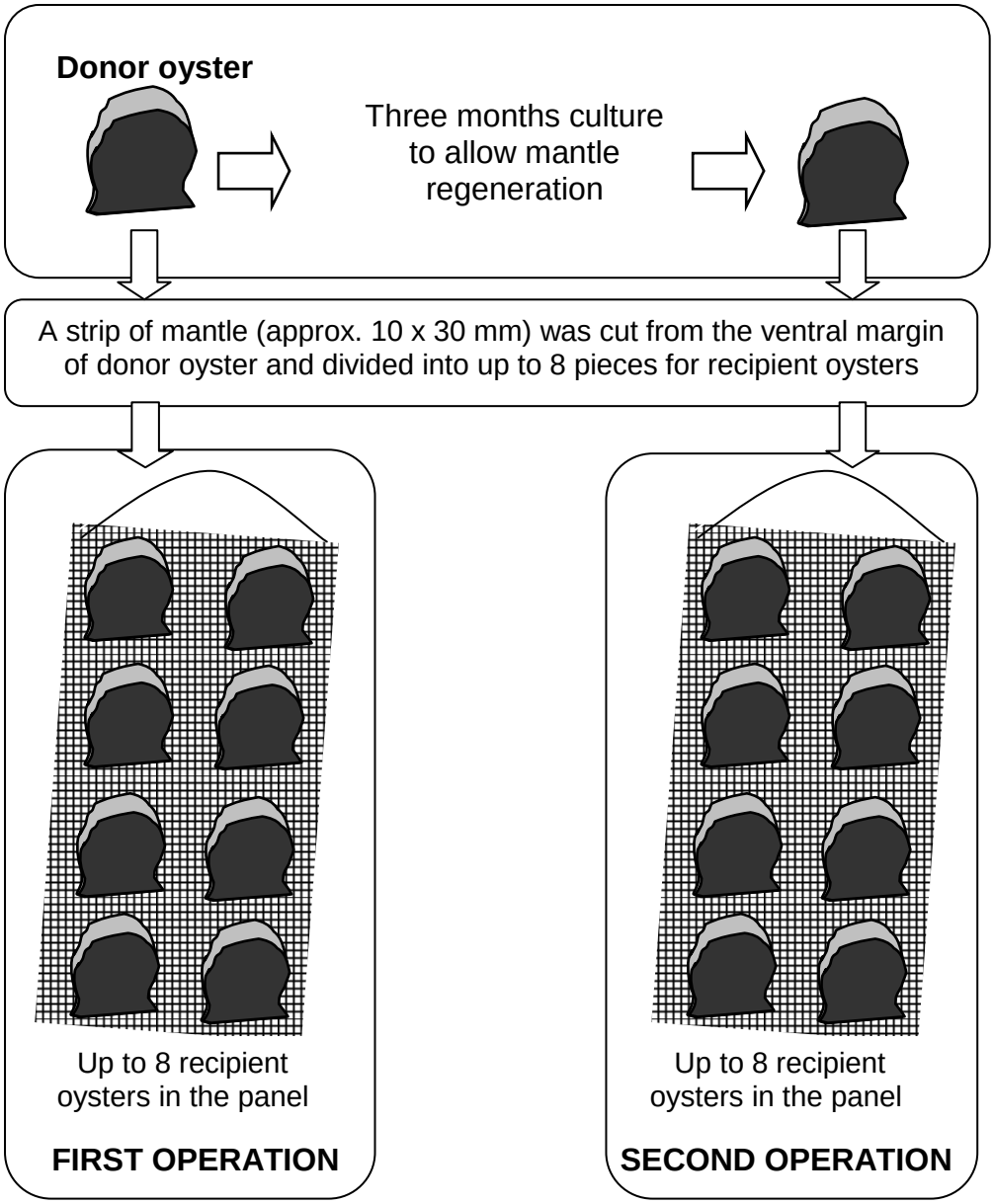

Fig. 6.1. A scheme showing the use of donor pearl oysters in two pearl operations conducted in this study.

For initial grading of the pearls resulting from this study, nucleated pearls together with keshi pearls were sorted into 'Accepted' and 'Rejected' groups. 'Accepted' pearls are valuable pearls for jewellery. 'Rejected' pearls are not valuable for jewellery and this category included small keshi (less than 3 mm) or pearls that appeared chalky, with no lustre or covered by conchiolin only (usually dark in colour). Accepted pearls were graded on the

basis of five criteria used to determine pearl quality: size and nacre thickness, shape, colour, surface complexion and lustre (Matlins, 2002; Strack, 2006; Taylor & Strack, 2008; Ward, 1995) and followed the guide for assessing quality in south sea pearl by Taylor (2007).

6.2.4.1 Pearl shape

Pearl shape was recorded visually only and classification followed the scheme proposed by Taylor (2007) (Table 6.2). Six main shape categories were recognised in this study: round, drop, oval, button, baroque, and circle. The proportion of pearls in each shape category was compared between two types of saibo used in this study: relaxed and regenerated using a Chi Square Test for Independence. Pearls resulting from normal non-anaesthetised, non-regenerated saibo were excluded from the test because of small sample size.

6.2.4.2 Size and nacre thickness

The size of all nucleated and keshi pearls was measured to the nearest 0.5 mm. The size was based on the diameter of the pearl taken from the widest point of the various shapes (Taylor, 2007). Nucleated pearls were grouped into three size classes: small pearls (< 9 mm), medium pearls (9-12 mm) and large pearls (>12 mm). The Chi Square Test for Independence was used to determine whether of the proportion of pearls in each of the size categories was influenced by the types of saibo used to produce them.

Nacre thickness was also determined by subtracting the nucleus diameter from the pearl diameter. This was done with pearls from the ‘round’ group only (round, near round and semi round) which were all nucleated pearls.

Table 6.2. Categories based on shape characters in grading of *Pinctada maxima* pearls modified from Taylor (2007).

Category	Description
Round (This category covers Round, Near Round and Semi Round)	Round: Spherical with maximum variation in measure on any round pearl should not exceed 3%
	Near Round: Spherical with minor elongation or flattening on one face. A spherical pearl with a very small 'bump' on one end. The variation in measure is 3 – 7.5%
	Semi Round: Appear round when viewed from a specific angle. This pearl may be slightly oval, button or baroque but will appear round when set with other pearls. The variation in measure exceeds 7.5 – 10%
Drop (This category includes Classic Drop, Drop and Semi Drop)	Classic Drop: A tear-drop shaped pearl. The pearl apex should be tapered to a fine end without any obvious distortion. The opposing end of the pearl should be rounded and not flat
	Drop: A short drop shaped pearl. The pearl tapers cleanly to the apex but is shorter than classic long drop
	Semi Drop: A conical shape drop pearl, drops with a flattened base, drops with deformation at the apex, drops with a single ring in the top 25% of the pearl (greater than this and the pearl is categorised as a circle pearl)
Oval (This category includes Oval and Semi Oval)	Oval: Oval shapes that cannot be placed in a Semi Round category because they are too long. These pearls have more or less the same taper or width either end of the pearl.
	Semi Oval: An oval shaped pearl with a flattened base, ovals with deformation at the apex, drops with a single ring in the top 25% of the pearl (greater than this and the pearl is a 'circle')
Button (This category covers High Button, Flat Button and Semi Button)	Button: These pearls are well rounded for the main with a slightly flattened base or with lesser curvature on one side compared with the other. Viewed from above, these type of pearls will appear to be round
	Flat Button: Either a distinctly flat on one side or disc like
	Semi Button: Conical, squat pearls similar to cone shaped drops but wider than they tall
Baroque (This category includes Baroque and Semi Baroque)	Baroque: Asymmetrical pearls with no clearly defined shape characteristic
	Semi Baroque: Loss of symmetry due to large bumps on one side or end of the pearl. These pearls generally still show the shape characteristics of other categories
Circle (This category includes Classic Circle and Semi Circle)	Classic Circle: A pearl with one to three distinct symmetrical rings. At least one of the rings will be more than 30% down the length (top to bottom for drops) of the pearl or there is more than one ring. If the ring does not run the entire diameter of a pearl the pearl will not be classed as a circle
	Semi Circle: A pearl with three or more rings or with asymmetrical rings or rings that give the pearl a scratched or scored appearance

One-Way Analysis of Variance (ANOVA) was used to determine any differences in nacre thickness among pearls produced by the different types of saibo used in this study. A Tukey HSD post-hoc analysis was used to identify significant differences between treatments following data transformation. Correlation analysis was then used to determine the degree of correlation between individual pearl size and nacre thickness or nucleus size using S-Plus ® Ver. 8 for Windows after passing a normality test.

6.2.4.3 Colour

Pearl colour and overtone were estimated using the classification of Taylor (2007) shown in Table 6.3. Pearl colours were described qualitatively to compare between the pearl produced by the different types of siabo used in this study and between saibo from the different colour saibo donors (i.e. silver/white and gold/yellow).

6.2.4.4 Surface complexion

The surfaces of the pearls produced in this study were inspected to identify imperfections such as spots, scratches, wrinkles, small blisters and cracks (Matlins, 2002; Strack, 2006; Taylor, 2007). Grading based on surface characteristics followed the classification of Taylor (2007) shown in Table 6.4.

6.2.4.5 Lustre

Pearl lustre was evaluated on the basis of the sharpness of reflections with clearer reflection indicating higher lustre (Matlins, 2002; Strack, 2006). Grading on the basis of lustre followed the classification of Taylor (2007) which is detailed in Table 6.5.

Table 6.3. Categories based on colour in grading pearls of *Pinctada maxima* (Taylor, 2007).

Category	**Description**
WW	Typical soft white colour with no obvious overtone
WS	A white pearl with a light silver overtone
WC	A white pearl with a cream overtone
WP	A white pearl with a pink overtone
WF	A white pearl with ’fancy’ overtone in green or blur
SS	A distinctly silver pearl
SP	A silver pearl with pink overtone
SF	A silver pearl with ‘fancy’ overtone in green or blue
PP	A pink pearl
CC	A cream pearl
CP	A cream pearl with pink overtone
CF	A cream pearl with fancy overtone in green or blue
YY	A yellow pearl
GL	A light gold pearl
GG	A metallic gold pearl
GF	A metallic gold pearl with ‘fancy’ overtone in red or copper
FF	A ‘fancy’ colour with high intensity: apricot, purple, blue, green, chocolate, platinum
FL	A ‘fancy’ colour of moderate intensity: apricot, purple, blue, green, chocolate, platinum

Table 6.4. Categories based on surface complexion used for grading pearls of *Pinctada maxima* (Taylor, 2007).

Category	Description
A1	No blemishes or one very small blemish that can be removed by drilling
B1	One to three very small blemishes in close proximity with the majority of the pearl surface being clear
B2	3 or more blemishes but with at least one clean face visible on the pearl
C1	Minor blemishes all over the pearl surface (no clean face) or one to two large blemishes that affect over 70% of the pearl surface. Wrinkled or Scratched pearls fall in this category
C2	Blemished on entire surface, spots are calcified
D1	A commercially reject pearl. A pearl that does not fall into above categories

Table 6.5. Categories based on lustre used for grading pearls of *Pinctada maxima* (Taylor, 2007).

Category	Description
Brilliant (1)	A very bright pearl. Light appears to reflect from within the pearl (referred to as orient or inner glow). Pin points of light are very clearly and sharply reflected. It appears like a mirror
Excellent (2)	A bright pearl but has a slightly blurred reflection
High (3)	A pearl with minimal inner reflection but blurred
Modest (4)	A pearl that appears slightly opaque, the reflection is not clear
Poor (5)	Opaque to the point appearing milky. Commercially, it is not for retail sale.

6.3 Results

6.3.1 Oyster survival

Survival of operated pearl oysters among technicians ranged from 90% to 94 % (Fig. 6.2). Oyster survival did not vary significantly between technicians (χ^2= 6.589906, p=0.159213). The percentage of pearl oyster survival based on the types of saibo used in this study (relaxed, regenerated and normal) varied from 90% (normal saibo) to 92% (regenerated saibo) and 95% (relaxed saibo) (Fig. 6.3). These values did differ significantly (χ^2= 8.990048, p=0.01116441).

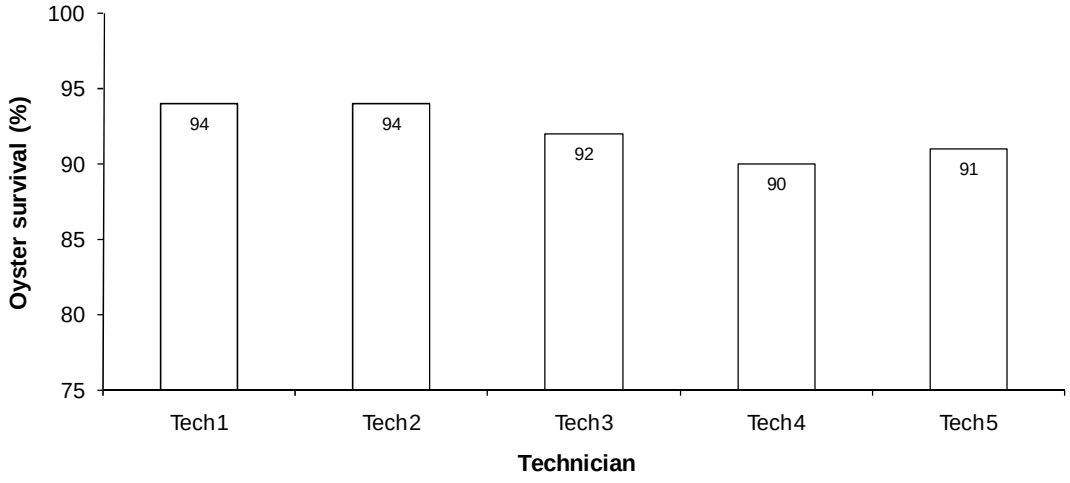

Fig 6.2. Survival (%) of *Pinctada maxima* two years after operation by five technicians (recorded two weeks before harvest). Tech 1 (n= 570 oysters), Tech 2 (n= 401 oysters), Tech 3 (n= 82 oysters), Tech 4 (n= 183 oysters) and Tech 5 (n=179 oysters).

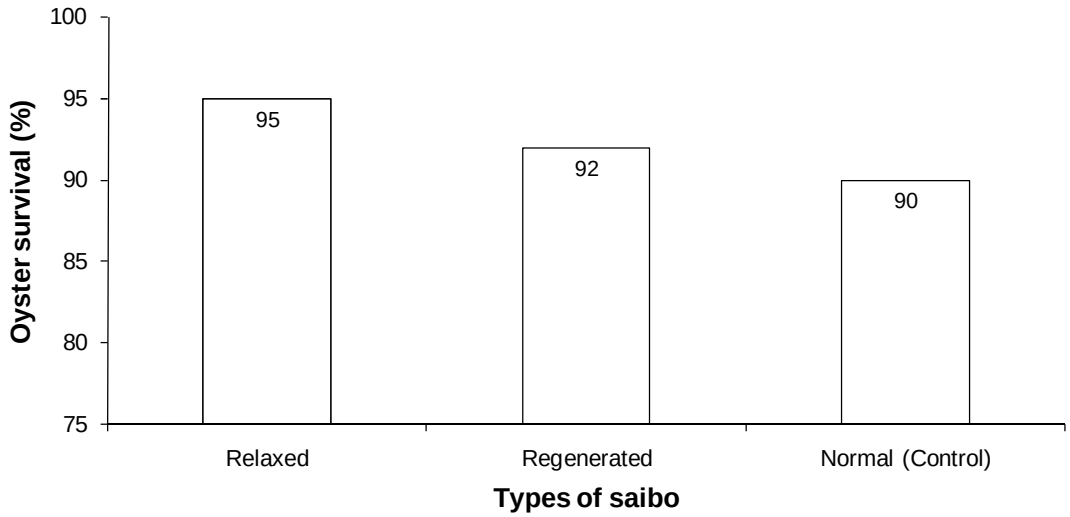

Fig 6.3. Survival (%) of *Pinctada maxima* two years after implantation with relaxed saibo (n = 753 oysters), regenerated saibo (n = 572 oysters) and normal saibo (control)(n = 90 oysters)(recorded two weeks before harvest). (χ^2= 8.990048, p=0.01116441).

6.3.2 Nucleus retention

Nucleus retention among technicians for all surviving oysters implanted with all three types of saibo (relaxed, regenerated and normal) varied from 26% (Tech 2) to 41% (Tech 4)(Fig. 6.4A). These values differed significantly (χ^2= 31.00954, p=0.0001399552). To determine whether the type of saibo contributed to these differences in nucleus retention between technicians, another Chi Square Test was conducted for oysters implanted with relaxed and regenerated saibo only. Retention of nuclei by oysters implanted with relaxed saibo was not significant (χ^2= 1.567007, p=0.457). The percentage of nucleus retention for this category was between 25% and 29% for two of the technicians (Fig. 6.4B). The same test conducted for retention of nuclei by oysters implanted with regenerated saibo was also not significant (χ^2= 11.9484, p=0.153) where the percentage of nucleus retention ranged from 32% to 41% across the five technicians (Fig. 6.4C). Overall nucleus retention for oysters implanted with

the three types of saibo varied from 27% for those implanted with relaxed and normal saibo to 37% for those implanted with regenerated saibo (Fig.6.5). Type of saibo had a very significant effect on nucleus retention ($\chi^2$34.01114, p=0).

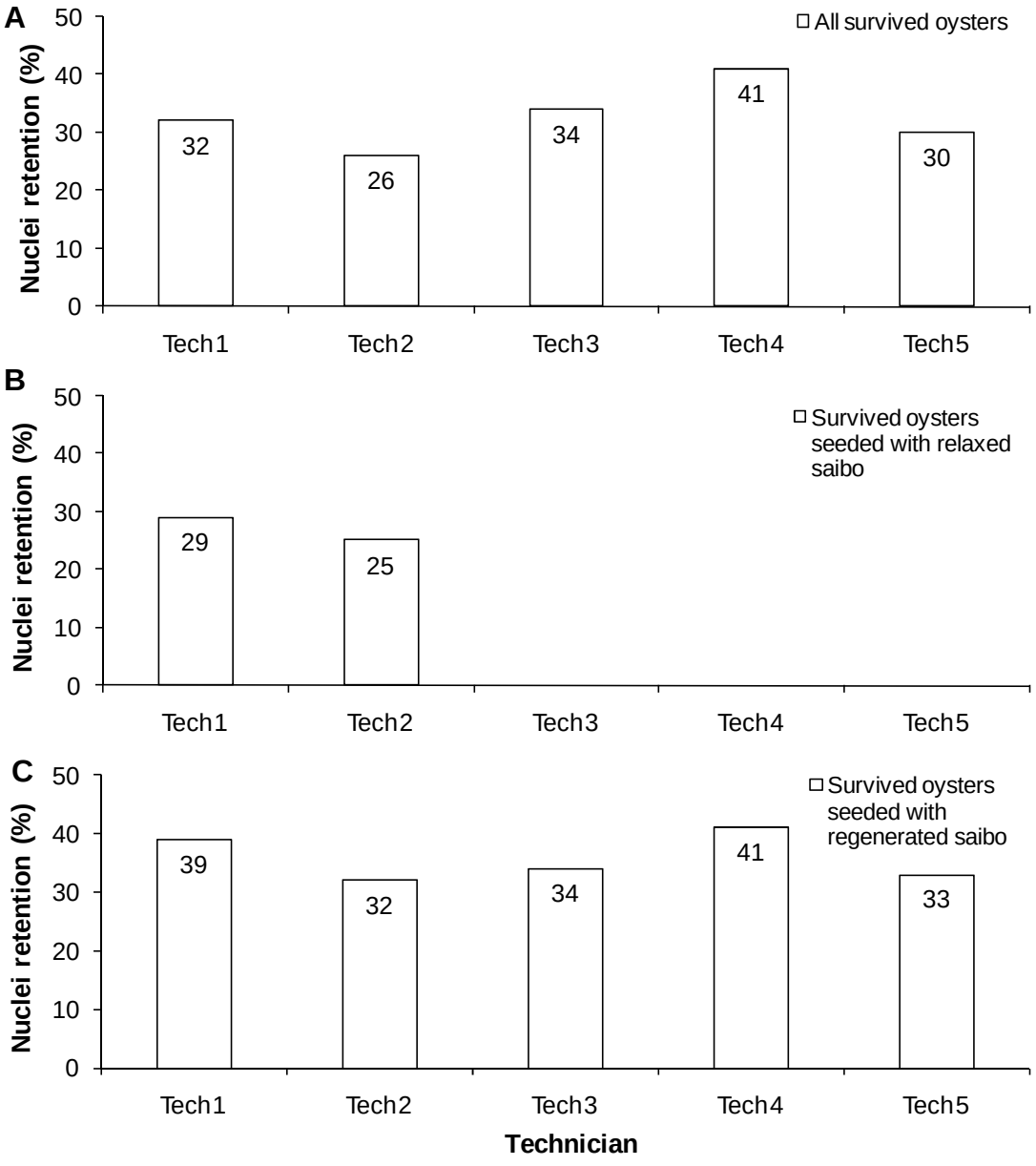

Fig 6.4. Percentage of nucleus retention by *Pinctada maxima* implanted by five technicians in (A) all surviving oysters, (B) surviving oysters implanted with relaxed saibo and (C) surviving oysters implanted with regenerated saibo.

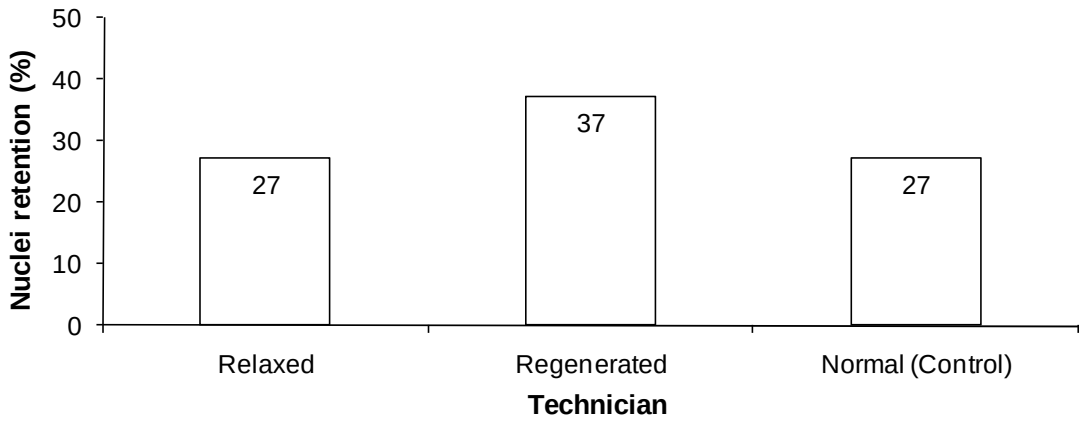

Fig 6.5. Percentage of nucleus retention by *Pinctada maxima* implanted with different types of saibo (relaxed, regenerated and normal). Relaxed saibo (n = 206 pearls), regenerated saibo (n = 214 pearls) and normal saibo (control)(n = 24 pearls)(χ^2= 34.01114, p=0).

6.3.3 Pearl quality

The total number of both nucleated and keshi pearls produced by oysters implanted with relaxed, regenerated and normal saibo was 240, 165 and 19, respectively. The proportion of these that were considered to be of acceptable commercial quality was 99%, 62% and 53% for oysters implanted with relaxed, regenerated and normal saibo, respectively (Fig. 6.6). There was a highly significant difference between these values (χ^2= 112.3091, p=0).

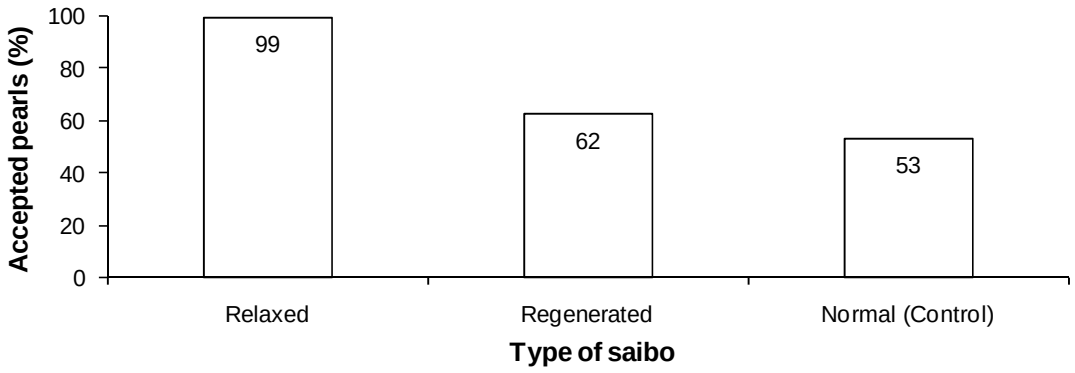

Fig 6.6. The percentage of accepted pearls as a proportion of total pearls produced by *Pinctada maxima* implanted with three types of saibo (relaxed, regenerated and normal). Relaxed saibo (n = 240 pearls), regenerated saibo (n = 166 pearls) and normal saibo (control)(n = 19 pearls) (χ^2= 112.3091, p=0).

6.3.3.1 Pearl shape

The proportions of pearls in each of the major categories of shape resulting from implantation of oysters with relaxed, regenerated and normal saibo are shown in Fig. 6.7. The majority of pearls were graded into the 'round' shape category (34.8% of a total of 425 pearls)(Fig. 6.7A). The majority of pearls in this category were produced by oysters implanted with relaxed saibo (47% of category total)(Fig. 6.7B), while those from oyster implanted with normal saibo made up the smallest proportion of pearls in this category (28%)(Fig. 6.7D). Pearls in the 'drop' category made up 20.2% of the total number of pearls produced. Again, the majority of pearls in this category were produced by oysters implanted with relaxed saibo (24% of category total) while those from oyster implanted with normal saibo made up the smallest proportion of pearls in this category (11%). Pearls in the 'oval' and 'button' categories made up 10.1% and 13.6% of the total number of pearls produced, respectively,

where the highest proportions of the totals were made up by pearls from oysters implanted with normal saibo (Fig. 6.7D). Pearls in the ‘circle’ and ‘baroque’ categories made up only 4% of the total number of pearls produced and were produced by oysters implanted with relaxed or regenerated saibo only (Fig. 6.7B,C). Keshi pearls made up 13.2% of the total number of pearls produced and again were produced by oysters implanted with relaxed or regenerated saibo only. Because of the low number of pearls produced by oysters implanted with normal saibo (19), statistical tests into the effects on types of saibo on pearl shape were conducted for relaxed and regenerated saibo only. There was shown to be a highly significant effect (χ^2= 15.32797, p=0.018).

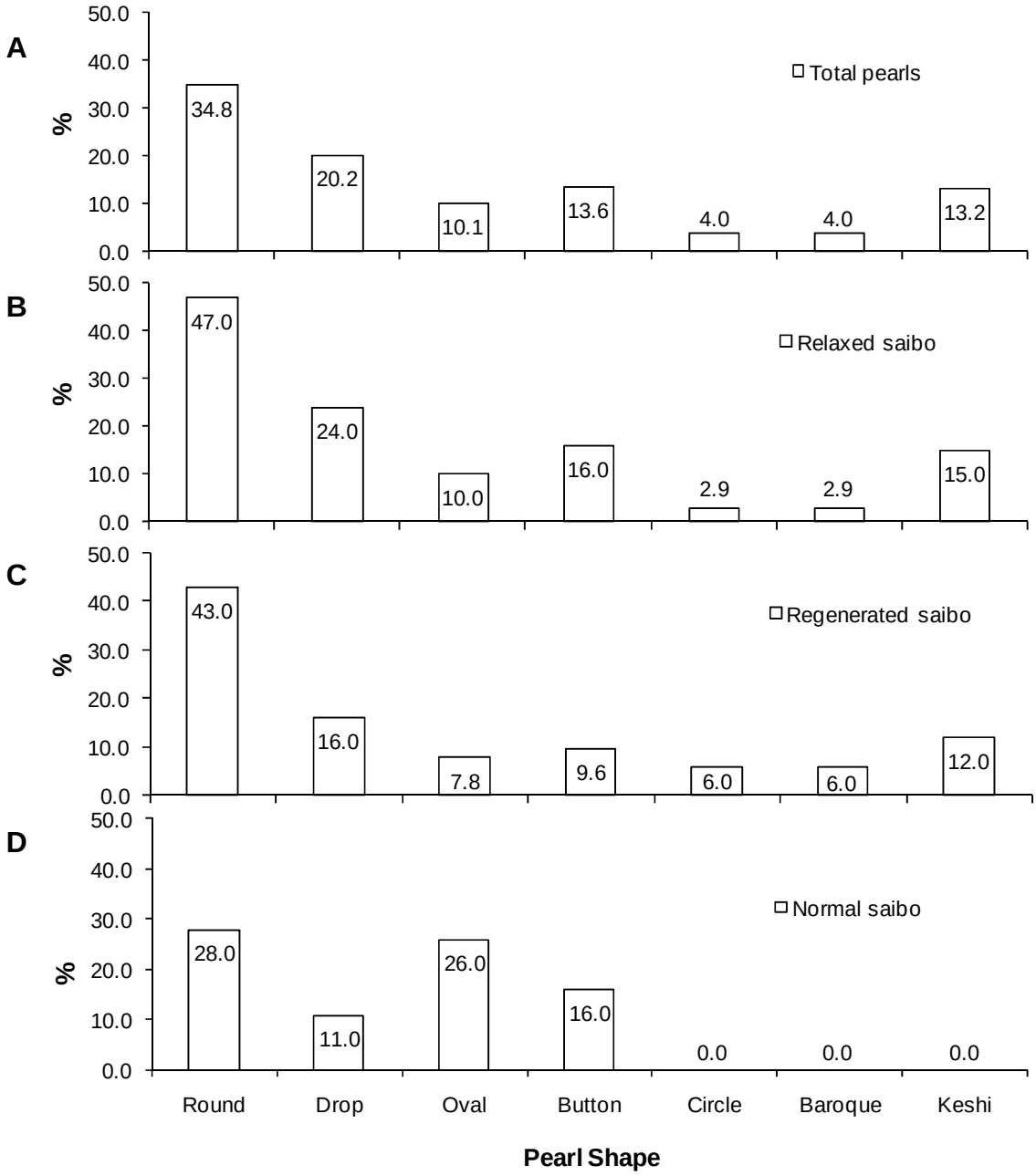

Fig 6.7. The proportions of various pearl shape categories produced by *Pinctada maxima*. A: total pearls (n= 425), B: pearls from oysters implanted with relaxed saibo (n = 240 pearls), C: pearls from oysters implanted with regenerated saibo (n = 166 pearls) and, D: pearls from oysters implanted with normal saibo (n = 19 pearls).

6.3.3.2. Pearl size and nacre thickness

The size frequency of both nucleated and keshi pearls produced by oysters implanted with relaxed, regenerated and normal saibo are shown in Fig. 6.8. Pearls produced by relaxed saibo ranged from 3-14 mm in size. The highest proportions of pearls produced by relaxed saibo were in the 10-11 mm categories which collectively made up 46% of the total (Fig. 6.8A). Pearls produced by relaxed saibo attained a larger size than those resulting from both regenerated and normal saibo (Fig. 6.8B,C); those in the 12.5 mm to 14 mm size range made up 3% of the total number of pearls produced by relaxed saibo. Pearls produced by regenerated saibo ranged in size from 4 mm to 12 mm with the majority (64.2%) in the 8-9 mm size classes (Fig. 6.8B). The largest pearls produced by regenerated saibo were in the 12 mm size class but only 3.6% of the total number of pearls fell into the 10.5 mm to 12 mm size categories. Pearl produced by normal saibo ranged from 8 mm to 11 mm in size (Fig. 6.8C) and did not attain the larger sizes of pearls produced by relaxed and regenerated saibo. The majority (57.9%) of pearls produced by normal saibo were in the 8.5 mm to 9 mm size categories and, unlike the pearls produced by relaxed and regenerated saibo, no small pearls (< 5 mm including keshi) were produced.

Further sorting of nucleated pearls only (i.e. without keshi) into three size group categories (< 9 mm, 9-12 mm and > 12 mm) showed that the majority of pearls in the former were produced by regenerated saibo while the majority of pearls produced by relaxed and normal saibo were captured by the 9-12 mm size category (Fig. 6.9). The > 12 mm size category contained mostly pearls produced by relaxed saibo (Fig. 6.9A) but they made up only 8.3% of the total pearls produced from this saibo.

There was a very significant effect of type of saibo on pearl size (χ^2= 44.57578, p=0).

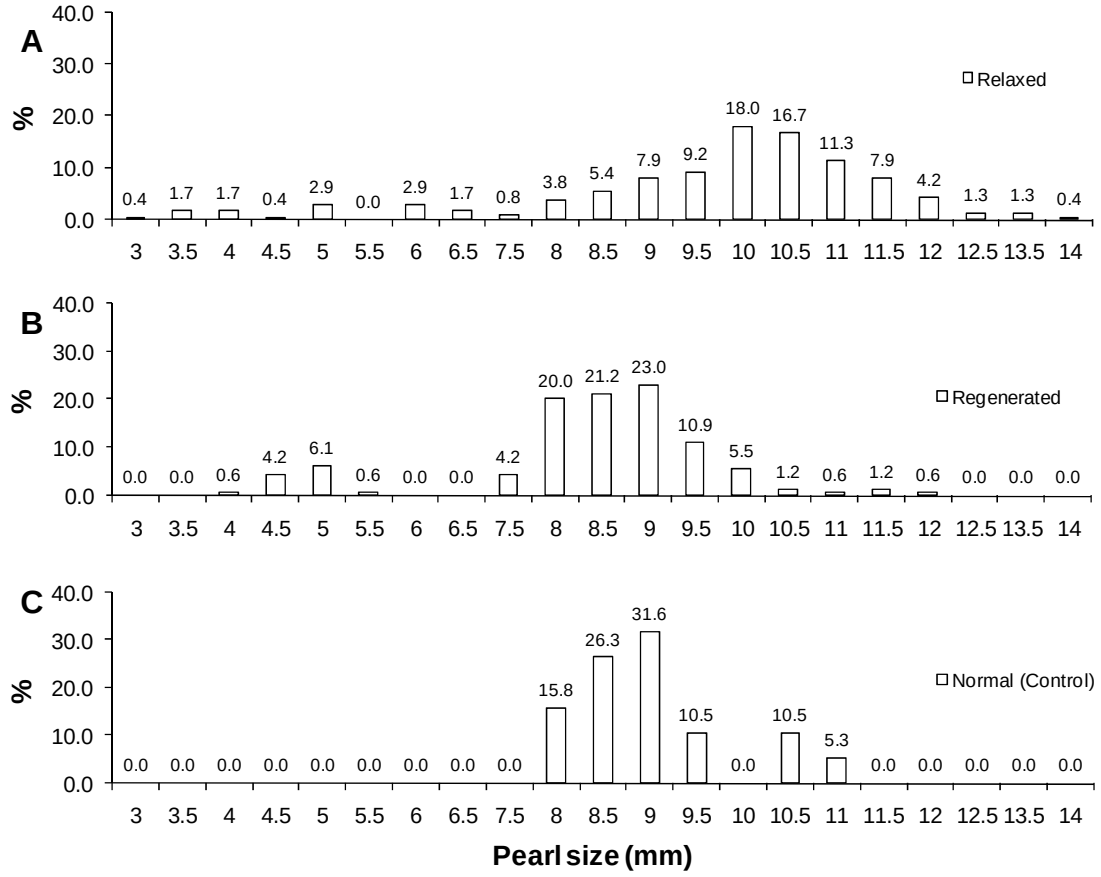

Fig 6.8. Size-frequency distributions of pearls produced by *Pinctada maxima* implanted with (A) relaxed, (B) regenerated and (C) normal saibo. Relaxed saibo (n = 239 pearls), regenerated saibo (n = 165 pearls) and normal saibo (n = 19 pearls).

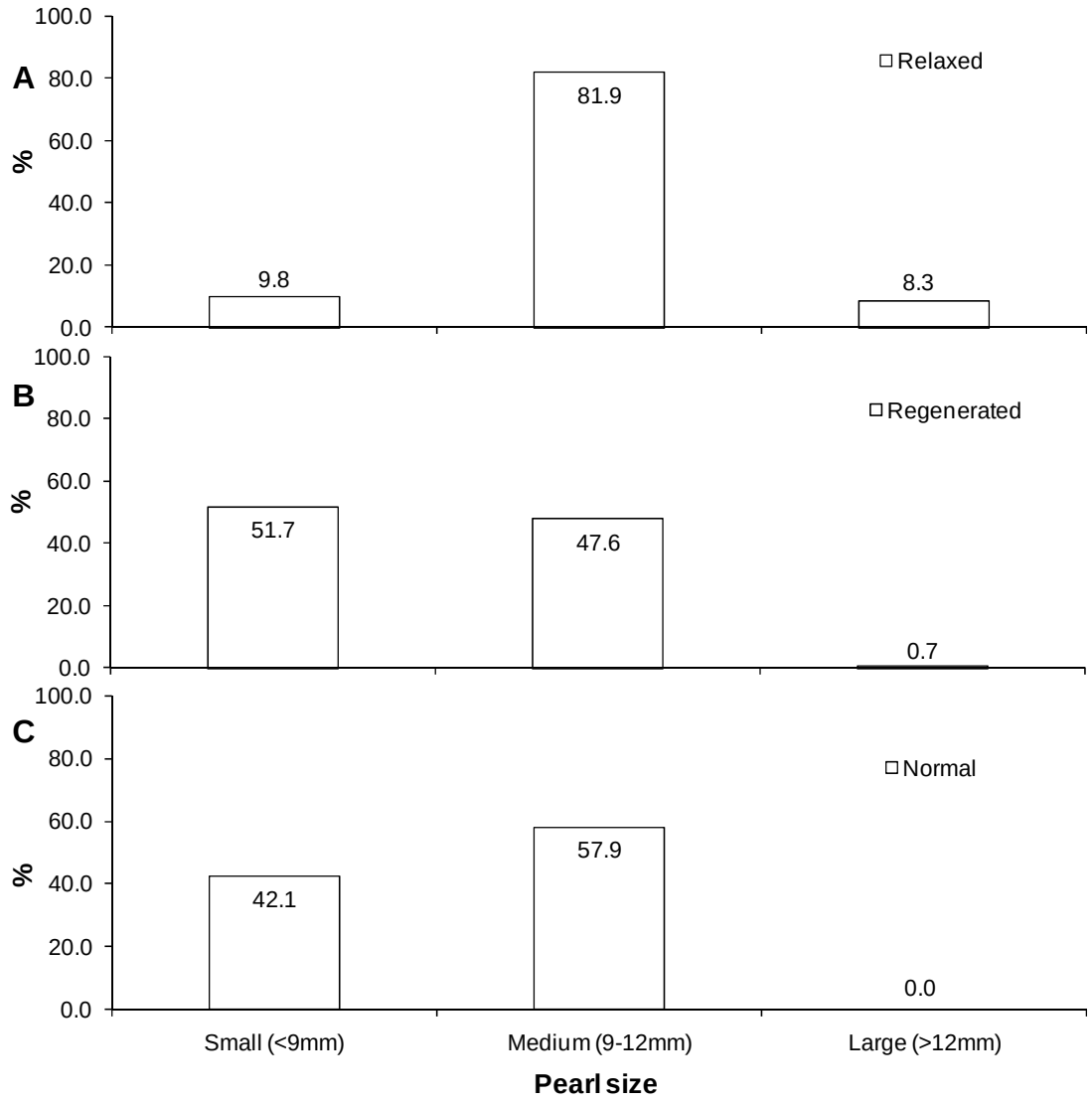

Fig 6.9. Proportions of nucleated pearls produced by *Pinctada maxima* within three different sizes classes following implantation with (A) relaxed, (B) regenerated and (C) normal saibo. Relaxed saibo (n = 204 pearls, regenerated saibo (n = 147 pearls) normal saibo (n = 19 pearls).

Mean pearl size and nacre thickness of nucleated pearls produced by oysters implanted with relaxed, regenerated and normal saibo from the 'round' shape category is shown in Fig. 6.10. Mean (± SE) sizes of pearls produced by relaxed, regenerated and normal saibo were 10.3 ± 0.14 mm, 8.7 ± 0.11 mm and 9.2 ± 0.33 mm, respectively. Nacre thickness ranged from 0.7 to

6.5 mm. The average nacre thickness among pearls produced by the different types of saibo was 3.4 ± 0.12 mm for relaxed saibo, 1.8 ± 0.11 mm for regenerated saibo and 2.4 ± 0.30 mm for normal saibo (Fig. 6.10). One-Way Analysis of Variance (ANOVA) was conducted to analyse the effect of types of saibo on nacre thickness after square root transformation of the data to achieve normal distribution. The result of the test was very significant (Table 6.6). A post-hoc Tukey HSD test showed that relaxed saibo produced significant greater nacre thickness than both regenerated ($p = 0.000$) and normal saibo ($p = 0.013$), while nacre thickness from regenerated saibo did not differ significantly from that of normal saibo ($p = 0.120$).

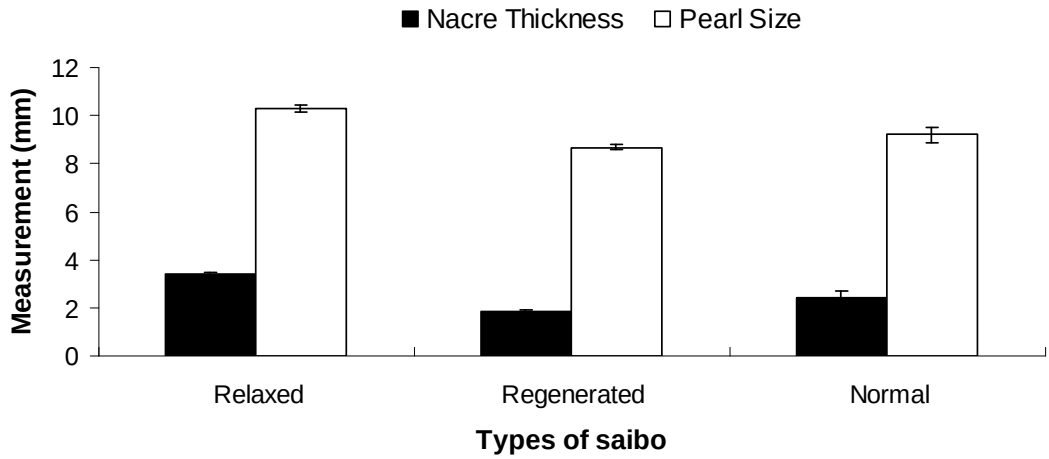

Fig 6.10. Mean (mm ± SE) pearl size and nacre thickness of round nucleated pearls produced by *Pinctada maxima* implanted with relaxed, regenerated and normal saibo.

Table 6.6. Effect of types of saibo (normal, relaxed, regenerated) on nacre thickness tested with One-Way Analysis Variance (ANOVA).

	df	Sum of Square	Mean Square	F Value	PR (F)
Types of saibo	2	8.76166	4.380830	50.73521	0
Residuals	143	12.34761	0.086347		

To determine the relative contributions of nucleus size and nacre thickness to pearl size, a correlation test was conducted on the transformed data. Using the categorisation of Fowler et al. (2004), the correlation between pearl size and nucleus size was 'weak' (r = 0.31, n = 146) while the correlation between size and nacre thickness was 'very strong' (r = 0. 95, n =146) (Fig. 6.11).

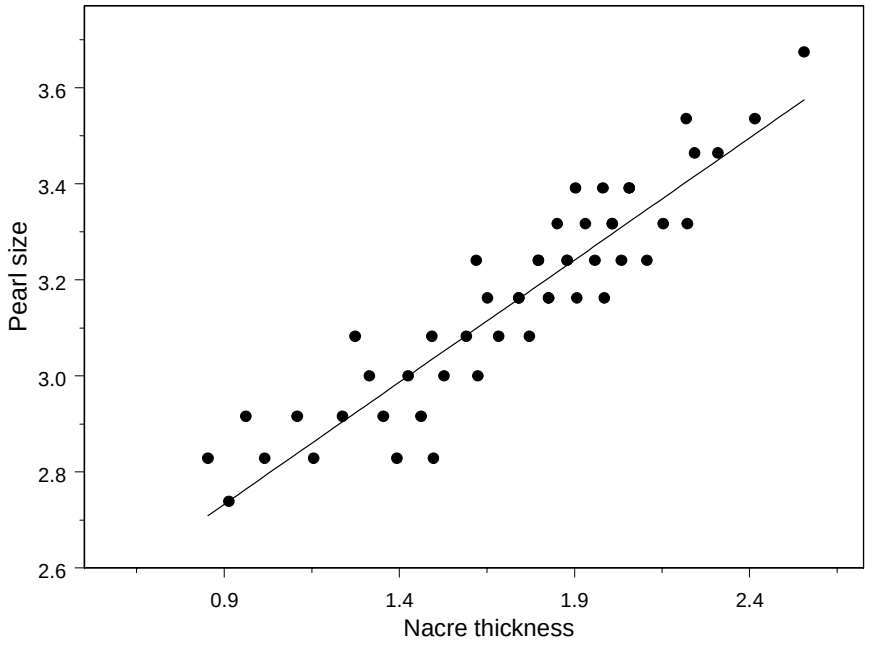

Fig. 6.11. Correlation between pearl size and nacre thickness (r = 0. 9460512) of round nucleated pearls from *Pinctada maxima*.

6.3.3.3 Pearl colour

White/silver colours (WW, WS, WC, WP, SS, SP; see Table 6.3) were dominant in pearls produced by oysters implanted with white/silver donor saibo of all types (relaxed, regenerated and normal saibo) (Fig. 6.12). This range of pearl colours made up 96% of total pearls produced by relaxed saibo and 83.1% and 84.2% of the total for regenerated and normal saibo, respectively (Fig. 6.12). Pearls with transition colours (PP, CC, CP and CF; see Table 6.3) were also produced by all types of saibo (4.2% for relaxed saibo, 10.8% for regenerated saibo and 15.8% for normal saibo). However, only regenerated saibo produced

pearls with various colours (FF and FL) which made up 6.1% of total pearls produced by regenerated saibo.

The frequency of the various pearl colours produced by yellow/gold oysters implanted with relaxed, regenerated and normal saibo from yellow/gold donors is shown in Fig. 6.13. There was much greater variability in pearl colour shown when compared to pearls produced by saibo from white/silver donors. White/silver colours (WW, WS, WC, WP, SS, SP; see Table 6.3) shared about the same percentage in both relaxed and regenerated saibo (53.1 and 52.6%, respectively) but the proportion of pearls in the yellow/gold colour range (YY, GL, GG, GF; see Table 6.3) was greater from relaxed saibo (22.5%) than from regenerated saibo (11%). However, regenerated saibo produced a higher proportion of pearls in both transition and various colour groups.

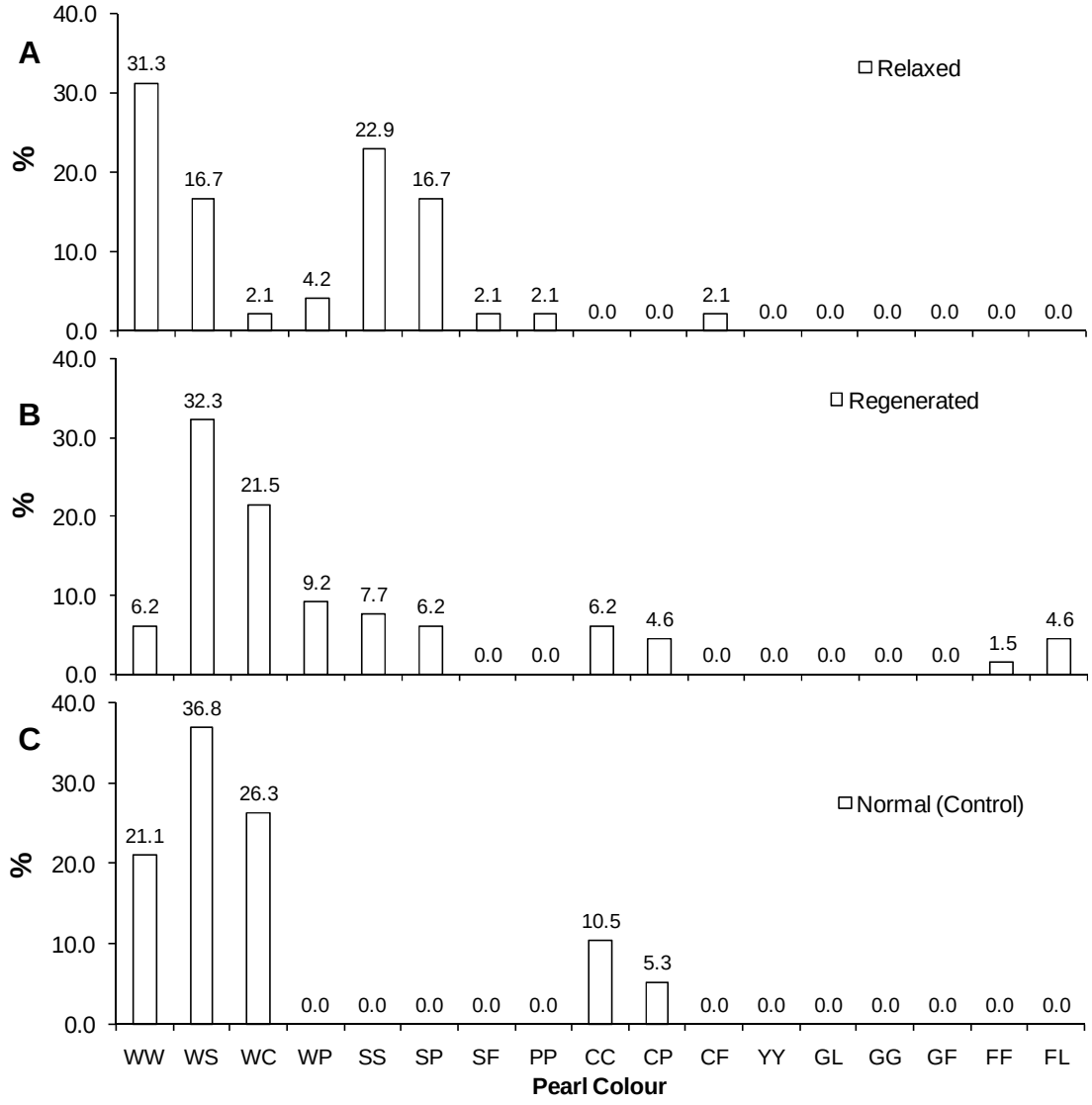

Fig 6.12. Percentage of pearls from *Pinctada maxima* implanted with white/silver saibo into white/silver oyster. A: implanted with relaxed saibo, B: implanted with regenerated saibo and C: implanted with normal saibo (control) (see Table 6.3 for colour categories and their abbreviations).

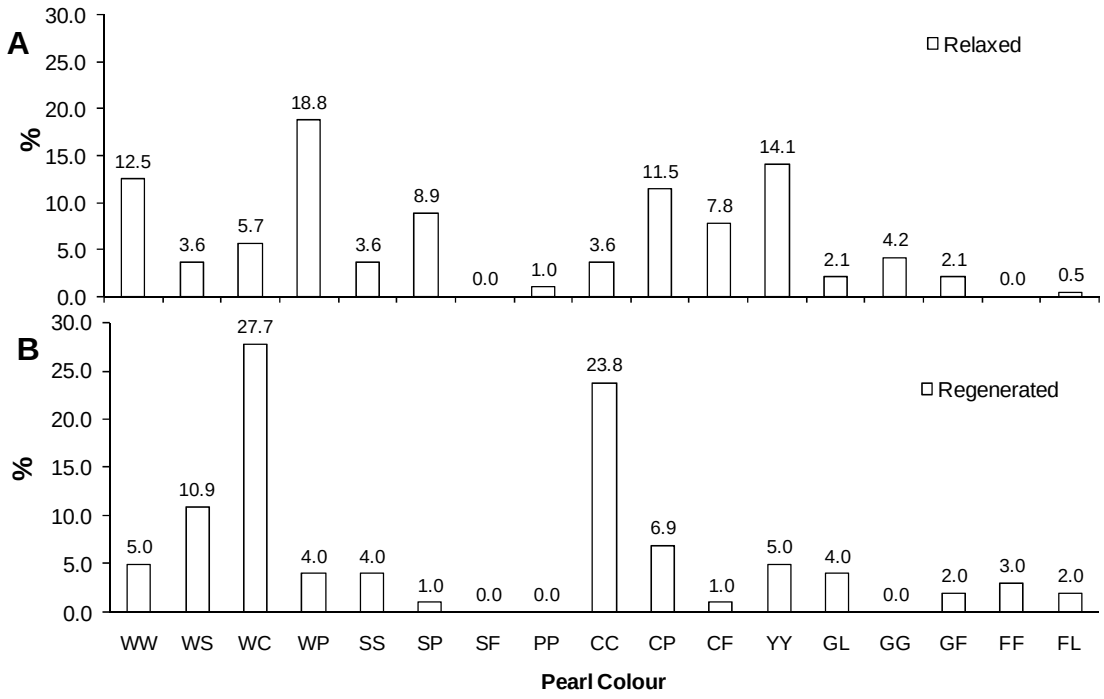

Fig 6.13. Percentage of pearls from *Pinctada maxima* implanted with yellow/gold saibo into yellow/gold oyster. A: implanted with relaxed saibo and B: implanted with regenerated saibo (see Table 6.3 for colour categories and their abbreviations).

6.3.3.4 Surface complexion

There was considerable variation in the proportion of pearls in each of the major categories of surface complexion shown in Table 6.4 (Fig. 6.14). Only 12.9% of the total number of pearls produced in this study were categorised within the A1 category (Fig. 6.14A) which are characterised by no blemishes or a small blemish that can be removed by drilling. Within the A1 category, pearls produced by relaxed, regenerated and normal saibo made up 11.7%, 15.1% and 10.5% of the total, respectively (Fig. 6.14B-D). Pearls in category B1 made up 13.9% of the total pearls produced, made up of 17.1%, 8.4% and 21.1% of the pearls produced by relaxed, regenerated and normal saibo, respectively. Most of pearls produced from both relaxed (45.4%) and normal (47.4%) saibo were graded within the B2 category

which is characterised by having three or more blemishes with at least a half sphere free of blemishes. Pearls in category B2 comprised 38.6% of the total number of pearls produced. Pearls in category C1, which have minor blemishes affecting 70% of the surface, made up 24.7% of the total number of pearls produced and included 33.1% of those produced by regenerated saibo. Pearls in category C2 with blemishes on the entire surface made up 9.9% of the total number of pearls produced and was composed of pearls produced by relaxed and regenerated saibo only (Fig. 6.14B,C). There was a highly significant effect of saibo type (relaxed and regenerated saibo only) on surface complexion of pearls produced (χ^2= 26.99977, p=0).

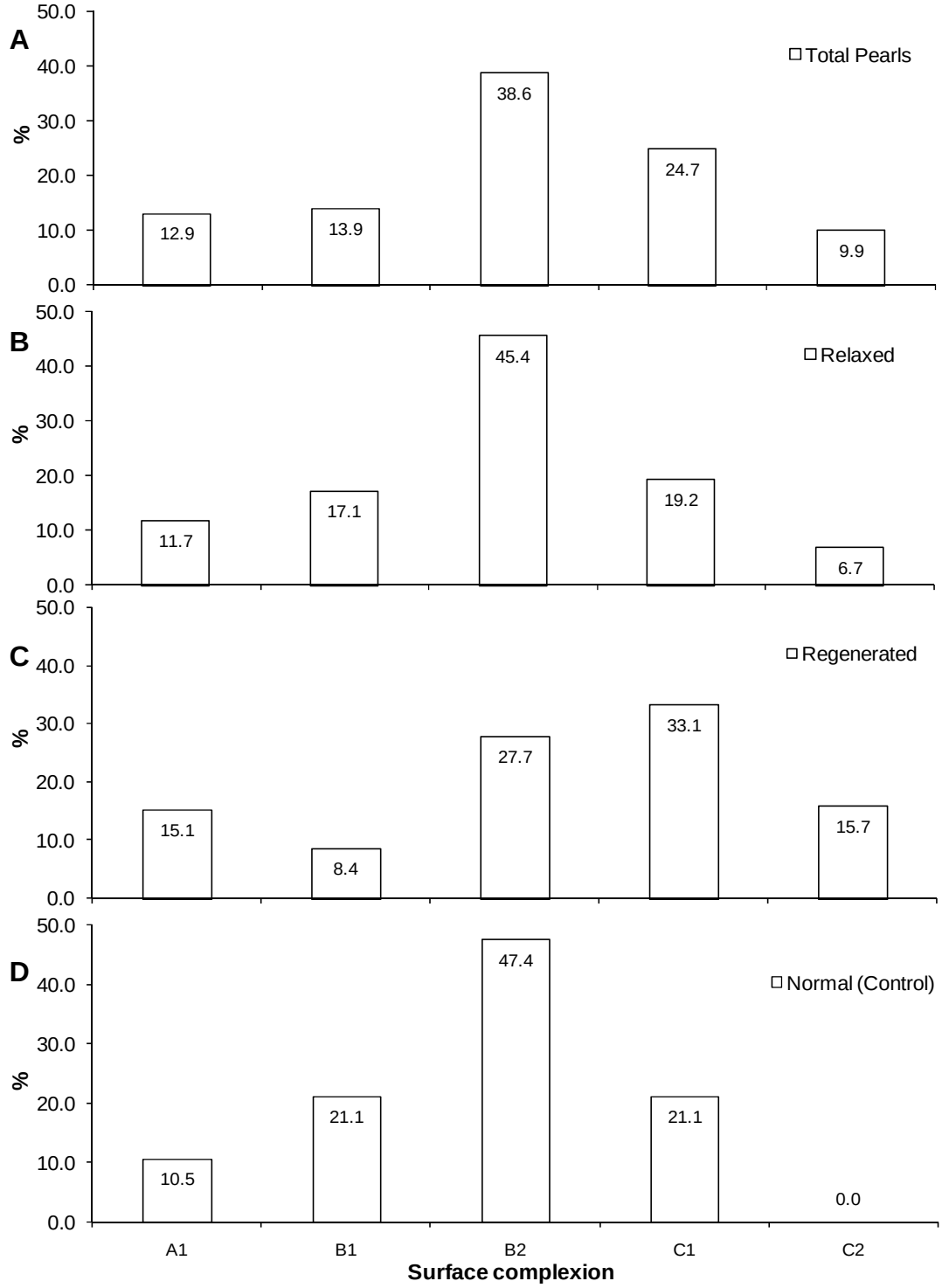

Fig. 6.14. Proportions of pearls within five surface complexion categories (see Table 6.4). A: total pearls (n = 425), B: pearls produced by relaxed saibo (n = 240), C: pearls produced by regenerated saibo (n = 166) and D: pearls produced by normal saibo (control)(n = 19).

6.3.3.5 Lustre

Only 10.6% of the pearls produced in this study were graded within the highest category for lustre (Fig. 6.15). Pearls produced by relaxed saibo made up the majority (14.2%) of these. The majority of the pearls produced from all treatments were placed into category 2 and were characterised as being bright pearls with a slightly blurred reflection. Pearls from regenerated saibo made up the majority of pearls in this category which contained 69.9% of pearls from regenerated saibo, 57.9% of pearls from normal saibo and 54.6% of those from relaxed saibo. Pearls in category 3 which have minimal but blurred inner reflection made up 30% of the total number of pearls produced and the major proportion of them (36.8%) was made up by pearls produced by normal saibo. Pearls in categories 1-3 made up 99% of the total pearls produced in this study (Fig. 6.15). The effect of saibo type (relaxed and regenerated only) on pearl lustre within categories 1-3 was very significant (χ^2= 10.07011, p=0.006).

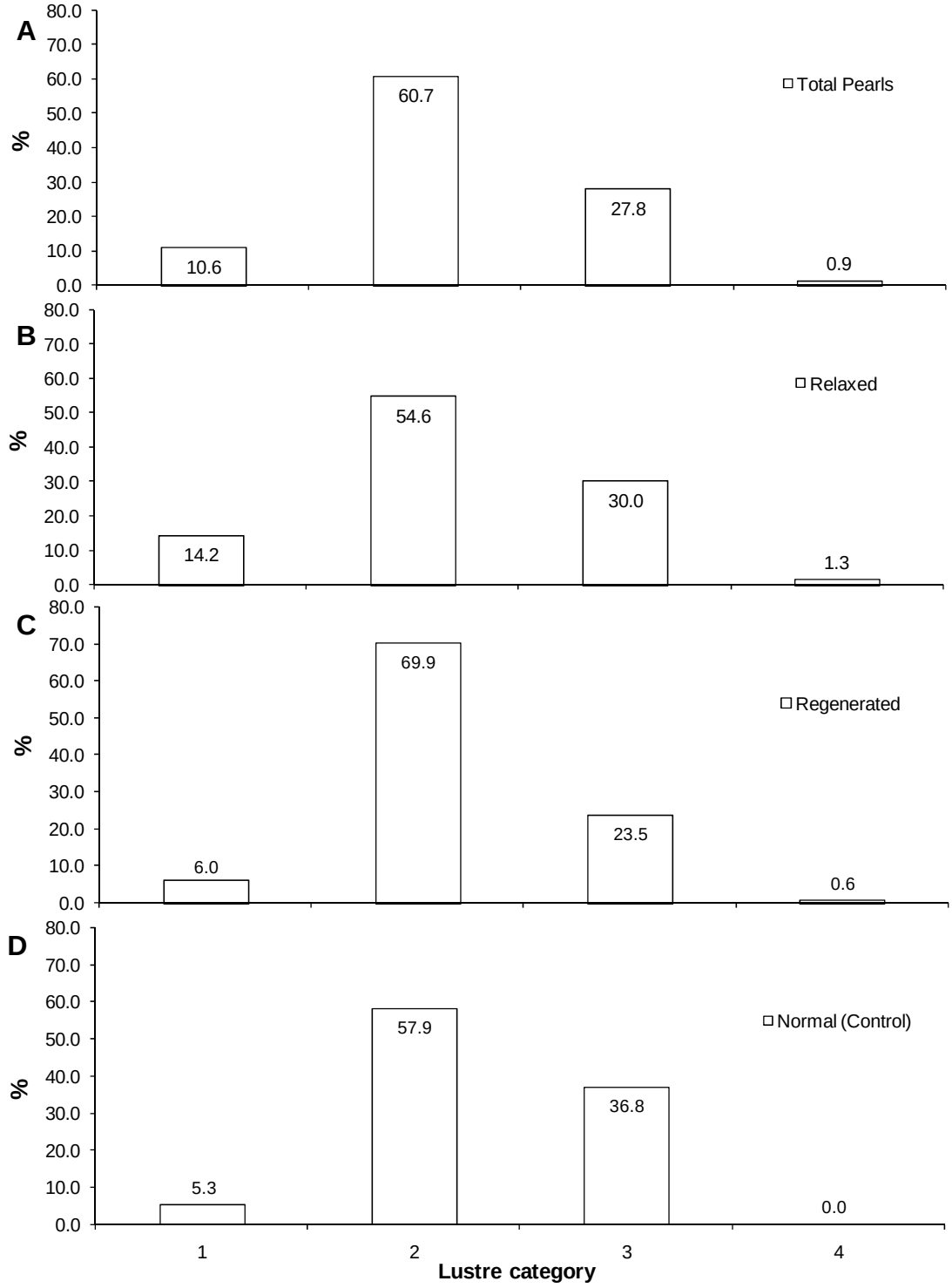

Fig 6.15. The proportion of pearls produced by *Pinctada maxima* based on lustre category (see Table 6.5). A: total pearls produced (n=425), B: pearls produced by relaxed saibo (n = 240), C: pearls produced by regenerated saibo (n = 166) and D: pearls produced by normal saibo (control, n = 19).

6.4 Discussion

Cultured pearl production requires sacrifice of high quality oysters to provide the donor saibo tissue that is implanted into recipient, pearl producing oysters (Taylor & Strack, 2008; Wada & Komaru, 1996). Acosta-Salmon et al. (2004) showed that saibo excision from anaesthetised *Pinctada margaritifera* could be undertaken without mortality and that the oysters could regenerate excised mantle tissue within 3 months of excision. They hypothesised that anaesthetising donor oysters for saibo excision, rather than killing them, provided potential benefits to the cultured pearl industry including the use of regenerated saibo for pearl implantation (Acosta-Salmon & Southgate, 2005; Acosta-Salmon et al., 2004). This hypothesis however is based on the assumptions that mantle tissue from anaesthetised oysters, and regenerated mantle tissue, perform well when compared to normal saibo. This study is the first to test these assumptions. Furthermore, this study is unique as it was conducted at a commercially relevant scale at a commercial pearl farm using standard commercial pearl culture methods.

The results of this study indicate that relaxed and/or regenerated saibo is as good as, or possibly better than, normal saibo for cultured pearl production. Based on the major criteria used to assess the performance of both oysters and pearls after implanting with relaxed, regenerated and normal mantle (Table 6.7), the results indicate that relaxed saibo from anaesthetised pearl oysters performed better than both regenerated and normal saibo.

The three types of saibo used in this study supported high oyster survival (90-95%) which is within the range normally associated with commercial pearl production of 90-98% (Taylor & Strack, 2008). The type of saibo used in this study did influence nucleus retention with greater retention resulting from the use of regenerated saibo compared to relaxed and normal

saibo (Table 6.7). However, nucleus retention in this study was relatively low (approx. 35%) compared to that of 70-90% achieved in commercial farms of *Pinctada maxima* (Taylor & Strack, 2008). The normal saibo treatment in this study showed particularly low retention. This was unexpected and perhaps indicates that technicians performances were affected by 'research conditions' which may have produced some nervousness during the operations, even though it was done as normally as possible, under identical conditions to those used by the same technicians during commercial pearl implanting operations. All implantations using normal saibo were conducted by the same technician, but the results show that the performance of the same technician in implanting other types of saibo (i.e. regenerated mantle) did not differ significantly from that of the other technicians. This particular technician therefore had no or little effect in the difference of nucleus retention among types of saibo.

When grading the pearls produced in this study using standard grading criteria, regenerated saibo was shown to produce more rejected (not acceptable for jewellery) pearls as well as lower quality pearls compared to those produced by relaxed saibo (Table 6.7). However, pearls produced by normal saibo showed a greater proportion of rejects than those resulting from regenerated saibo (Table 6.7). Most of the rejected pearls were covered with periostracum and were dark in colour indicating that the pearl-sac formed from the saibo could only produce the organic matrix, not nacre, as the base for mineral deposition (Addadi & Weiner, 1997). This may result from an inability of the pearl-sac to secrete nacre because secretory cells did not function properly nor did not exist. Regenerated mantle is morphologically identical to normal mantle tissue and is assumed to have equivalent secretory abilities (Acosta-Salmon & Southgate, 2005). However, production of nuclei coated with organic matrix only by oysters implanted with regenerated saibo indicates that some of

the regenerated mantle used in this study may not have achieved full functionality within the three months regeneration period prior to implantation. It may also indicate that a greater period is required for complete mantle regeneration or that regenerated mantle may not always achieve full functionality. Further indication that regenerated mantle may not achieve the same level of secretory function as non-regenerated mantle is shown by the relatively high proportion of pearl with poor surface complexion (category C1) and the relatively low nacre thickness of pearls produced by this mantle type. Both nacre thickness and pearl size of round nucleated pearls produced by regenerated saibo were the lowest among the three treatments (Table 6.7) with the average nacre thickness about half that of pearls resulting from relaxed saibo and three quarters that of pearls resulting from normal saibo.

Size is one of the major criteria when determining pearl values with large pearls having much greater value than small pearls (Matlins, 2002; Strack, 2006; Ward, 1995). Pearls produced by relaxed saibo from anaesthetised donor oysters made up the highest proportions of pearls in the medium and large size ranges (Table 6.7).

Table 6.7. Numerical ranking of the performance of oysters and pearls resulting from implantation of *Pinctada maxima* with three types of saibo (relaxed, regenerated and normal) against standard criteria where 1 = most favourable and 3 = least favourable.

Criteria		**Types of saibo**		
		Relaxed	**Regenerated**	**Normal**
Oyster survival		1	1	1
Nucleus retention		2	1	2
Less rejected pearls		1	2	3
Pearl shape (round preferred)		1	1	2
Pearl size (% larger pearls)		1	3	2
Pearl colour	White/Silver	1	1	1
	Gold	1	2	No data
Nacre thickness		1	3	2
Surface complexion		1	2	1
Pearl lustre		1	2	3
Total:		**11**	**18**	**17**

Of the other important criteria used in determining pearl quality (pearl shape, pearl colour, nacre thickness, surface complexity and lustre), pearls resulting from relaxed saibo ranked highest, when compared to those from regenerated and normal saibo (Table 6.7). Round pearls from relaxed and regenerated saibo made up almost half of the total pearls produced by each type of saibo but made up less than 30% of pearls produced by normal saibo.

‘Gold’ is the most valuable pearl colour but value decreases considerably when pearls have a ‘yellow’ colour. Silver pearl colour, however, is the second most valuable pearl colour in South Sea pearls. The colours of pearls resulting from this study, show that regardless of saibo type, saibo from white/silver nacre donor oysters produced pearls predominantly within the white/silver colour range (Table 6.7). However, both relaxed and regenerated saibo originating from yellow/gold nacre donor oysters differed in the colours of pearls that they produced. Relaxed saibo tended to produce more yellow/gold colour pearls than regenerated saibo from yellow/gold saibo donors (Table 6.7). This result is interesting as it indicates that pearl colour was influenced by the regeneration process; i.e., is not determined solely by the genetic make-up of the saibo. This aspect requires more research.

The surface complexion of pearls produced by regenerated saibo was the poorest of produced by the three types of saibo used in this study (Table 6.7). Most of the pearls produced by both relaxed and normal saibo were graded into the B2 category with three or more blemishes and at least a clean face visible. The majority of pearls produced by regenerated saibo however were graded into the C1 category with no clean face on the pearl surface or one to two large blemishes that affect 70% of the pearl surface.

Pearl lustre was also influenced by saibo type in this study. Relaxed saibo produced a greater proportion of pearls within category 1 with ‘brilliant’ lustre. However, for each type of saibo, more than half of the pearls were graded into category 2 with ‘excellent’ lustre, and regenerated saibo produced more pearls in this category than relaxed or normal saibo. Therefore, both relaxed and regenerated saibo have their potential in this regard (Table 6.7).

In conclusion, relaxed saibo performed considerably better than regenerated and normal (non-relaxed, non-regenerated) saibo in this study with regard to overall oyster performance and

pearl quality based on the criteria outlined in Table 6.7. The results confirm that relaxed mantle tissue can be used for pearl production with similar or better results than normal mantle. On this basis, pearl farmers could confidently consider anaesthesia of donor oysters prior to mantle excision so that they may be retained alive for future use. While one such use is subsequent saibo donation (Acosta-Salmon & Southgate, 2005; Chapter 3), the results of this experiment have shown that regenerated mantle tissue was inferior to normal saibo in supporting production of good quality pearls. Some of the potential shortcomings of regenerated mantle are discussed above and our results suggest that high quality donor oysters, in which mantle is allowed to regenerate, should probably not be used as multiple saibo donors.

Prior studies have considered potential practical difficulties in applying anaesthetics for pearl production at a large scale (Acosta-Salmon et al., 2005; Norton et al., 1996). However, much of this discussion has related to difficulties in anaesthetising the larger numbers of oysters used as nucleus recipients. Much smaller numbers of donor oysters are required because each may produce sufficient saibo to implant around 20-30 recipient oysters (Taylor & Strack, 2008). Anaesthetising donor oysters prior to mantle excision could probably be accommodated with relatively minor changes to saibo preparation practices. However, timing may be a crucial aspect of this process because over-exposure to anaesthetics can adversely affect the response of oyster tissues (Chapter 2) and may affect the subsequent function of relaxed saibo. The results of this experiment show clearly that there can be positive benefits to pearl quality from using relaxed saibo for pearl production. The reasons for this require further study.

Chapter 7

Assessing pearl quality using Reflectance UV-Vis Spectroscopy: does the same donor produce similar pearl quality?

7.1 Introduction

Pearls resemble the inner part of the nacreous molluscan shell (Dall, 1883). Nacre is composed of calcium carbonate crystallized in the form of aragonite platelets on an organic matrix scaffold of conchiolin (Gre´goire, 1957; Kobayashi & Samata, 2006; Simkiss & Wilbur, 1989; Weiner et al., 1984). Conchiolin is a dark-colored organic protein secreted during the initial phases of pearl formation. Generally it is the first layer deposited by the pearl-sac. The quality of nacre composing the pearl is generally assumed to resemble that of the donor oyster, and donor oysters are chosen primarily on this basis (Wada & Komaru, 1996). Therefore, good donor selection is assumed to contribute to good quality of pearls (Taylor, 2002). However, no prior study has compared the quality of pearls resulting from the same donor.

Pearl quality is determined by five primary factors: lustre, colour, shape, surface contour and size (Matlins, 2002; Southgate et al., 2008; Strack, 2006; Ward, 1995). Many of these characteristics are subjective and may depend on individual perceptions (Ward, 1995). Pearl grading is also labour intensive and requires skill and experience. A pearl grader has to be able to quantify and collate all visual observations and allocate each pearl to a defined grading level. However, techniques are available that may assist in reducing subjectivity in assessing some aspects of pearl quality. For example, UV-visible (UV-Vis) spectrophotometry utilising a diffuse reflectance accessory can be used to assess material

properties based on reflected light. This technique has been extensively applied in a wide-range of chemical analyses of gaseous, liquid and solid materials (Agatonovic-Kustrin et al., 1999; Burger et al., 1997; Huang et al., 2003; Ito et al., 2008; Kemper & Sommer, 2002). As UV-Vis light passes through, or is reflected by a material, specific groups of wavelengths are absorbed and the remaining light is reflected and interpreted by the eye as colour. Based on light wavelengths detected within the ultraviolet and eye-visible regions, UV-Vis spectroscopy can rapidly identify chemical properties and colour pigments in a non-destructive way. Because pearl colour and overtone is determined by the way in which light is reflected through the various outer layers of nacre, this technique has obvious potential application in quantification of pearl colour. UV-visible (UV-Vis) spectrophotometry has been used to record the reflectance of pearls from *Pinctada maxima* and *P. margaritifera* (Elen, 2001; Elen, 2002) as well as freshwater pearls (Karampelas, 2009). These studies showed that pearl colour could be clearly characterised by peaks in reflectance spectra which are correlated with the presence of particular pigments in the nacre layers (Elen, 2001; Elen, 2002).

The research described in this Chapter used UV-visible (UV-Vis) spectrophotometry, utilising a diffuse reflectance accessory, to analyse some of the pearls produced by *Pinctada maxima* earlier in this study (Chapter 6). Of particular interest was a comparison between pearls from the same mantle donors and several individual pearls in various colours (from white/silver to gold) and overtones.

7.2 Material and methods

Eight pearls from *Pinctada maxima* (seven ‘acceptable’ pearls and one ‘rejected’ pearl) ranging from white to gold in colour together with one white pearl nucleus were used in this

study (Fig. 7.1, Table 7.1). ‘Acceptable pearls’ are pearls used for jewellery purposes while ‘rejected pearls’ are not; they have no commercial value. The rejected pearl used in this study was mainly covered with periostracum not nacre. The studied pearls (Fig. 7.1) were harvested from a commercial pearl farm in Bali, Indonesia and graded using the South Sea Pearl Grading System developed and used by Atlas Pacific, Ltd and detailed in Chapter 6 (Tables 6.2, 6.3, 6.4, 6.5). Pearl samples with labels RDG 5-3, 5-4 and 5-6 as well as RDG 13-2 and 13-6 resulted from the same saibo donor while two other pearls were from different donors (RD 2-2, RD 7-7). All pearls were produced by oysters implanted with relaxed siabo (Chapter 6). No treatment was conducted on the pearls to enhance colour or lustre after harvest with the exception that they were cleaned with a soft cloth. Nacre thickness was also recorded by subtracting the nucleus diameter from the pearl diameter.

Because of the opaque nature of pearls, spectroscopy measurements were performed using diffuse reflectance techniques; Diffuse Reflectance UV-Visible spectroscopy. The reflectance UV-visible spectra were collected using a Cary 50 UV-Vis spectrophotometer equipped with an external remote Diffuse Reflectance Accessory (DRA) probe (Barrelino^{TM}, Harrick Scientific) that can scan an area less than 1.5 mm in diameter. UV-Vis spectra of pearls were acquired in the region 200-800 nm using appropriate baseline correction at approximately 100%. The UV-Vis scan rate was 9600 nm min^{-1}. Prior to scanning, white level was calibrated with Wavelength Reflectance Standard (Labsphere®) in which approximately 100% reflectance across the entire spectrum is designated as white reference standard. The spectra were acquired at two different locations on each sample to assess surface homogeneity. To conduct the scanning, each sample was put on a stand and scanned with the DRA probe connected to the spectrophotometer. The spectral data were then analysed and the graphs were smoothed to reduce the noise using the Moving Average method by counting the

average of twenty data sets in every fifth wavelength record (Fuller, 1996). The analysis was based mainly on the visible region of the graph (380-750 nm).

Fig. 7.1. Pearls from *Pinctada maxima* analysed in this study. Above (left to right): RD 2-2, RD 7-7, RDG 5-3, and RDG 5-4; bottom (left to right): RDG 5-6, RDG 12-6, RDG 13-2, and RDG 13-6. Five pearls were from two donors, RDG 5-3, RDG 5-4 and RDG 5-6 were from the first donor and RDG 13-2 and RDG 13-6 were from the second donor.

Table 7.1. Characteristics of tested South Sea pearls produced by *Pinctada maxima* implanted with relaxed saibo.

Code	Pearl colour	Pearl lustre[1]	Pearl Size (mm)	Nacre thickness (mm)	Donor colour[2]	Recipient colour[3]
RD2-2	White	3	10.5	3.2	White	White
RD7-7	White	3	9.5	2.8	White	Yellow
RDG5-3	Cream, various overtone	2	10	3.6	Yellow	Yellow
RDG5-4	White, pink overtone	2	8.5	2.7	Yellow	Yellow
RDG5-6	Gold	2	10.5	4.4	Yellow	Yellow
RDG12-6	Rejected pearl (brown to dark brown)	-	-	-	White	Yellow
RDG13-2	White with cream overtone	3	10.5	3.5	Yellow	Yellow
RDG13-6	Cream, various overtone	1	5	Keshi [4]	Yellow	Yellow

[1]Pearl lustre grading factor: 1=mirror reflection lustre, 2=somewhat mirror reflection, 3=chalky appearance
[2]Yellow consists of yellow to gold, and white as white or silver
[3]Yellow consists of yellow to gold, and white as white or silver
[4]A cultured pearl without nucleus, mostly nacreous.

7.3 Results

Spectral analysis of pearls originating from two donors, represented by the upper and lower curves in Fig. 7.2, respectively, showed the same reflectance peak recorded in the UV region at 260 nm, followed by absorptions (converse of reflectance peaks) ranging from 270 to 280 nm, then a second peak in the region of 320 to 340 nm. The upper curve in Fig. 7.2 showed variation of spectra of three different pearls from the same gold donor. Cream and gold coloured pearls (RDG 5-3 and RDG 5-6) showed a wide absorption from 320 to about 460 nm while it was just a slight reflectance around 400 nm in white pearls with pink overtone (RDG 5-4). The cream and gold pearls (RDG 5-3 and RDG 5-6) reached a reflectance peak at 560 to 590 nm while the white pearl with pink overtone had a slightly wider absorption in the area (RDG 5-4). Both cream and gold pearls showed an absorption peak after the reflectance peak; at about 700 nm for the cream pearl and 750 nm for the gold pearl. The bottom graph of Fig. 7.2 shows absorption spectra of two pearls resulting from another gold donor. These spectra are similar but differ in their intensity, the slight absorption after 330 nm and in the slight reflectance peak at around 600 nm for the cream pearl with various overtones (RDG 13-6).

The spectra of two white pearls resulting from different silver nacre donors (RD 2-2 and RD 7-7), a rejected pearl (mostly covered with periostracum) and a pearl nucleus are shown in Fig. 7.3. The spectra of the two white pearls showed a reflectance at 260 nm, followed by absorption at 280 nm and another reflectance peak at 340 nm. After this peak the spectra for these pearls remained flat until a slight absorption peak around 700

nm. Spectral data recorded for the ‘rejected pearl’ showed that the reflectance was not obvious in the UV area (<380 nm) (Fig. 7.3). After sloping down to 330 nm, the spectral line rose steadily, passing through the visible region until reaching a maximum at 780 nm. It showed a wide range of absorption. The spectrum of the pearl nucleus showed a slight reflectance peak at 260 nm with a slight absorption in the UV region (Fig. 7.3). The spectral line for the pearl nucleus increased gradually up to a wavelength of 530 nm then decreased only very slightly passing through the visible region.

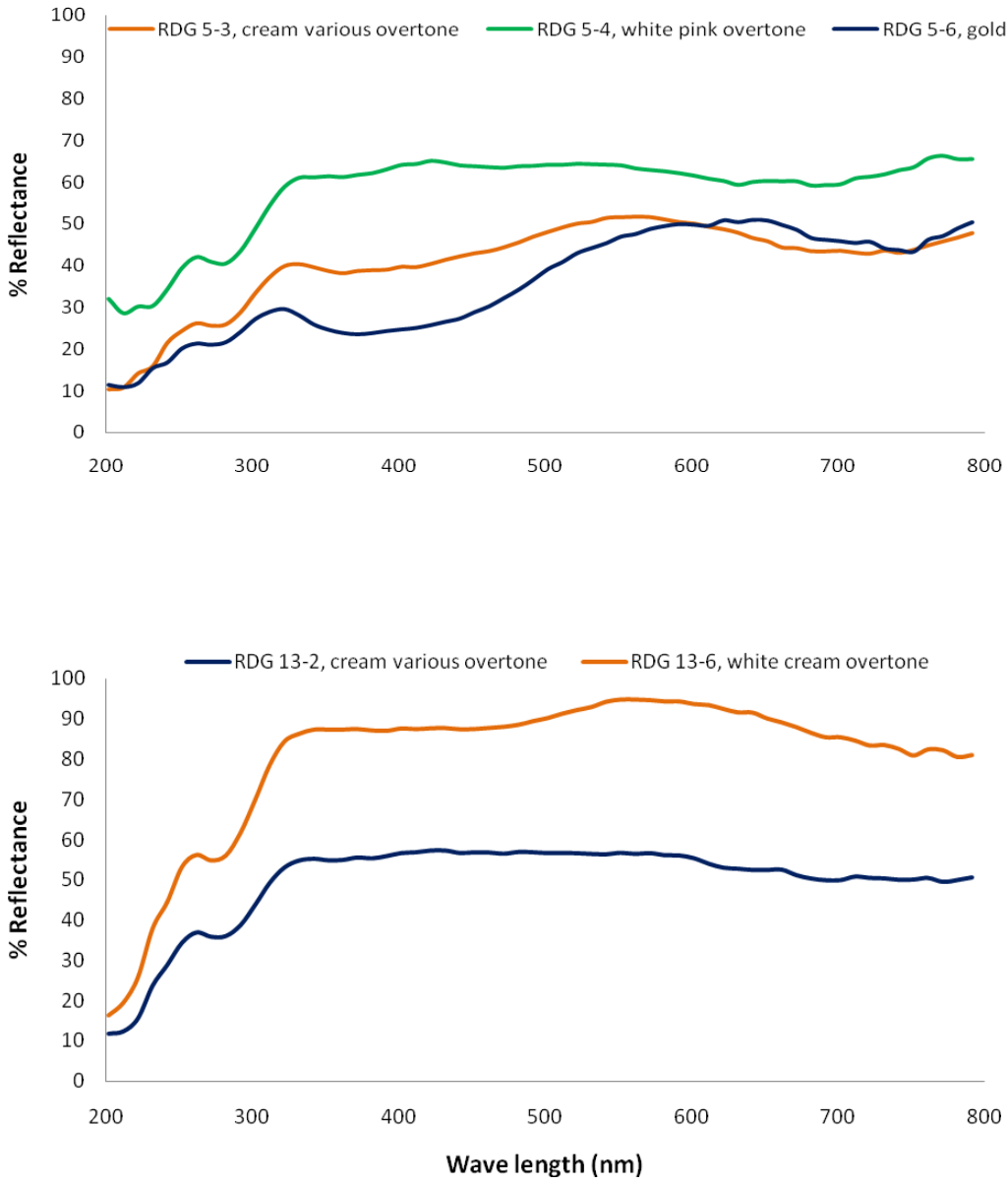

Fig. 7.2. UV-Vis spectral data (reflectance) of three pearls from the same gold donor (top) and two pearls from another gold donor (bottom) from *Pinctada maxima*.

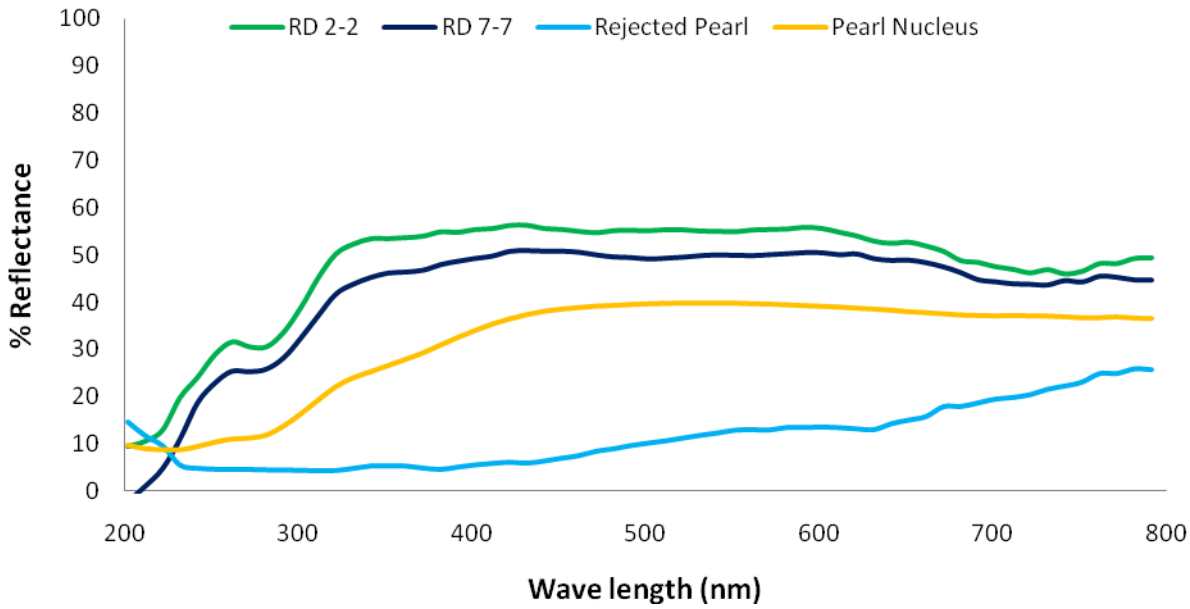

Fig. 7.3. UV-Vis spectral data (reflectance) of two white pearls from different silver donors and a rejected pearl from *Pinctada maxima*, and a pearl nucleus.

7.4 Discussion

UV-Vis spectral analysis of pearls has been shown to have considerable potential in characterising pearl colour. For example, yellow-coloured pearls and yellow shell nacre from *P. maxima* showed characteristic maximum absorption between 330 and 385 nm while 'heat-treated' yellow pearls from *P. maxima* did not (Elen, 2001) allowing treated pearls to be distinguished from natural pearls of similar colour. Similarly, vis spectrophotometry was also used to identify the presence of chromatophores which show characteristic absorption at 700 nm and provide natural colour to Tahitian black

pearls from *P. margaritifera* (Komatsu & Akamatsu, 1978). Absorption at 700 nm is not shown by *P. margaritifera* pearls that are treated to assume similar dark colour (Elen, 2002) which again provides a means to distinguish between naturally coloured and treated pearls. UV-Vis absorption spectra can also be used to distinguish between the cultured pearls of *P. margaritifera* and *P. maxima* (Elen, 2002). This study is the first to compare pearls from the same donor of *P. maxima*. It shows similar maximum absorption of yellow and gold pearls at around 330-385 nm to those recorded by Elen (2001). The yellow and gold pearls from the same donor (RDG 5-3 and RDG 5-6) in this study also show similar absorption spectra. The maximum absorption at 330-385 nm is the peak of the wide absorption of 320-460 nm recorded in yellow and gold pearls in this study. Elen (2001) reported an absorption similarity in the area of 320-460 nm between yellow shell nacre and yellow/gold pearls. These absorption wavelengths also characterise the differences between yellow shell nacre and white shell nacre from *P. maxima*. Yellow shell nacre showed high absorption between 330 – 460 nm which was not evident in white shell nacre (Elen, 2001). The result of study also confirms that there is a wide absorption in the same area (330-460 nm) recorded in yellow/gold pearls but no absorption in white pearls. Therefore it can be concluded that the area between 330-460 nm characterises yellow-gold pearls from *P. maxima.*

One similarity shown by all nacre covered pearls analysed in this study (i.e., except the rejected pearl) was the same reflectance peak at 260 nm as well as absorptions from 270 to 280 nm in the UV region. This characteristic appeared on absorption at this range from the studied pearls is a typical spectral curve that was also recorded in other pearls. A recent study conducted with diffuse reflectance UV-Vis-NIR showed that the

absorption in the UV region of freshwater pearls collected from two freshwater pearl mussel species (*Hyriopsis cumingi* and *H. schlegeli*) was at 280 nm (Karampelas et al., 2009). However, the spectral curve recorded from both the rejected pearl and the pearl nucleus in this study does not comply with the result from the pearls. There was wide absorption across the whole UV and visible ranges by the rejected pearl but just a small absorption by the pearl nucleus. This may indicate that the nacreous part of pearls is primarily responsible for absorption in the UV region. Based on the amount of absorption shown by the rejected pearl, it is likely that the amount of organic matrix plays an important role in the variation of spectral formation in the UV region. The amount of matrix protein composing the surface of the rejected pearl created the wide absorption in both UV and visible regions. The pronounced absorption recorded in the UV region in the pearls from this study, although not in the wide area as in the rejected pearl, indicates that the amount of organic matrix in the nacre of those pearls is lower than in the rejected pearl. Furthermore, a similar reflectance and absorbance in the UV region recorded for the nucleus, although it is just a little, may indicate a trace of organic matrix.

Throughout the visible region, all white pearls used in this study showed similar reflectance spectra although there were differences in reflectance intensity. Unlike the spectral results from those white pearls, the results from yellow and gold pearls varied according to colour saturation of the pearl. A weak absorption in the violet region of the visible part marks the spectra of both RDG 5-3 and RDG 13-6. It is followed with a rather strong reflectance of yellow-green colour at around 560 to 590 nm. These spectral characteristics confirm the existence of yellow colour in the pearls used in this study.

Unlike the yellow pearls, strong absorption in the violet region characterised the gold pearl (RDG 5-6). This was followed with a strong reflectance of yellowish orange colour which is visible to the eye. A similar result was also recorded by Elen (2001) when he compared the reflectance of natural-colour and heat-treated golden cultured pearls that he assumed were from *P. maxima*. He reported broad absorption in the violet region for both natural and treated pearls with yellow and golden colours but not for the white pearls. The absorptions were, however, lower in treated pearls. The same range of absorption was also recorded on the inner shell of the yellow colour area (lip) of *P. maxima* (Elen, 2001).

The colour of pearls may come from either the structure of the nacre or the pigments lying in the nacre (Snow et al., 2004; Karampelas et al., 2007; Karampelas et al., 2009). In the study conducted by Snow et al. (2004) with pearls from *Pinctada maxima*, the colour variations seen in pearls from *Pinctada maxima* were shown to result not only from pigments but also as a result of their structure. They reported that white and gold colours were influenced by differences in the thickness of the edge-band (matrix between the platelets). They stated that white pearls had a 70 nm edge-band thickness while the edge-band of gold pearls can be up to 90 nm. They also reported that colour saturation is determined by the uniformity of nacre structure; the more uniform it is then the more saturate the colour. This colour appearance has also been detected through UV-Vis reflectance in this study. The more saturated pearl colour is indicated by a big variation in wavelengths such as that shown by the gold pearl. Other pearls, i.e., yellow pearls or even white pearls, however, showed small variation in the wavelengths.

The results of this study show that similarities between absorption and reflectance spectra of cultured pearls resulting from the same saibo donor are negligible and could not be detected with UV Vis spectrophotometry. Although such pearls showed the same absorbance at 280 nm in the UV region, this characteristic was also seen in other pearls tested. Furthermore, this study cannot confirm similarities of certain reflectance intensity characteristics in pearls seeded with saibo from the same donor since there were different reflectance wavelengths and intensities among pearls originating from the same saibo donor. However, this is only one part of the equation; further study is needed to compare the structural and elemental composition of donor shell nacre and the nacre of pearls resulting from saibo from that donor, before firmer conclusions can be made.

UV Vis spectrophotometry has proven to be a useful tool in prior studies which have used this technique to distinguish between naturally coloured and treated cultured pearls and to determine absorption spectra characteristics associated with particular pearl colours (Elen, 2001; Elen, 2002). While the present study could not identify spectra characteristics of pearls resulting from the same saibo origin, the data indicate that UV Vis spectrophotometry may also have value in quantifying another pearl 'virtue' – lustre. Two pearls used in this study originated from the same saibo donor (RDG 13-2 and RDG 13-6) and showed very similar absorption spectra. They differed however in their lustre and this is clearly shown in the intensity of reflectance recorded for each pearl. RDG 13-2 was the only pearl in this study with the highest lustre grading and it showed considerably greater reflectance than all other pearls.

This study assessed a non-qualitative method for determining the characteristics of pearl colour. UV-Vis spectroscopy is non-destructive and can be used to determine different pearl colours, pearl origin, colour enhancements and treatment, and as a quantitative measure of pearl lustre. This study is the first conducted with a record of the origin of the pearls used, as well as the first to compare pearls produced by saibo resulting from the same donor. This technique could become increasingly important as the pearling industry seeks to develop less subjective methods of assessing pearl quality (grading), it may also become a valuable tool in further studies of the relationships between pearl quality and that of the donor and recipient oysters.

Chapter 8

General Discussion and Conclusions

8.1 Background to the study

The method used for cultured round pearl production was developed in the 1920's (Taylor & Strack, 2008) and has changed little since. It utilises mantle tissue grafts from donor oysters ('saibo') which are killed. The quality of resulting pearls is highly influenced by the nacre quality of the donor (Taylor, 2002) and, because of this, a pearl farm's best oysters are sacrificed for pearl production and cannot be used as broodstock to improve stock quality.

My research was based on the results of a prior study in this laboratory with *Pinctada margaritifera* and *P. fucata* which showed that: (1) saibo tissue can be removed from donor pearl oysters for cultured pearl production without killing them using anaesthetics; and (2) excised mantle tissue can regenerate within three months and appeared to be fully functional (Acosta-Salmon & Southgate, 2005). These findings offered promising potential benefits for the cultured pearl industry: (1) donor oysters producing high quality pearls could be identified and used as potential breeding stock; and (2) high quality donors could potentially be used as saibo donors on more than one occasion.

The results of this novel research were preliminary and these potential benefits can only be realised if the mantle tissue of anaesthetised pearl oysters and regenerated mantle

tissue perform in a similar manner to ‘normal’ mantle when used as saibo for cultured pearl production. Assessing this new approach and testing of the above assumptions was the basis of this study. It was conducted with *P. maxima* which supports the largest sector of the marine cultured pearl industry with around 46% of production by volume and value (Southgate et al., 2008).

8.2 Major findings of this study

The major findings of this study are:

1. *Pinctada maxima* can be anaesthetised to facilitate mantle excision without mortality.

2. The site of mantle excision heals rapidly and regeneration of excised mantle is complete within 3 months, apparently with full nacre secreting ability.

3. When used as saibo, relaxed mantle tissue and regenerated mantle tissue are able to proliferate to form a pearl-sac with a structure similar to that formed by normal (non-relaxed and non-regenerated) saibo, and with similar secretory function.

4. Relaxed mantle tissue and regenerated mantle tissue can be used successfully as saibo for culture pearl production.

    - Relaxed mantle tissue produced pearls of higher quality than those produced by normal mantle tissue.

- Regenerated mantle tissue produced pearls of inferior quality to normal mantle and results indicate that this may result from incomplete secretory function of regenerated mantle

5. Similarities between absorption and reflectance spectra of cultured pearls resulting from the same saibo donor are negligible and could not be detected using UV-Vis spectrophotometry.

8.3 Implications of these findings

8.3.1 Economic benefits

Once pearls are graded according to the commercial criteria described in Chapter 6, their individual values are calculated using a 'pricing matrix' which derives a single dollar value on the basis of the five grading factors (shape, size, surface complexion, colour and lustre) described in detail in Chapter 6. The 'retail price matrix' used by the commercial partner in this study (Atlas Pacific Ltd.) was made available to me for the purpose of estimating values of the pearls produced in this study (Chapter 6). For commercial reasons, however, the price matrix was not available for reproduction in this thesis. The collective and average values of the pearls produced in Chapter 6 of this study from relaxed, regenerated and normal saibo were calculated using the 'retail price matrix' and are shown in Table 8.1. The 171 pearls produced by relaxed saibo had a collective value of US$40,019.82 and an average value of US$234.0. This average value is almost three-times higher than the average value of pearls produced by regenerated and normal saibo of around US$86. Based on these results, there is very clear incentive to pearl farmers to use relaxed saibo for cultured pearl production.

Table 8.1 Total and average values of pearls produced in this study using relaxed, regenerated and normal saibo calculated using the Atlas Pacific Ltd 'retail price matrix' based on the grading criteria outlined in Chapter 6.

Type of saibo	Number of pearls	Value (US$)*
Relaxed	171	40,019.82 (234.0)
Regenerated	108	9,317.9 (86.3)
Normal	12	1,033.3 (86.1)

*Average value per pearl in parentheses

8.3.2 Modification of pearl implanting methods

This study has shown that the use of mantle tissue from anaesthetised pearl oysters as saibo for pearl production resulted in a greater pearl yield and pearls of generally higher quality when compared to the use of normal mantle as saibo. In contrast, regenerated mantle produced inferior results to normal mantle when used as saibo. These results have major implications for the pearling industry and indicate that minor changes to the pearl seeding process (i.e., use of relaxed mantle as saibo) could bring about improvements in pearl yield and pearl quality. They provide a basis for modification of current cultured pearl production practices.

As outlined in section 1.4.2, cultured pearl production is currently based on identification of oysters with high quality nacre which are sacrificed to provide saibo. Suggested modifications to this method, based on the results of this study, are shown in Fig. 8.1. Briefly, high quality oysters are still identified for saibo production but, instead of being killed, are anaesthetised prior to mantle excision. Excised mantle is used as saibo for pearl production and the anaesthetised donor oysters are retained as future broodstock. They are not used again for saibo donation.

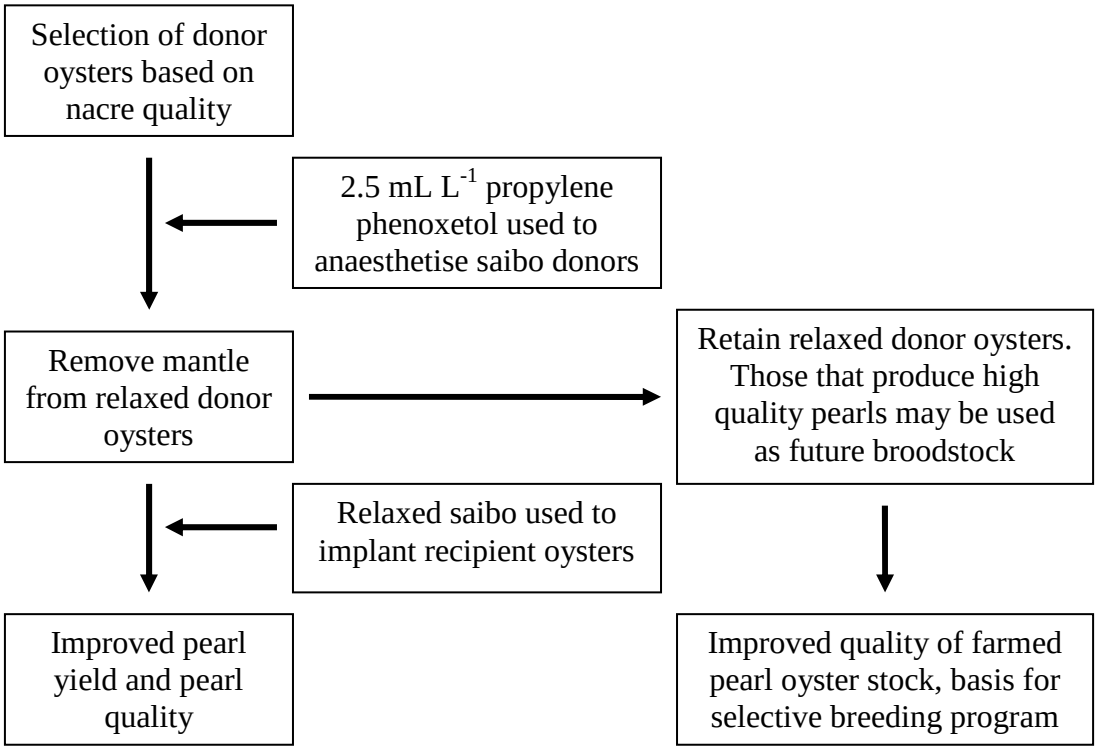

Fig. 8.1 Proposed modifications to the methods for cultured pearl production based on the results of this study.

In summary, the results of this thesis have major implications for the pearling industry and indicate that minor changes to the pearl seeding process (i.e., use of relaxed mantle as saibo) could bring about beneficial improvements to pearl yield and pearl quality and allow greater use of perhaps the most important resource of commercial pearl farms – high quality donor oysters. Some of these results have already been taken-up by the commercial collaborator in this research and used to modify culture practices. The past few years have seen considerable research effort directed towards pearl oyster biology, development and improvement of husbandry practices and a greater understanding of the mechanisms of pearl or nacre production and factors affecting this. The research described in this thesis is part of this effort and will hopefully provide a basis for further evolution of the pearling industry and sustainable development of the industry into the future.

References

Acosta-Salmon, H., Southgate, P.C., 2005. Mantle regeneration in the pearl oysters *Pinctada fucata* and *Pinctada margaritifera*. Aquaculture 246, 447-453.

Acosta-Salmon, H., Southgate, P.C., 2006. Wound healing after excision of mantle tissue from the Akoya pearl oyster, *Pinctada fucata*. Comp. Biochem. Physiol., A 143, 264–268.

Acosta-Salmon, H., Davis, M., 2007. Inducing relaxation in the queen conch *Strombus gigas* (L.) for cultured pearl production. Aquaculture 262, 73-77.

Acosta-Salmon, H., Martinez-Fernandez, 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.

Acosta-Salmon, H., Martinez-Fernandez, E., Southgate, P.C., 2005. Use of relaxants to obtain saibo tissue from the blacklip pearl oyster (*Pinctada margaritifera*) and the Akoya pearl oyster (*Pinctada fucata*). Aquaculture 246, 167-172.

Acosta-Salmón, H., 2004. Broodstock management and egg quality of the pearl oysters *Pinctada margaritifera* and *Pinctada fucata.* PhD Thesis, James Cook University. Townsville, Australia. 142 pp.

Addadi, L., Weiner, S., 1997. Biomineralization - A pavement of pearl. Nature 389, 912.

Agatonovic-Kustrin, S., Tucker, I.G., Schmierer, D., 1999. Solid state assay of ranitidine HCl as a bulk drug and as active ingredient in tablets using DRIFT spectroscopy with artificial neural networks. Pharmaceutical Research 16, 1477-1482.

Alagarswami, K., 1976. Results of multiple implantation of nuclei in production of cultured pearls. Indian J. Fish. 21, 601-604.

Alvarado, A.S., 2000. Regeneration in the metazoans: why does it happen? Bioessays 22, 578-590.

Alvarado, A.S., 2004. Regeneration and the need for simpler model organsims. Philos Trans R Soc Lond B Biol Sci 359, 759-763.

Alvarado, A.S., 2006. Planarian regeneration: Its end is its beginning. Cell 124, 241-245.

Anon, 2007a. The pearl book: natural, cultured & imitation pearls — terminology & classification. CIBJO/GEM materials, Pearl Comission. CIBJO, The World Jewellery Confederation, Milano, Italy. 47 pp.

Anon, 2007b. Pearl market expands. Modern Jeweler 106, 20.

Aquilina, B., Roberts, R., 2000. A method for inducing muscle relaxation in the abalone, *Haliotis iris*. Aquaculture 190, 403-408.

Araujo, R., Remon, J.M., Moreno, D., Ramos, M.A., 1995. Relaxing techniques for fresh-water mollusks - trials for evaluation of different methods. Malacologia 36, 29-41.

Armstrong, D.A., 1971. Experimental wound repair in the black abalone, *Haliotis cracherodii*. J Invertebr Pathol 17, 216-227.

Awaji, M., Suzuki, T., 1995. The pattern of cell proliferation during pearl sac formation in the pearl oyster. Fish Sci 61, 747-751.

Barthelat, F., Espinosa, H.D., 2007. An experimental investigation of deformation and fracture of nacre-mother of pearl. Experimental Mechanics 47, 311-324.
Bauer, G., 1988. Threats to the freshwater pearl mussel *Margaritifera margaritifera* L. in Central Europe. Biol. Conserv. 45, 239-253.
Beasley, C.R., Roberts, D., 1996. The current distribution and status of the freshwater pearl mussel *Margaritifera margaritifera* L. 1758 in north-west Ireland. Aquat. Conserv.: Mar. Freshwat. Ecosyst. 6, 169-177.
Behzadi, S., Parivar, K., Roustaian, P., 1997. Gonadal cycle of pearl oyster, *Pinctada fucata* (Gould) in Northeast Persian Gulf, Iran. J. Shellfish Res. 16, 129-135.
Bekkum, D.W.V., 2004. Phylogenetic aspects of tissue regeneration: role of stem cells, a concise overview. Blood Cells Mol Dis 32, 11-16.
Bely, A.E., 2006. Distribution of segment regeneration ability in the Annelida. Integr Comp Biol 46, 508–518.
Bevelander, G., Nakahara, H., 1966. Correlation of lysosomal activity and ingestion by the mantle epithelium. Biol Bull 131, 76-82.
Blank, S., Arnoldi, M., Khoshnavaz, S., Treccani, L., Kuntz, M., Mann, K., Grathwohl, G., Fritz, M., 2003. The nacre protein perlucin nucleates growth of calcium carbonate crystals. Journal of Microscopy-Oxford 212, 280-291.
Bondad-Reantaso, M.G., McGladdery, S.E., Berthe, F.C.J., 2007. Pearl oyster health management: a manual. FAO Fisheries Technical Paper, Rome, pp. 119.
Brockes, J.P., Kumar, A., Velloso, C.P., 2001. Regeneration as an evolutionary variable. J Anat 199, 3-11.
Bubel, A., 1984. Mollusca: Epidermal Cells. In: Bereiter-Hahn, J., Matolsty, A.G., Richards, K.S. (Eds.), Biology of the Integument 1, Invertebrates. Springer-Verlag, Berlin, pp. 400-440.
Burger, T., Ploss, H.J., Kuhn, J., Ebel, S., Fricke, J., 1997. Diffuse reflectance and transmittance spectroscopy for the quantitative determination of scattering and absorption coefficients in quantitative powder analysis. Applied Spectroscopy 51, 1323-1329.
Carlson, B.M., 2007. Principles of Regenerative Biology. Elsevier, Michigan, 379 pp.
Carriker, M.R., Zandt, D.V., 1972. Regeneration of the accessory boring organ of muricid gastropods after excision. Transactions of the American Microscopical Society 91, 455-466.
Che, L.M., Campion-Alsumard, T.L., Boury-Esnault, N., Payri, C., Golubic, S., Bezac, C., 1996. Biodegradation of shells of the black pearl oyster, *Pinctada margaritifera* var. *cumingii*, by microborers and sponges of French Polynesia. Marine Biology (Berlin) 126, 509-519.
Checa, A.G., Rodriguez-Navarro, A.B., 2005. Self-organisation of nacre in the shells of Pterioida (Bivalvia : Mollusca). Biomaterials 26, 1071-1079.
Chen, L., Xie, L., Dai, Y., Xiong, X., Fan, W., Zhang, R., 2004. Cloning and characterization of an mRNA encoding a novel G protein (alpha)-subunit abundant in mantle and gill of pearl oyster *Pinctada fucata*. Comp Biochem Physiol B Biochem Mol Biol 139, 669-679.
Cosgrove, P., Hastie, L., Young, M., 2000. Freshwater pearl mussels in peril. Br. Wildl. 11, 340-347.
Cosgrove, P.J., Hastie, L.C., 2001. Conservation of threatened freshwater pearl mussel populations: river management, mussel translocation and conflict resolution. Biol. Conserv. 99, 183-190.

Culling, C.F.A., Allison, R.T., Barr, W.T., 1985. Cellular pathology technique. 4th Ed., Butterworths, London, 642 pp.
Dakin, W.J., 1913. Pearls. Cambridge University Press, Cambridge, vi, 143 pp.
Dall, W.H., 1883. Pearls and Pearl Fisheries. The American Naturalist 17, 579-587.
De Nys, R., Ison, O., 2008. Biofouling. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 527-554.
DesVoigne, D.M., 1968. The process of wound healing in the pacific oyster, *Crassostrea gigas*. J Invertebr Pathol 12, 53-65.
Dix, T.G., 1973. Histology of the mantle and pearl sac of the pearl oyster *Pinctada maxima* (Lamellibranchia). J Malacol Soc Aust 2, 365-375.
Doroudi, M.S., 1994. Infestation of the pearl oyster by the boring and fouling organisms in the pearl culture farm and natural beds from northern coast of Persian Gulf. J. Shellfish Res. 13, 333.
Elen, S., 2001. Spectral reflectance and fluorescence characteristics of natural-color and heat-treated "golden" south sea cultured pearls. Gems & Gemology 37, 114-123.
Elen, S., 2002. Identification of yellow cultured pearls from Black-lipped oyster *Pinctada margaritifera*. Gems & Gemology 38, 66-72.
Falini, G., Albeck, S., Weiner, S., Addadi, L., 1996. Control of aragonite or calcite polymorphism by mollusk shell macromolecules. Science 271, 67-69.
Fang, Z., Feng, Q.L., Chi, Y.Z., Xie, L.P., Zhang, R.Q., 2008. Investigation of cell proliferation and differentiation in the mantle of *Pinctada fucata* (Bivalve, Mollusca). Mar Biol 153, 745-754.
Fiske, D., Shepherd, J., 2007. Continuity and change in Chinese freshwater pearl culture. Gems & Gemology 43, 138-145.
Fleury, C., Marin, F., Marie, B., Luquet, G., Thomas, J., Josse, C., Serpentini, A., Lebel, J.M., 2008. Shell repair process in the green ormer *Haliotis tuberculata*: A histological and microstructural study. Tissue and Cell 40, 207-218.
Fong, Q.S.W., Ellis, S.C., Haws, M., 2004. Economic feasibility of small-scale, commercial culture of black pearls in rural communities in the Central Pacific. J. Shellfish Res. 23, 291.
Fougerouse, A., Rousseau, M., Lucas, J.S., 2008. Soft tissue anatomy, shell structure and biomoneralization. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 77-102.
Fowler, J., Cohen, L., Jarvis, P., 2004. Practical statistics for field biology, second edition. John Wiley & Sons, Chichester, England, 259 pp.
Franchini, A., Ottaviani, E., 2000. Repair of molluscan tissue injury: role of PDGF and TGF-β. Tissue Cell 32, 312-321.
Fromont, J., Craig, R., Rawlinson, L., Alder, J., 2005. Excavating sponges that are destructive to farmed pearl oysters in Western and Northern Australia. Aquacult. Res. 36, 150-162.
Fuller, W.A., 1996. Introduction to statistical time series. Wiley, New York, 698 pp.
Garcia-Gasca, A., Ochoa-Baez, R.I., Betancourt, M., 1994. Microscopic anatomy of the mantle of the pearl oyster *Pinctada mazatlanica* (Hanley, 1856). J Shellfish Res 13, 85-91.
George, C.D., 1967. The cultured pearl: Its history and development to the present day. South Pacific Post, Port Moresby, 16 pp.
George, C.D., 1996. The pearling industry in Australia and Papua New Guinea, 1949-1977, Cairns, 128 pp.

Gervis, M.H., Sims, N.A., 1992. The biology and culture of pearl oysters (Bivalvia: Pteriidae). ICLARM, Manila (Philippines), 49 pp.
Goss, R.J., 1969. Principles of Regeneration. Academic Press, Inc, New York, 287 pp.
Gre´goire, C., 1957. Topography of the organic components in mother-of-pearl. The Journal of Biophysical and Biochemical Cytology 3, 797-808.
Guenther, J., Southgate, P.C., de Nys, R., 2006. The effect of age and shell size on accumulation of fouling organisms on the Akoya pearl oyster *Pinctada fucata* (Gould). Aquaculture 253, 366-373.
Haws, M., 2002. The basic methods of pearl farming: a layman's manual. Hawaii Sea Grant.
Haws, M.C., 1998. Nucleus implantation for cultivation of black pearls: review of current technology and constraints for development of the industry. J Shellfish Res 17, 328.
He, M.X., Huang, L.M., Shi, J.H., Jiang, Y.P., 2005. Variability of ribosomal DNA ITS-2 and its utility in detecting genetic relatedness of pearl oyster. Marine Biotechnology 7, 40-45.
Hernandez-Olalde, L., Garcia-Dominguez, F., Arellano-Martinez, I., Ceballos-Vazquez, B.P., 2007. Reproductive cycle of the Pearl oyster *Pteria sterna* (Pteriidae) in the Ojo de Liebre lagoon, BCS, Mexico. J. Shellfish Res. 26, 543-548.
Hillman, R.E., 1961. Formation of the periostracum in *Mercenaria mercenaria*. Science 134, 1754-1755.
Hodgson, A.N., 1982. Studies on wound healing, and an estimation of the rate of regeneration, of the siphon of *Scrobicularia plana* (da Costa). J Exp Mar Bio Ecol 62, 117-128.
Huang, Y., Cavinato, A.G., Mayes, D.M., Kangas, L.J., Bledsoe, G.E., Rasco, B.A., 2003. Nondestructive determination of moisture and sodium chloride in cured Atlantic salmon (Salmo salar) (Teijin) using short-wavelength near-infrared spectroscopy (SW-NIR). Journal of Food Science 68, 482-486.
Humphrey, J.D., 2008. Disease and predation. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 367-436.
Humphrey, J.D., Norton, J.H., 2005. The pearl oyster *Pinctada maxima* (Jameson, 1901); An atlas of functional anatomy, pathology and hispathology. Northern Territory Department of Primary Industry, Fisheries and Mines, Queensland Department of Primary Industries and Fisheries Research and Development Corporation, 111 pp.
Hynd, J.S., 1955. A revision of the Australian pearl shells, genus *Pinctada* (Lamellibranchia). Australian Journal of Marine and Freshwater 6, 98-137.
Ito, M., Suzuki, T., Yada, S., Kusai, A., Nakagami, H., Yonemochi, E., Terada, K., 2008. Development of a method for the determination of caffeine anhydrate in various designed intact tables by near-infrared spectroscopy: A comparison between reflectance and transmittance technique. Journal of Pharmaceutical and Biomedical Analysis 47, 819-827.
Jones, J.B., 2007. Review of pearl oyster mortalities and disease problems, pp. 59–68. In: Bondad-Reantaso, M.G., McGladdery, S.E. and Berthe, F.C.J. Pearl oyster health management: a manual. FAO Fisheries Technical Paper No. 503. Rome, FAO. 2007. 119p.
Joyce, K., Addison, S., 1992. Pearls : ornament & obsession. Thames and Hudson, London, 253 pp.

Karampelas, S., Fritsch, E., Mevellec, J.Y., Sklavounos, S., Soldatos, T., 2009. Role of polyenes in the coloration of cultured freshwater pearls. Eur. J. Mineral. 21, 85-97.
Karampelas, S., Fritsch, E., Mevellec, J.Y., Gauthier, J.P., Sklavounos, S., Soldatos, T., 2007. Determination by Raman scattering of the nature of pigments in cultured freshwater pearls from the mollusk *Hyriopsis cumingi*. Journal of Raman Spectroscopy 38, 217-230.
Katti, K.S., Katti, D.R., 2006. Why is nacre so tough and strong? Materials Science and Engineering C, 1317– 1324.
Kemper, T., Sommer, S., 2002. Estimate of heavy metal contamination in soils after a mining accident using reflectance spectroscopy. Environ. Sci. Technol. 36, 2742-2747.
Kennedy, S.J., 1998. Pearl Identification. Australian Gemmologist 20, 2-19.
Kobayashi, I., Samata, T., 2006. Bivalve shell structure and organic matrix. Mater. Sci. Eng. C-Biomimetic Supramol. Syst. 26, 692-698.
Komatsu, H., Akamatsu, S., 1978. Studies on differentiation of true and artificially coloured black and blue pearls. Journal of Gemmological Society of Japan 5, 3-8.
Kremkow, C., 2005. Pearl production down, prices up. Modern Jeweler 104, 20.
Kunz, G.F., Stevenson, C.H., 1908. The book of the pearl: The history, art, science, and industry of the queen of gems. Macmillan & Co Ltd, London, 548 pp.
Lamprell, K., Healy, J., 1997. Bivalves of Australia, vol. 2. Backhuys Publisher, Leyden, 288 pp.
Lin, A.Y.M., Chen, P.Y., Meyers, M.A., 2008. The growth of nacre in the abalone shell. Acta Biomaterialia 4, 131-138.
Lowenstam, H.A., Weiner, S., 1989. On biomineralization. Oxford University Press, New York, 324 pp.
Mao Che, L., Le Campion-Alsumard, T., Boury-Esnault, N., Payri, C., Golubic, S., Bezac, C., 1996. Biodegradation of shells of the black pearl oyster, *Pinctada margaritifera* var. *cumingii*, by microborers and sponges of French Polynesia. Mar. Biol. 126, 509-519.
Matlins, A.L., 2002. The Pearl Book: The definitive buying guide; how to select, buy, care for & enjoy pearls. GemStone Press, Woodstock, Vermont, 198 pp.
Matsushiro, A., Miyashita, T., Miyamoto, H., Morimoto, K., Tonomura, B., Tanaka, A., Sato, K., 2003. Presence of protein complex is prerequisite for aragonite crystallization in the nacreous layer. Marine Biotechnology 5, 37-44.
McGinty, E.L., Evans, B.S., Taylor, J.U.U., Jerry, D.R. (2010). Xenografts and pearl production in two pearl oyster species, *P. maxima* and *P. margaritifera*: Effect on pearl quality and a key to understanding genetic contribution. Aquaculture (in press).
Mills, D., Tlili, A., Norton, J., 1997. Large-scale anesthesia of the silver-lip pearl oyster, *Pinctada maxima* Jameson. J. Shellfish Res. 16, 573-574.
Mount, A.S., Wheeler, A.P., Paradkar, R.P., Snider, D., 2004. Hemocyte-mediated shell mineralization in the Eastern oyster. Science 304, 297-300.
Norton, J.H., Dashorst, M., Lansky, T.M., Mayer, R.J., 1996. An evaluation of some relaxants for use with pearl oysters. Aquaculture 144, 39-52.
Norton, J.H., Lucas, J.S., Turner, I., Mayer, R.J., Newnham, R., 2000. Approaches to improve cultured pearl formation in *Pinctada margaritifera* through use of

relaxation, antiseptic application and incision closure during bead insertion. Aquaculture 184, 1-17.
O'Connor, W.A., Lawler, N.F., 2002. Propylene phenoxetol as a relaxant for the pearl oysters *Pinctada imbricata* and *Pinctada albina*. Asian Fish. Sci. 15, 51-57.
O'Sullivan, D., 1992. A review of oyster farming techniques in Australia. J. Shellfish Res. 11, 204-205.
O'Sullivan, D., Cropp, D., 1994. An overview of pearl production techniques in Australia. J. Shellfish Res. 13, 348.
Pan, C.-M., Watabe, N., 1989. Periostracum formation and shell regeneration in the lingulid *Glottidia pyramidata* (Brachiopoda: Inarticulata). Trans Am Microsc Soc 108, 283-298.
Pauley, G.B., 1966. Observations on experimental wound repair in the adductor muscle and the Leydig cells of the oyster *Crassostrea gigas*. J Invertebr Pathol 9, 298-309.
Pouvreau, S., Prasil, V., 2001. Growth of the black-lip pearl oyster, *Pinctada margaritifera*, at nine culture sites of French Polynesia: synthesis of several sampling designs conducted between 1994 and 1999. Aquatic Living Resources 14, 155-163.
Reed-Miller, C., 1983. Scanning electron microscopy of the regenerated shell of the marine archaeogastropod, *Tegula*. Biological Bulletin 165, 723-732.
Rousseau, M., Lopez, E., Coute, A., Mascarel, G., Smith, D.C., Naslain, R., Bourrat, X., 2004. Multi-scale structure and growth of nacre: a new model for bioceramics. Bioceramics 16 254-2, 1009-1012.
Ruiz-Rubio, H., Acosta-Salmon, H., Olivera, A., Southgate, P.C., Rangel-Davalos, C., 2006. The influence of culture method and culture period on quality of half-pearls ('mabe') from the winged pearl oyster *Pteria sterna*, Gould, 1851. Aquaculture 254, 269-274.
Ryan, M., O'Sullivan, D., 2001. Culture techniques of the Australian pearl industry. Austasia Aquacult. June/July, 32-37.
Saucedo, P., Rodriguez-Jaramillo, C., Monteforte, M., 2002a. Microscopic anatomy of gonadal tissue and specialized storage cells associated with oogenesis and spermatogenesis in the Calafia mother-of-pearl oyster, *Pinctada mazatlanica* (Bivalvia: Pteriidae). J. Shellfish Res. 21, 145-155.
Saucedo, P., Racotta, I., Villarreal, H., Monteforte, M., 2002b. Seasonal changes in the histological and biochemical profile of the gonad, digestive gland, and muscle of the Calafia mother-of-pearl oyster, *Pinctada mazatlanica* (Hanley, 1856) associated with gametogenesis. J. Shellfish Res. 21, 127-135.
Saucedo, P.E., Southgate, P.C., 2008. Reproduction, development and growth. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 131-186.
Scarratt, K., Moses, T.M., Akamatsu, S., 2000. Characteristics of nuclei in Chinese freshwater cultured pearls. Gems & Gemology 36, 98-109.
Scoones, R.J.S., 1996. The development of the pearl sac in *Pinctada maxima* (Jameson, 1901) (Lamellibranchia: Pteriidae) and the implications for the quality of cultured pearls. Unpublished Master Thesis, The University of Western Australia, Perth. 89 pp.
Shirai, S., 1994. Pearls and pearl oysters of the world. Marine Planning Co., Nagano, Japan, 108 pp.

Simkiss, K., 1988. Molluscan skin (excluding Cephalopods). In: Wilbur, K.M., Trueman, E.R., Clarke, M.R. (Eds.), The Mollusca, pp. 11-35.
Simkiss, K., Wada, K., 1980. Cultured pearls- commercialised biomineralisation. Endeavour 4, 32-37.
Simkiss, K., Wilbur, K.M., 1989. Biomineralization: cell biology and mineral deposition. Academic Press, San Diego, 337 pp.
Snow, M.R., Pring, A., Self, P., Losic, D., Shapter, J., 2004. The origin of the color of pearls in iridescence from nano-composite structures of the nacre. American Mineralogist 89, 1353-1358.
Southgate, P.C., 2007. Overview of the cultured marine pearl industry. In: Bondad-Reantaso, M.G., McGladdery, S.E., F.C.J., B. (Eds.), Pearl oyster health management: a manual. FAO Fisheries Technical Paper No. 503, Rome, pp. 7-17.
Southgate, P.C., 2008. Pearl oyster culture. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 231-272.
Southgate, P.C., Lucas, J.S., Torrey, R.D., 2008a. Future developments. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 555-566.
Southgate, P.C., Strack, E., Hart, A., Wada, K.T., Monteforte, M., Cariño, M., Langy, S., Lo, C., Acosta-Salmón, H., Wang, A., 2008b. Exploitation and culture of major commercial species. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 303-356.
Spencer, G.E., Syed, N.I., Lukowiak, K., Winlow, W., 1995. Halothane-induced synaptic depression at both in vivo and in vitro reconstructed synapses between identified *Lymnaea neurons*. J. Neurophysiol. 74, 2604-2613.
Strack, E., 2006. Pearls. Ruhle-Diebener-Verlag, Stuttgart, Germany, 707 pp.
Streeter, E.E., 1886. Pearls and pearling life. George Bell & Sons, Yorks Street, Covent Garden.
Taylor, J., Strack, E., 2008. Pearl production. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 273-302.
Taylor, J.J.U., 1999. Juvenile production and culture of the silver-lip pearl oyster, *Pinctada maxima* (Jameson). PhD Thesis, James Cook University. Townsville, Australia. 222 pp.
Taylor, J.J.U., 2002. Producing golden and silver South Sea pearls from Indonesian hatchery reared *Pinctada maxima*. POIB 15, 30.
Taylor, J.J.U., 2007. Atlas south sea pearl grading and coding matrix. Internal Company Document. 7 pp.
Tsonis, P.A., 2000. Regeneration in vertebrates. Dev Biol 221, 273-284.
Ubukata, T., 1994. Architectural constraints on the morphogenesis of prismatic structure in Bivalvia. Palaeontology 37, 241-261.
Wada, K.T., Komaru, A., 1996. Color and weight of pearls produced by grafting the mantle tissue from a selected population for white shell color of the Japanese pearl oyster *Pinctada fucata martensii* (Dunker). Aquaculture 142, 25-32.
Wada, K.T., Tëmkin, I., 2008. Taxonomy and Phylogeny. In: Southgate, P.C., Lucas, J.S. (Eds.), The pearl oyster. Elsevier, Amsterdam, pp. 37-76.
Wang, Q., Wu, H., 1994. Pearl culture in China. J. Shellfish Res. 13, 353.
Ward, F., 1995. Pearls. Gem Book Publishers, Bethesda, 64 pp.
Watabe, N., 1988. Shell structure. In: Trueman, E.R., Clark, M.R. (Eds.), The Mollusca Vol. 11; Form and Function. Academic Press, Inc., San Diego, pp. 69-104.

Webster, R., 1994. Gems: their sources, descriptions and identification, 1026 pp.
Weiner, S., Traub, W., Parker, S.B., 1984. Macromolecules in mollusc shells and their functions in biomineralization. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 304, 425-434.
White, H.I., Hecht, T., Potgieter, B., 1996. The effect of four anaesthetics on *Haliotis midae* and their suitability for application in commercial abalone culture. Aquaculture 140, 145-151.
Woodall, A.J., Naruo, H., Prince, D.J., Feng, Z.P., Winlow, W., Takasaki, M., Syed, N.I., 2003. Anesthetic treatment blocks synaptogenesis but not neuronal regeneration of cultured *Lymnaea neurons*. J. Neurophysiol. 90, 2232-2239.
Xie, Y., 1990. A study on maximum pearl oysters *Pinctada maxima*. Bull. Mar. Sci. 47, 262-263.
Young, M.R., 1991. Conserving the freshwater pearl mussel (*Margaritifera margaritifera* L.) in the British Isles and continental Europe. Aquat. Conserv.: Mar. Freshwat. Ecosyst. 1, 73-77.

Appendix 1: Publications resulting from this thesis

Mamangkey G.H., Acosta-Salmon, H., Southgate, P.C. 2009. Use of anaesthetics with the silver-lip pearl oyster, *Pinctada maxima* (Jameson). Aquaculture 288, 280-284.

Mamangkey, G.H., Southgate, P.C. 2009. Regeneration of excised mantle tissue by the silver-lip pearl oyster, *Pinctada maxima* (Jameson). Fish and Shellfish Immunology 27, 164-174.