Improving the quality of cultured round pearls produced by *Pinctada margaritifera* in Fiji (Linnaeus, 1758)

Thesis submitted by

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Statement of contribution of others

Publication details

Chapter No.	Details of publications	Nature and extent of the intellectual input of each author, including the candidate
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6	**Kishore, P**., Hunter, J., Zeng, C. & Southgate, P.C., 2014. The effects of different culture apparatuses and current velocities on byssus production by the black-lip pearl oyster, *Pinctada margaritifera*. *Aquaculture* 434, 74-77.	Kishore and Southgate co-developed the research question. Kishore carried out the experiment, collected the data and performed statistical tests. Hunter provided technical input while Zeng assisted in performing statistical analysis. Kishore wrote the first draft of the paper which was revised with editorial input from Southgate.
7	**Kishore, P.** & Southgate, P.C., 2015. The effect of different culture methods on the quality of round pearls produced by the black-lip pearl oyster *Pinctada margaritifera* (Linnaeus 1758). *Aquaculture* (accepted).	The authors co-developed the research question. Kishore carried out the experiment, collected the data and performed statistical tests. Kishore wrote the first draft of the paper which was revised with editorial input from Southgate.

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Abstract

The pearl industry is recognised as one of the most profitable in the aquaculture sector. In many countries it makes up a large segment of the aquaculture sector and in French Polynesia for example, it is the second highest export earner (after tourism) and makes a substantial contribution to the country's economy and to the livelihoods of its people. The major products of the marine pearl industry are Akoya pearls, white South Sea Pearls, and 'black' South Sea Pearls pearls, otherwise known as Tahitian pearls that are produced almost exclusively in the Pacific. 'Black' South Sea Pearls produced by the black-lip pearl oyster *Pinctada margaritifera* are the focus of this study. At present, the proportion of high quality 'black' pearls in any pearl farm is very low making up to only around 3%-5% of the total harvest, yet earning around 95% of farm revenue. A major influence on the high proportion of low quality pearls is the presence of 'circles' or concentric depressions or grooves on surfaces that reduce pearl values considerably. Reducing the proportion of these low quality pearls by a small proportion would significantly increase the profit margin of a 'black' pearl farm. This study aimed to identify the causes of low quality 'black' pearls, particularly circles, and provide a basis for improved pearl grafting and oyster husbandry practices supporting increased production and revenue for pearl farmers.

In Chapter 2, pearl-sac development after grafting in *P. margaritifera* was studied in detail for the first time. A total of 110 *P. margaritifera* with a mean (± SE) antero-posterior measurement of 110.82 ± 0.41 mm and dorso-ventral measurement of 112.06 ± 0.45 mm were grafted to allow histological examination and chronological description of pearl-sac development in this species. Beginning two days after grafting, oysters were sacrificed regularly until the 48th day and the pearl-sacs of sampled

oysters were sectioned and analysed. The graft tissue proliferated and developed into a complete pearl-sac within 14 days of grafting when the epithelial cells responsible for nacre secretion were fully developed. However, first nacre secretion onto the nucleus was not observed until 32 days after grafting. Furthermore, the presence of byssus in close proximity of developing pearl-sac was demonstrated in this study; a factor that has the potential to impact pearl-sac development affecting even nacre deposition and resulting pearl quality. Haemocytes were also present with clumps or aggregations noted in some pearl-sacs. The findings reported in this Chapter provide a more detailed understanding of pearl-sac development in *P. margaritifera* and a basis for future research towards developing improved pearl culture practices and pearl quality.

A detailed examination of haemocyte accumulation during pearl-sac formation provided the basis for Chapter 3. The level of haemocytes present in the pearl-sacs decreased overtime in many oysters with the samples from day two showing the highest levels. Such a trend generally supports the development of a spherical shaped pearl-sac that would form a regular shaped pearl. However, in some oysters, clumps of haemocytes persisted for a period longer than expected causing a bulge in the pearl-sacs. The pearl-sacs grew over the clumps that resulted in a deformity to what should have been spherical shaped pearl-sacs. Pearls produced from such misshapen pearl-sacs often have calcified "tails" or be of baroque shapes with much reduced values. The exact cause(s) of varying levels of haemocyte accumulation during pearl-sac development in *P. margaritifera* is not known. However, it is reasonable to assume that haemocyte production is positively related to the degree of damage caused to host oyster tissues during the grafting procedure. While haemocytes have an important

wound healing role in pearl oysters, excessive haemocyte presence may be detrimental to maximizing pearl quality.

The feasibility of using regenerated graft tissue for pearl production in *P. margaritifera* was investigated in Chapter 4. Twelve days after grafting with regenerated graft tissue, there was complete encapsulation of the nucleus by the fully developed pearl-sac and the first layer of organic matrix had been secreted. Sixteen days after grafting, the pearl-sac was completely integrated with host tissue and could no longer be distinguished as foreign. The epithelial cells in the pearl-sac continued to secrete the organic matrix layer but there were no signs of nacre deposition at this stage. However, after three months of culture, nuclei in oysters grafted with regenerated mantle tissue were completely covered with nacre. The average nacre thickness on pearls produced from regenerated (0.55 ± 0.01 mm, $n = 8$) and normal (0.53 ± 0.01 mm, $n = 8$) mantle tissue did not differ significantly ($p > 0.05$). Nacre secretion rates, over the 80 day period subsequent to pearl-sac formation were 6.84 ± 0.1 μm day^{-1} and 6.66 ± 0.1 μm day^{-1} for oysters grafted with regenerated and normal mantle tissue, respectively. Again, these means were not significantly different ($p = 0.258$). These results clearly showed that regenerated mantle tissue can function successfully as saibo for pearl production in *P. margaritifera*. This finding could provide significant benefits to pearl farmers and provide a basis for further development of current pearl grafting practices.

It is widely assumed that *P. margaritifera* producing low quality pearls with circles are unlikely to produce pearls with improved quality if grafted again for pearl production. Such oysters are often discarded. However, if these oysters are capable of improved pearl quality when re-grafted, then this would provide opportunities for improved

income for pearl farmers. Chapter 5 aimed to determine whether oysters producing circled pearls are able to produce pearls with improved quality after re-grafting. A total of 100 oysters that produced circled pearls and would have normally been discarded were re-grafted and the quality of successive pearls produced by individual oysters was compared in terms of shape, size, lustre, colour, surface perfection and overall quality. The proportion of pearls with circles decreased from 95% of first graft pearls to 48% after the second graft, and 18% of second graft pearls were classified as 'semi-round' and superior in shape to all first graft pearls. There was a significant improvement (p = 0.04) in the overall shape of second graft pearls compared to first graft pearls. The highest proportion of pearls (63%) from the first graft were 10-11 mm in size while the majority of second graft pearls (51%) were 11-12 mm in size, and the differences in pearl size between first and second graft were significant (p = 0.04). Second graft pearls had poorer lustre than first graft pearls with a higher proportion of dull pearls, a lower proportion of medium lustre pearls and no pearls with high lustre. Despite this, the number of pearls in different lustre categories after the first and second graft did not differ significantly (p = 0.07). For overall grading, most first graft pearls (83%) were assessed as 'C' grade with 17% categorised as 'D' grade. Similarly, most second graft pearls (78%) were assessed as 'C' grade and 20% as 'D' grade; however, 2% of pearls were assessed as 'B' grade which were not present in first graft pearls. Nonetheless, the number of pearls belonging to different grades was not significant (p = 0.08). The data in chapter show for the first time that that production of circled pearls after second graft is not obligatory for *P. margaritifera* that produced circled pearls after first graft. The data further show that marketable pearls can be produced from oysters that are normally discarded after the first pearl harvest and this has potential to generate increased revenue.

The potential effects of byssus production on the development or function of normal pearl-sacs was determined in Chapter 6. This was done after byssus was observed in close proximity to developing pearl-sacs in the experiment reported in Chapter 2. This Chapter investigated the impacts of relative current strength and different culture units on byssus secretion by *P. margaritifera*. Oysters were either 'ear–hung' or housed in panel nets before being transported to low (Nawi) and high (Raviravi) current sites. The quantity of new byssus produced by oysters in the two culture units at the two sites was counted 5, 10, 15 and 20 days after deployment. At the end of the experiment, the thicknesses and tensile strengths of randomly selected byssal threads from ear-hung oysters and oysters held in panel nets were determined. Ten days after deployment, there was no significant difference in the quantity of byssus produced by oysters in the two types of culture units at both sites. An average of around two threads per byssus secreting oyster was recorded by the tenth day. However, after 15 and 20 days, ear-hung oysters had produced significantly more byssus ($p < 0.01$) than those housed in panel nets at the high current site. On the twentieth day, ear-hung oysters had an average of six byssal threads while those housed in panel nets had an average of around three per oyster at the Raviravi site. In contrast, production of byssus by oysters in the two types culture units did not differ significantly for the same period at the low current site. Furthermore, ear-hung oysters produced significantly thicker byssus than those held in panel nets ($p = 0.01$) which had significantly high tensile strengths ($p = 0.01$). It is hypothesised that secretion of an increased number of byssal threads by ear-hung oysters is a response to a greater degree of agitation than those held in panel nets. This could be one of the reasons for anecdotal commentary relating to the production

of a high proportion of pearl with inferior quality by oysters cultured using the 'ear-hanging' method.

With oysters cultured using chaplets producing more byssus compared to oysters housed in panel nets, the experiment described in Chapter 7 was designed to determine if oysters held in panel nets produced higher quality pearls with fewer circles compared to oysters that were ear-hung on chaplets. Six hundred *P. margaritifera* were grafted for the first time and cultured using panel nets or chaplets at three commercial farm sites to determine if these different culture methods influence resulting pearl quality. The pearls produced were compared in terms of size, shape, lustre, colour, surface perfection and overall quality. The highest proportion of pearls produced in all treatments was in the 10-11 mm size category (37% to 54%) but culture method did not significantly (p = 0.211) influence the size of pearls produced. Oysters held on chaplets produced more pearls with concentric surface grooves or circles (47% to 60%) compared to oysters in held panel nets (43% to 45%) at all three culture sites. Oysters held in panel nets produced higher proportions of pearls in the more desirable 'round' and 'semi-round' shape categories (6% and 25%, respectively) than oysters held on chaplets (5% and 15%, respectively) at all three culture sites, and culture methods had a significant impact (p = 0.031) on pearl shape overall. Higher proportions of pearls in the 'very high' and 'high' lustre categories (8% and 40%, respectively) were produced by oysters held in panel nets compared to those on chaplets (3% and 16%, respectively) at each of the three culture sites. However, the overall impact of culture methods on pearl lustre was not significant (p = 0.100). At all three culture sites, higher proportions of pearls assigned to grades 'A' (6%) and 'B' (46%) were produced by oysters in panel nets compared to those held on chaplets where 3% and 29% of pearls were assigned to

grade ‘A’ and grade ‘B’, respectively. Oysters held on chaplets produced higher proportions of grade ‘C’ (49%) and grade ‘D’ (19%) pearls than those in panel nets (39% and 9%, respectively) at all three culture sites. The grades of pearls were significantly influenced (p = 0.035) by culture method. The results of this experiment clearly demonstrated the benefits of pearl production using panel nets compared to the traditional chaplet-based system used by the majority of pearl farmers in Fiji and throughout the Pacific. Pearls production using panel nets will provide better returns with higher profit margins for pearl farmers but requires greater outlay for infrastructure and labour that may be beyond the scope of most pearl farmers in Fiji and the Pacific. A detailed cost-benefit analysis of the two husbandry options would be beneficial to pearl farmers.

This study addressed factors affecting the quality of cultured ‘black’ pearls through a number of experiments that assessed the impacts of both developmental and biological factors (e.g. pearl-sac development and function, oyster response to culture method and culture environment) as well as husbandry and culture conditions (e.g. culture method and current strength) on pearl production and pearl quality. The major applications of the results of this study are: (1) potential use of saibo donors producing high quality pearls for multiple saibo donations potentially improving the proportion of high quality pearls; (2) production of marketable pearls from oysters that are normally discarded after the first pearl harvest resulting in increased production and revenue; and (3) change to a panel net-based culture system resulted in higher pearl quality and a ~30% increase in the value of pearls produced. These findings provide a good basis for increased pearl production in Fiji and for future research in this field.

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Chapter 1

General Introduction

The cultured pearl industry is recognised as one of the most profitable in the aquaculture sector (Fassler, 1997; Bondad-Reantaso *et al.*, 2007; Southgate, 2007) and in a number of countries it contributes considerably to the national economy. In French Polynesia for example, it is the second highest export earner (after tourism) and makes a substantial contribution to the country's economy and the livelihoods of its people (Tisdell and Poirine, 2008; Ky *et al.*, 2014a; Ky *et al.*, 2015). The cultured pearl industry is made up of freshwater and marine pearls and encompasses individuals, cooperatives, families and large multinational companies in more than 30 countries (Southgate, 2007). Freshwater pearl production, mainly by China, was more than one billion pearls in 2012 (Anonymous, 2014a) while the estimated production of marine pearls was 46.7 tonnes in 2013 (Müller, 2013). The cultured pearl industry also includes half pearl (mabè) production (Southgate, 2007); however, the quantity produced is insignificant compared to other pearl types at present.

The marine cultured pearl industry utilises three major pearl producing oyster species; the silver or gold–lip pearl oyster *Pinctada maxima* (Jameson 1901) which produces white or gold 'South Sea' (or 'South Seas') pearls, the Akoya pearl oyster, *Pinctada fucata/martensii/radiata/imbricata* species complex (Gould 1850) and the 'black-lip' pearl oyster *Pinctada margaritifera* (Linnaeus 1758) which is used to produce 'black' South Sea or Tahitian pearls (Gervis and Sims, 1992; Strack, 2006; Southgate, 2007; Strack, 2008; Wada and Tëmkin, 2008). The term "South Sea" pearl refers to pearls produced by *P. margaritifera* and *P. maxima* that are cultured south of Japan

(Southgate, 2007). The international pearl market differentiates pearls from *P. margaritifera* and *P. maxima* as "black" and "white" pearls, respectively (Strack, 2006; Southgate, 2007). South Sea pearls from *P. margaritifera* are the target of this study and will be referred to as "black pearls" (Goebel and Dirlam, 1989; Elen, 2002; Shor, 2007).throughout this thesis.

The marine cultured pearl industry has experienced significant economic alterations over recent years (Tisdell and Poirine, 2000; Haoatai and Monypenny, 2011). The industry's total value dropped from US$800 m in 1993 to US$500 m in 1999 (Müller, 2005, 2009). It dropped further to US$ 390 m in 2009 (Müller, 2013). Pearl prices remained under pressure until recently when some stability and a slight increase in total turnover was estimated for 2013. Forecasted production value for 2013 was approximately US$397 m (Müller, 2013). Trends in the production values of the three major marine pearl producing species are shown in Table 1.1.

Table 1.1. Production values of the three major components of the marine pearl industry from 1983 to 2013. (Source: Muller, 2009; Müller, 2013)

Year	Akoya pearls	White/Gold South Sea pearls	'Black' pearls
1983	US$400 m	US$24 m	US$5 m
1993	US$600 m	US$120 m	US$75 m
1999	US$131 m	US$217 m	US$125 m
2005	US$135 m	US$248 m	US$125 m
2009	US$65 m	US$172 m	US$130 m
2013	US$111 m	US$192 m	US$ 94 m

1.1. Cultured 'black' pearls

The focus of this study are cultured pearls produced by *P. margaritifera* that are dominantly produced by French Polynesia and the Cook Islands over the last 45 years (Ponia, 2010). Annual production decreased from 10 tonnes (t) in 2002 and 2003 to 8.5 t in 2004 and later to 8.1 t in 2005 (Torrey and Sheung, 2008; Ponia, 2010). This was mainly due to increasing production of low quality pearls in French Polynesia that were prevented from entering the pearl market (by export) by the government after it imposed a series of regulatory measures (Torrey and Sheung, 2008). However, in 2006, an increase in the production volume from other 'black' pearl producing countries, mainly the Cook Islands and Fiji, stimulated an increase in the total value of 'black' pearl production. The total production volume continued to increase after an improvement in the French Polynesia pearl sector and a total of 15.75 t was estimated for 2013. French Polynesia still dominates the industry and had an approximate production of 15 t with a value of US$90 m estimated for 2013. The other cultured 'black' pearl producing countries; the Cook Islands, Fiji, Marshall Island and Federated States of Micronesia had a total estimated production value of US$4 m in 2013 (Müller, 2013; Anonymous, 2014a) (Table 1.2).

Table 1.2. Total volume and value of cultured 'black' pearl production between 1983 and 2013. (Source: Anon, 2007; Muller, 2009; Müller, 2013)

Year	Volume (tonnes)	Value (m)
1983	0.1	US$5
1992	1	US$44
1996	5	US$152
1999	6.3	US$141
2002	10	-
2004	8.5	US$120
2005	8.1	US$126
2009	12.5	US$130
2013	15.75	US$94

1.2. The Fijian cultured pearl industry

Cultured 'black' pearls generate the largest proportion of revenue compared to all other aquaculture commodities in Fiji. Pearl farming in Fiji was first initiated in 1966 by a company from Japan (Uwate *et al.*, 1984), however, it was not until 2000 that Fiji began commercial pearl farming after establishment of J. Hunter Pearls in Savusavu Bay on the island of Vanua Levu (Southgate *et al.*, 2008). Since then, Fiji has rapidly gained a reputation of producing high quality pearls with unique colours (Anon, 2007) that are mostly exported to the USA, Japan. Hong Kong, China and European Union countries (Chand *et al.*, 2015).

The Fijian cultured pearl industry has gradually expanded since 2000 and there were a total of eight farms in 2012. However, increasing production costs and limited

technical support lead to the loss of one of these farms and the seven remaining pearl farms in Fiji now provide employment to around 90 people. The largest of the farms is J. Hunter Pearls, which produces approximately 80% of Fiji's cultured pearl production (Chand *et al.*, 2015) and dominates the domestic and export market for Fiji pearls. Civa Pearls, Valili Pearls and Tokito Pearls are the other pearl farms that currently export cultured pearls from Fiji, while the rest produce pearls that are sold in domestic markets only. A new pearl farm, Desci Malolo Pearls, established in 2011 at Namarai on the main island of Viti Levu, has also shown every indication of reaching export level production by 2017.

The current production volume of cultured pearls in Fiji is unclear but in 2011 was estimated to be around 23,000 pearls with a value of around $1.4 m (Southgate, 2011; Chand *et al.*, 2015); however, increases in the number of farmed pearl oysters in Fiji resulting from establishment of a national spat collection program supporting industry expansion (Anon, 2014) indicates that production will be increased over the short-term to meet growing international demand and the total amount of revenue generated is expected to further increase this year compared to previous years (Justin Hunter, J. Hunter Pearls, *pers. comm*., 2014). This is partly due to increased domestic sales resulting from an expansion in the Fijian tourism industry and the high demand of the high quality uniquely coloured pearls by overseas markets.

The proportion of high quality pearls produced in any cultured pearl farm is relatively small generally making up around 3% (Ky *et al.*, 2015) to 5% (Haws, 2002) of the total harvest but generating around 95% of farm revenue (Haws, 2002). A high proportion of cultured pearls from a given crop are of low quality and pearls produced by *P.*

margaritifera are particularly renowned for symmetrical concentric grooves (generally called 'circles') on their surfaces which reduces pearl value significantly (Ito, 1996; Ito, 2009). An increase in the proportion of high quality pearls by just a small percentage could increase the profit margin of a pearl farm considerably (Haws, 2002) and, on this basis, this study focused on developing a better understanding of the factors that influence the quality of cultured pearls produced by *P. margaritifera*.

1.3. The Black-lip pearl oyster, *Pinctada margaritifera*

1.3.1. Distribution

Pinctada margaritifera has a broad Indo-Pacific distribution from east Africa, the Persian Gulf and Red Sea, through Australia and S.E. Asia to Polynesia including the majority of the south Pacific Ocean (Fig. 1.1) (Hynd, 1955; Gervis and Sims, 1992; Wada and Tëmkin, 2008).

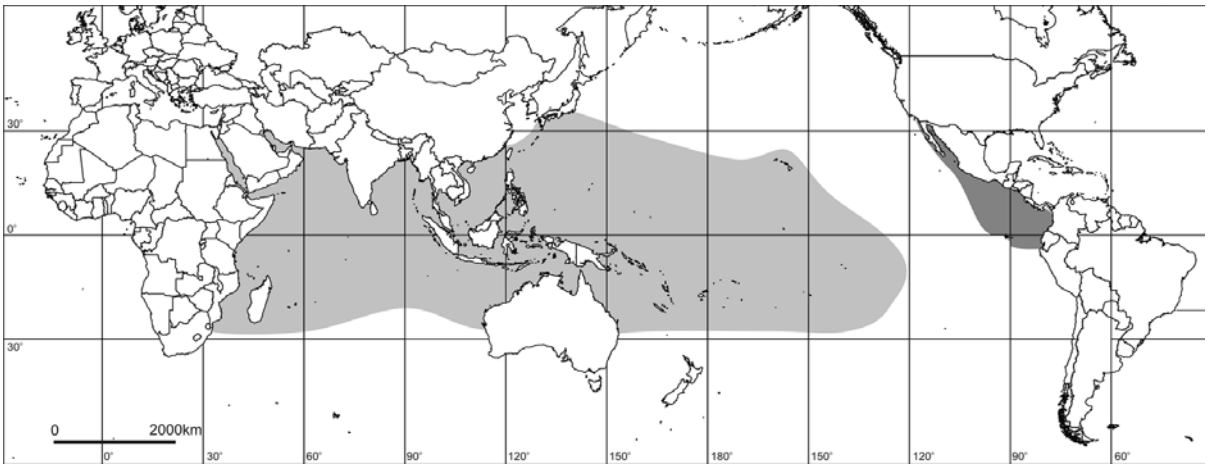

Figure 1.1. The distribution of *Pinctada margaritifera* around the globe shown by the light grey shade. (Source: Wada and Tëmkin, 2008)

1.3.2. Taxonomy of Pinctada margaritifera

Pearl oysters belong to family Pteriidae which contains three genera, *Pteria, Pinctada and Electroma.* The accepted taxonomic framework (Wada and Tëmkin, 2008) for *P. margaritifera* is as follows:

Kingdom: Animalia

Phylum: Mollusca

Class: Bivalvia

Order: Pterioida

Family: Pteriidae

Genus: *Pinctada*

Species: *margaritifera* (Linnnaues 1758)

1.3.3. Morphology

Shells of *P. margaritifera* are compressed laterally and are generally rounded in lateral outline (Poutiers, 1998; Wada and Tëmkin, 2008). External surfaces of shells are green (bronze), brown, or black with radial stripes of white spots (Wada and Tëmkin, 2008). The left valve is more convex than the right valve (Fig. 1.2b) and the width (anterior-posterior measurement, APM) is longer than the hinge (Gervis and Sims, 1992; Poutiers, 1998; Wada and Tëmkin, 2008). The auricle or 'ear' is well developed by an extension of the anterior shell border (Fig. 1.2c); it is used as a site for drilling a hole used for tying pearl oysters to ropes during culture. Byssus for attachment to substrates extends from a narrow byssus notch (Rao and Rao, 1974). Posterior shell meets the hinge perpendicularly (Hynd, 1955; Rao and Rao, 1974; Wada and Tëmkin, 2008) and the shell height (DVM) is either equal to or slightly longer than the length of the shell (Fig. 1.2a) (Wada and Tëmkin, 2008). A dark black marginal colouration is present

between the rim of distally black shell and the internal silvery nacreous layer (Fig. 1.2c), hence the common name of "black–lip" pearl oysters.

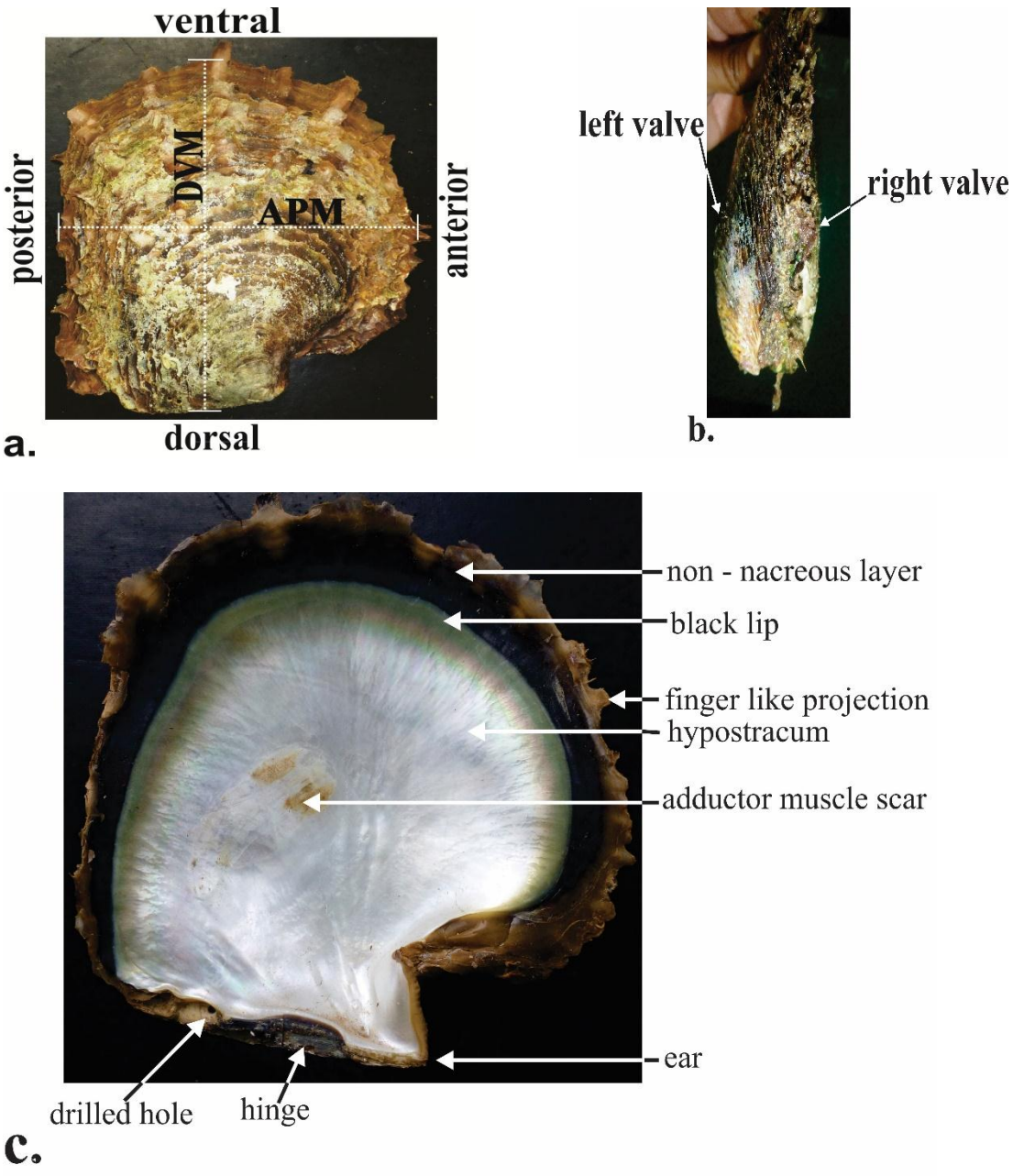

Figure 1.2. Shell morphology of *Pinctada margaritifera*: a, antero-posterior and dorso-ventral measurements; b, left and right valves; c, different shell features.

1.3.4. Shell structure

Shells of *P. margaritifera* have a primary anatomical structure that is present in all pearl oyster species (Gervis and Sims, 1992; Suzuki and Nagasawa, 2007; Fougerouse *et al.*, 2008). The shells are divided into three main layers: 1) the outer periostracum layer; 2) the prismatic or ostracum layer; and 3) the mother-of-pearl (MOP) nacreous layer or hypostracum (Fig. 1.3) (Miyoshi *et al.*, 1987; Gervis and Sims, 1992; Wilt *et al.*, 2003; Fougerouse *et al.*, 2008). The outer periostracum layer, mainly made up of proteins, protects oysters from fouling and facilitates shell formation (Dharmaraj *et al.*, 1987a; Gervis and Sims, 1992; Mao Che *et al.*, 1996; Fougerouse *et al.*, 2008). The prismatic layer is made from calcite polygonal prisms assembled in a lattice–like format with their axis perpendicularly meeting the shell surface (Gervis and Sims, 1992; Fougerouse *et al.*, 2008). The MOP nacreous layer differs considerably from these two layers. It is made up of extra-crystalline and intra-crystalline organic networks that are arranged in flat polygonal tablets (Gervis and Sims, 1992; Wang *et al.*, 2001; Rousseau *et al.*, 2005; Strack, 2006; Dauphin *et al.*, 2008; Fougerouse *et al.*, 2008).

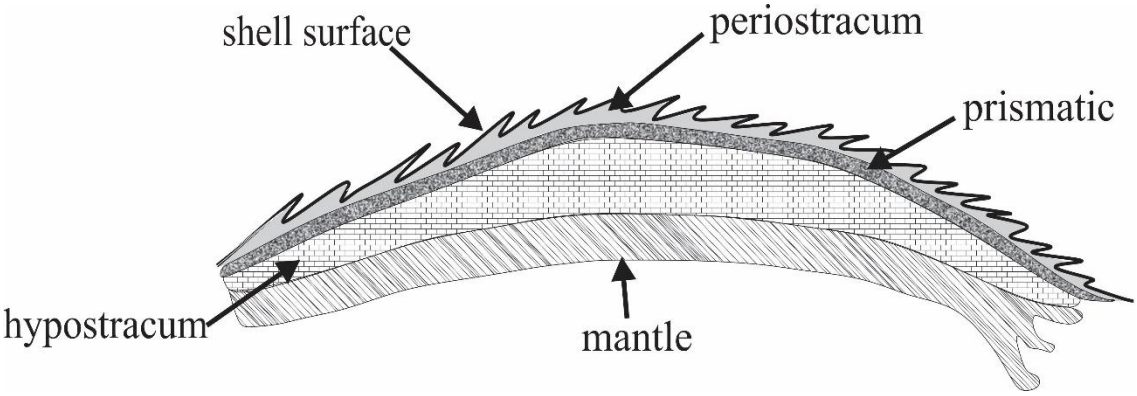

Figure 1.3. The three different shell layers of *Pinctada margaritifera.* (redrawn from Fougerouse et al., 2008)

1.3.5. Anatomy

Discussed and shown below (Fig. 1.4) are some of the organs directly related to pearl production and of particular interest to this study.

1.3.5.1. Mantle

The mantle lines the inside surface of both shells, It has numerous roles including protection of all the other organs which it encloses (Gervis and Sims, 1992; Wada and Tëmkin, 2008), aiding in respiration, reproduction and locomotion (Simkiss, 1988). It also produces ions and minerals used in the biomineralisation process during shell growth and nacre formation (Garcia-Gasca *et al.*, 1994; Kono *et al.*, 2000; Sud *et al.*, 2001; Blank *et al.*, 2003; Fougerouse *et al.*, 2008; Taylor and Strack, 2008) and the later process is utilised for cultured pearl production (see *section 1.6.1.)*. A mantle lobe is divided into four zones; isthmus, central area, distal or pallial area and a free marginal area (Jabbour-Zahab *et al.*, 1992; Blank *et al.*, 2003; Humphrey and Norton, 2005). The lobes are attached dorsally to the hinge at the isthmus zone, the central area covers most internal organs, the pallial area contains muscular bundles for mantle contraction and tissues for pearl production, and the free marginal area hosts mantle epithelium (Fougerouse *et al.*, 2008). The mantle epithelium has various types of secretory cells (Miyamoto *et al.*, 1996; Barik *et al.*, 2004) that manage and secrete different acids, proteins and nacre material required for shell (and pearl) formation (Garcia-Gasca *et al.*, 1994; Taylor and Strack, 2008).

1.3.5.2. Gonad

The gonad contains gametes that are released during the spawning process. Spawning in pearl oysters is generally triggered after a change in water temperature (Tranter,

1958; Southgate and Beer, 1997; Doroudi *et al.*, 1999; Haws and Ellis, 2000; Saucedo and Southgate, 2008). During sexual maturity, gametes move into the 'pearl pocket', an antero-ventral extension of the gonad (Fougerouse *et al.*, 2008) (Fig. 1.4) by entering the tissues between the two retractor muscles. The gonad should be empty during the pearl grafting procedure to initiate cultured pearl production; it hosts the nucleus and mantle grafts (see *section 1.5.2.4.*). For this to happen, pearl oysters are best grafted after spawning or conditioning which empties the gonads (Taylor and Strack, 2008).

1.3.5.3. Pearl pocket

This antero-ventral extension of the gonad is located beneath the retractor muscles between the gills and the ventral part of the adductor muscles (Fougerouse *et al.*, 2008). It is flattened laterally with a slim anterior zone.

1.3.5.4. Byssal gland

Situated at the mid zone of the retractor muscle, the double lobed byssal gland comprises of two bundles of grooves which end externally as byssus threads (Fougerouse *et al.*, 2008). The initially clear grooves get darkened gradually forming byssus (Fougerouse *et al.*, 2008). The flattened distal end of byssus threads attach *P. margaritifera* to substrates (Haws and Ellis, 2000; Fougerouse *et al.*, 2008).

1.3.5.5. Foot

The foot is situated between the mouth and the byssus in the anterior part of the oyster (Fougerouse *et al.*, 2008). It has a locomotory role during the early stages of life in pearl oysters (Doroudi and Southgate, 2002; Zhao *et al.*, 2003) and a site at its base is used for an excision through which the nucleus and mantle graft are inserted during

grafting (see *section 1.5.2.4.*) for pearl production in adult oysters (Taylor and Strack, 2008).

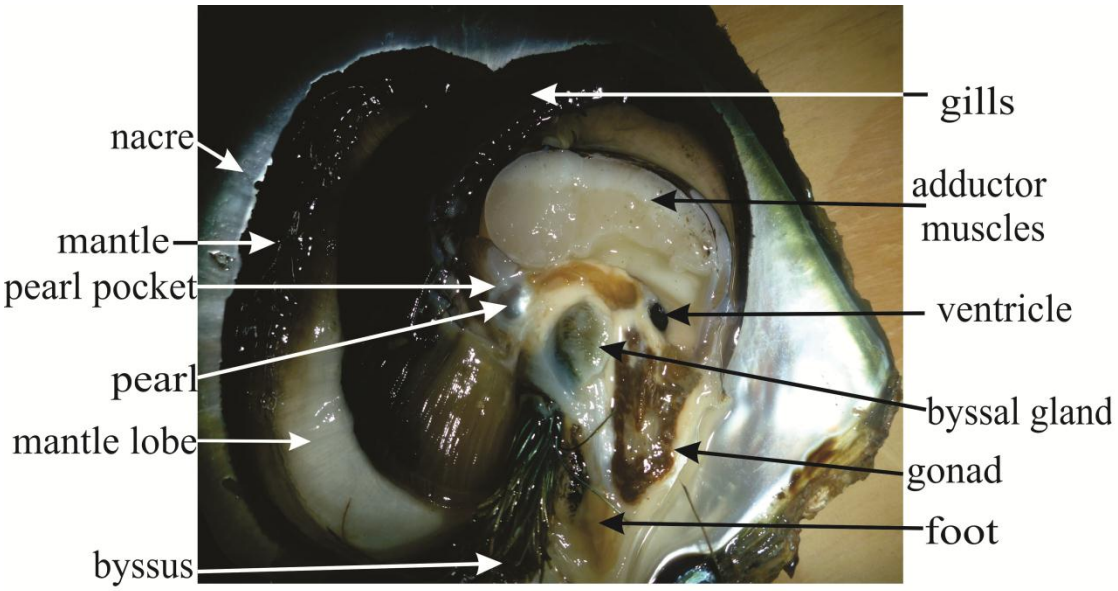

Figure 1.4. Anatomy of *Pinctada margaritifera* pearl oyster.

1.4. Culture of *Pinctada margaritifera*

1.4.1. Culture units

The preferred types of culture units for *P. margaritifera* depend on the various factors described by Southgate (2008). Wild spat of *P. margaritifera* can be recruited onto spat collectors made from materials such as tree branches, pearl oyster shells, corals and plastic materials (Southgate, 2008). The most commonly used is the "flower type" collector made from a plastic mesh (Southgate, 2008). Spat collector deployment coincides with spawning in oysters to maximise spat recruitment and spat are normally retained on collectors for 6-9 months before being removed to other types of culture units. Some of the culture units used include plastic mesh trays, mesh cages, lantern nets, panel nets, baskets, chaplets, box nets, pocket nets with and without frames, openable sandwich nets and circle nets (Gervis and Sims, 1992; Gaytan-Mondragon *et*

al., 1993; Southgate and Beer, 1997; Friedman and Southgate, 1999; Southgate and Beer, 2000; Urban, 2000). Hatchery produced spat are usually cultured in plastic mesh trays, panel nets or on spat collectors before being transferred to different culture units at a size of 60–90 mm (Southgate and Beer, 1997; Friedman and Bell, 2000; Pouvreau and Prasil, 2001; Southgate, 2008). The two most common types of culture method used for *P. margaritifera* use panel (pocket) nets or chaplets.

1.4.1.1. Panel (pocket) nets

Panel nets are made from strong steel or galvanised frames covered by mesh that is sewn to form pockets to hold the oysters (Fig. 1.5) (Southgate, 2008). Panel nets are available with different pocket and mesh sizes that increases with increasing oyster size (Gervis and Sims, 1992; Southgate, 2008).

Figure 1.5. *Pinctada margaritifera* housed in panel nets at J. Hunter Pearls (Fiji), pearl farm in Savusavu Bay, Vanua Levu, Fiji (photo: J. Hunter Pearls).

1.4.1.2. Chaplets

Chaplets are commonly used in French Polynesia, Cook Islands (Ellis and Haws, 1999; Haws and Ellis, 2000; Haws, 2002; Southgate, 2008) and Fiji for pearl oyster culture (Fig. 1.6). Tying a *P. margaritifera* to a chaplet involves drilling a small hole of approximately 1-2 mm diameter close to the hinge towards the posterior region or the 'ear'. Monofilament fishing line is then inserted through the hole and the oyster is tied to a single rope either individually or in pairs . This method is also known as "ear–hanging". Chaplets with oysters are then tied to long ropes used in suspended culture (Fig. 1.6). Use of chaplets for pearl oyster culture minimises cost and labour inputs needed for pearl production, however, oysters tied to chaplets are more susceptible to predation and wave agitation than those held in nets.

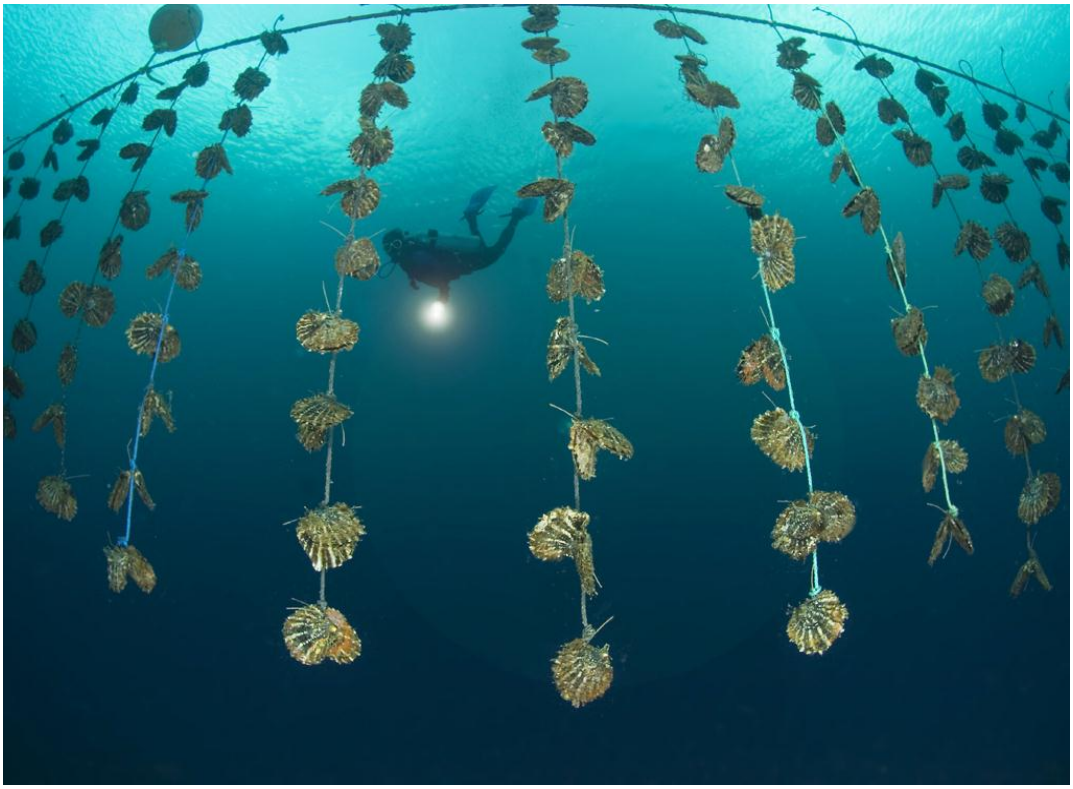

Figure 1.6. *Pinctada margaritifera* cultured on chaplets at J. Hunter Pearls (Fiji), pearl farm in Savusavu Bay, Vanua Levu, (photo: J. Hunter Pearls)

1.5. Cultured pearl production

Despite minor variations between the different pearl oyster species and farming practices, the grafting procedure used for cultured pearl production is still performed on the basis of the technique first developed by Kokichi Mikimoto in the early 1900s (Strack, 2008). Improvements have been made to this technique over the years by specialised pearl grafting technicians in an effort to maximise pearl production and pearl quality (Taylor and Strack, 2008). However, the skills developed and improvements made are confidential and highly guarded by grafting technicians. Described below are generalised methods for the preparation of pearl oysters for grafting, the grafting procedure and post-grafting care and culture of oysters.

1.5.1. Pre–grafting phase

1.5.1.1. Pre–grafting conditioning

Pre–operative conditioning techniques vary between different pearl oyster species. However, in all species, the intention is to generate reduced metabolic rate and to reduce or empty the gonad of gametes prior to grafting (Alagarswami, 1970; Dharmaraj *et al.*, 1991; Gervis and Sims, 1992; Haws, 2002; Taylor and Strack, 2008). Several methods are employed to condition tropical *P. margaritifera* that include: (i) covering the oysters with fine mesh covers; (ii) crowding and placing the oysters on the seabed with reduced access to food; and (iii) pulling the long lines containing oysters to the surface few weeks before grafting (Gervis and Sims, 1992; Taylor and Strack, 2008).

1.5.1.2. Anaesthetising

Use of anaesthetics has shown both positive and negative effects in pearl oyster culture (Norton *et al.*, 1996a; Mills *et al.*, 1997; Norton *et al.*, 2000; Acosta-Salmón *et al.*, 2005; Acosta-Salmón and Southgate, 2006). A trial to anaesthetise host oysters in attempts to reduce stress and damage caused to mantle and adductor muscles while opening their valves before grafting did not show promising signs because of high mortality, a high rate of nucleus rejection and low nacre secretion rate (Norton *et al.*, 2000). On the other hand, success has been achieved when anaesthetising donor oysters (see *section 1.5.2.1.*) before their mantles are excised for grafting (Acosta-Salmón *et al.*, 2004; Acosta-Salmón *et al.*, 2005; Acosta-Salmón and Southgate, 2006). Of the broad range of chemicals assessed, propylene phenoxetol is considered to be the most appropriate anaesthetic for pearl oysters as it provides rapid anaesthesia with short recovery times (Norton *et al.*, 1996b; Mills *et al.*, 1997; Acosta-Salmón *et al.*, 2005; Mamangkey *et al.*, 2009).

1.5.2. Grafting

Pearls are produced when a piece of mantle tissue (saibo) from a foreign (donor) pearl oyster and a round inorganic nucleus are inserted into the gonad of a host pearl oyster (Gervis and Sims, 1992; Taylor and Strack, 2008). This process is called ‘grafting’ or ‘seeding’. Pearl grafting is usually performed by skilled technicians that commonly work only with a single species of pearl oyster. After the graft operation, saibo tissue proliferates to form a pearl-sac, which envelopes the nucleus, and nacre secreting epithelial cells from the pearl-sac begin to deposit nacre onto the nucleus (Dix, 1972; Scoones, 1996; Taylor and Strack, 2008; Cochennec-Laureau *et al.*, 2010). For the *P. margaritifera,* a culture period of approximately 18 months is required before a

commercially acceptable nacre thickness of 0.35-0.8 mm (Haws, 2002; Matlins, 2002; Ruiz-Rubio *et al.*, 2006) is achieved and the resulting cultured pearl is ready to be harvested.

1.5.2.1. Donor oyster

Pearl oysters to be used as mantle graft or saibo donors are selected on the basis of their health and nacre colour (Taylor and Strack, 2008) because the graft used has an influence on the colour and quality of resulting pearls (Wada and Komaru, 1996; McGinty *et al.*, 2010; McGinty *et al.*, 2011). Obtaining saibo tissues involves sacrificing an oyster and removing the outer section of the mantle (marginal mantle) to expose pallial mantle (Fig. 1.7) which is carefully cleaned of dirt and mucus by wiping it with a clean wet cloth. The resulting strip of pallial mantle is then cut into small square pieces (ca. 3 mm^2) on a chopping board using a scalpel (Fig. 1.8). The number of saibo pieces generated from a donor oyster depends on its size but generally, a large *P. magaritifera* can provide approximately 20–30 pieces.

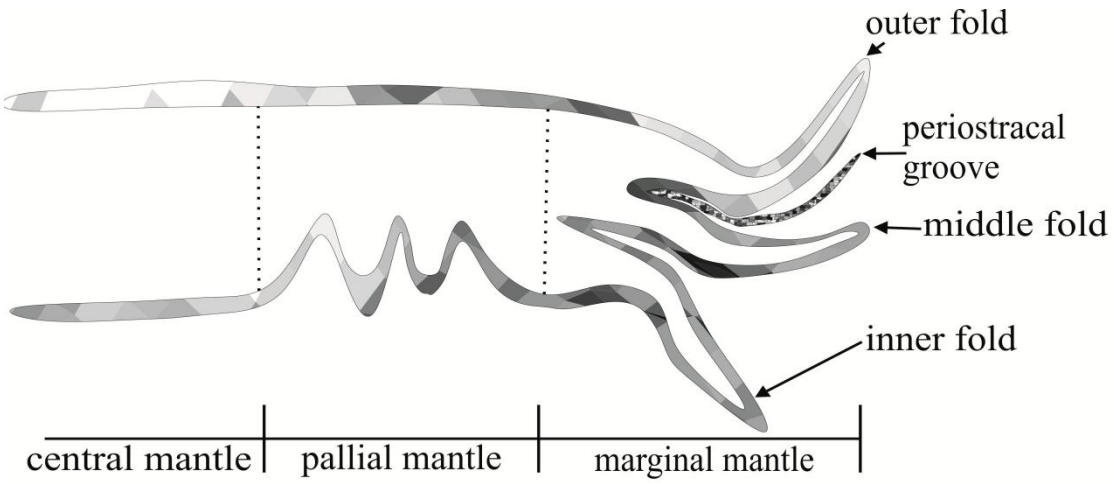

Figure 1.7. Cross-section of a mantle showing the pallial region from which saibo is excised for grafting (redrawn from Wada and Tëmkin, 2008)

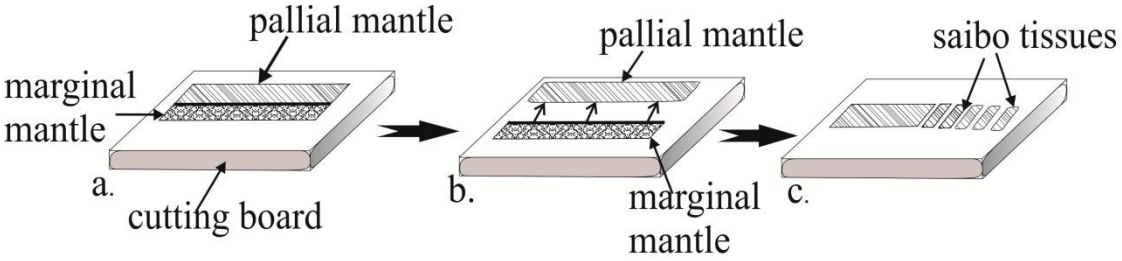

Figure 1.8. The process of saibo preparation for grafting in pearl oysters: a, mantle placed on the cutting board for sectioning; b, marginal mantle excised and removed; c, pallial mantle sectioned into small square pieces (ca. 3 mm^2) as saibo for grafting.

Donor oysters do not necessarily have to be sacrificed to obtain saibo tissue (Acosta-Salmón *et al.*, 2005). Saibo can be removed from anaesthetised oysters (Acosta-Salmón *et al.*, 2005) which have been reported to completely regenerate excised sections of mantle within a period of three months (Acosta-Salmón and Southgate, 2005; Acosta-Salmón and Southgate, 2006).

1.5.2.2. Host oysters

Host oysters preferably with empty gonads are cleaned before their valves are opened to a distance of approximately 25–30 mm with a speculum (Taylor and Strack, 2008). A specifically designed plastic wedge for pearl oysters is then inserted between the valves (to prevent closure) before the oyster is passed to the technician for grafting. The technician removes the wedge but maintains the opened valves using a speculum for grafting. The oyster is then placed in a clamp and positioned for a clear view of tissues before grafting begins (Taylor and Strack, 2008).

1.5.2.3. Nucleus

Nuclei used for pearl production are sourced from the shells of American freshwater mussels belonging to family Unionidae (Alagarswami, 1970; Fassler, 1991; Gervis and Sims, 1992; Fong *et al.*, 2005; Taylor and Strack, 2008). The shells are cut into cubes, tumbled in lapping machines and polished with hydrochloric acid (Gervis and Sims, 1992; Taylor and Strack, 2008). Nuclei from American freshwater mussel provide the required hardness, specific gravity and thermal conductivity needed to produce high quality pearls that are easily drilled for processing. However, due to declining stocks of American freshwater mussels, nuclei from other sources have also been investigated (Fassler, 2002; Taylor and Strack, 2008). Attempts to use nuclei made from giant clam shells as an alternative were not successful due to difficulty encountered while drilling the pearl for processing (Roberts and Rose, 1989); however, artificial nuclei manufactured from calcium carbonate have similar properties to nuclei from American freshwater mussels (Taylor and Strack, 2008) but are more expensive to produce.

The size of the nucleus to be used in a given grafting operation depends on the technicians' decision after the size of the pearl pocket (see *section 1.3.5.3)* and condition of the gonad has been examined. Unlike *P. fucata* (Alagarswami, 1987b), *P. margaritifera* as well as *P. maxima* only host one nucleus per grafting (Haws, 2002; Strack, 2006; Taylor and Strack, 2008).

1.5.2.4. Grafting procedure

After the oyster is placed in the clamp, the gills are pushed aside using a spatula before the foot is held securely by a hooked tool (Taylor and Strack, 2008; Mamangkey, 2009). An incision is made in the gonad along the foot retractor muscles using a

specifically shaped scalpel. The scalpel is then twisted downwards forming an arc towards the gonad ending (Mamangkey, 2009). The saibo and nucleus are then inserted using specially designed tools in an order which depends on the technician's choice. However, it is vital that the saibo is in contact with the nucleus once in position in the gonad (Fig 1.9). Failure to achieve this will result in the formation of a non–nucleated pearl or "keshi" (Taylor and Strack, 2008; Ito, 2009).

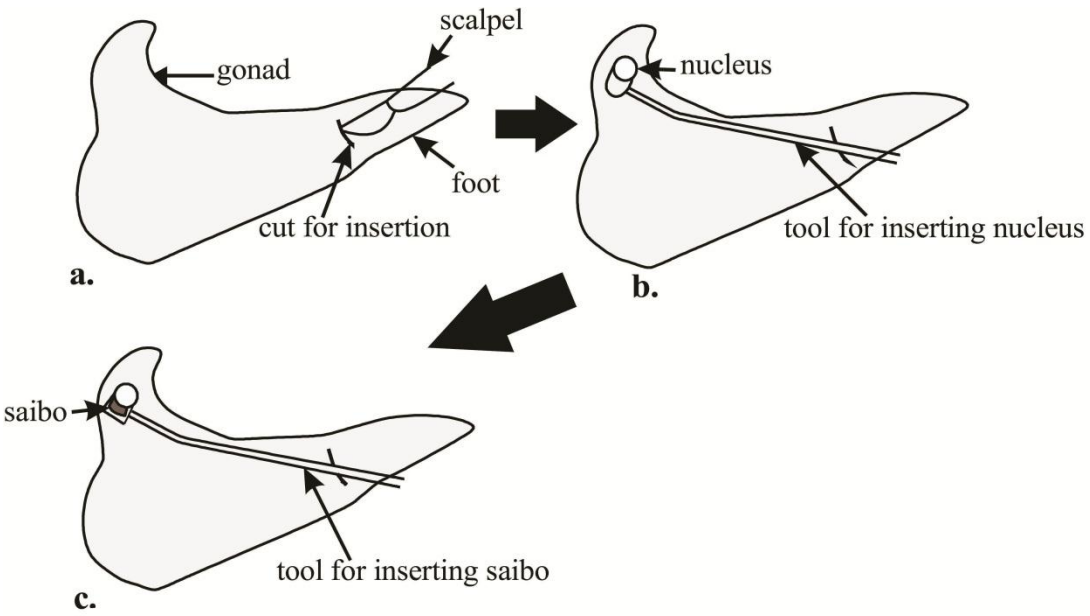

Figure 1.9. A schematic representation of the grafting procedure in pearl oysters showing: a. the cut made using a scalpel at the base of the foot; b. nucleus insertion through the cut using a special tool; and c. saibo inserted using a special tool and placed in contact with the nucleus.

1.5.3. Post-grafting care

Post-operative culture techniques vary between species and individual pearl farms. In normal circumstances, *P. margaritifera* are placed with their ventral side facing downwards in panel nets just after grafting. In some farms, grafted *P. margaritifera* are

first put into fine mesh bags (catcher bags) before being placed in panel nets (Taylor and Strack, 2008). The catcher bags trap any nuclei that may be rejected during the first six weeks after grafting enabling pearl farmers to have an idea on the approximate number of pearls to expect from the stock. Oysters are removed from the catcher bags after six weeks.

1.6. Pearl formation

A culture period of 1.5 to 2 years from grafting is required for pearls to fully develop in *P. maragatifera* (Ellis and Haws, 1999; Haws, 2002; Taylor and Strack, 2008). A minimum nacre thickness of 0.35-0.8 mm is required for round pearls to attain full commercial value (Haws, 2002; Matlins, 2002; Ruiz-Rubio *et al.*, 2006; Southgate *et al.*, 2008)

1.6.1. Cultured round pearl formation

After grafting the mantle graft proliferates to form a pearl-sac which encases the nucleus (Fig. 1.10). Scoones (1996) reported that pearl-sac development in *P. maxima* was completed 23 days after grafting and that nacre secretion was first observed after 30 days. However, varying periods of between 3 days to 65 days have been reported for pearl-sac formation in *P. fucata* (George, 1967; Wada, 1968; Achari, 1982). Earlier reports mentioned that pearl-sac development in *P. margaritifera* could take as long as six months (Haws, 2002) but a recent study confirmed that this occurred in only 14 days (Cochennec-Laureau *et al.*, 2010). Despite these two studies pearl-sac development in *P. margaritifera* has not been described in detail and there is a limited

basis for research investigating the potential impacts of pearl-sac structure and/or function of resulting pearl quality.

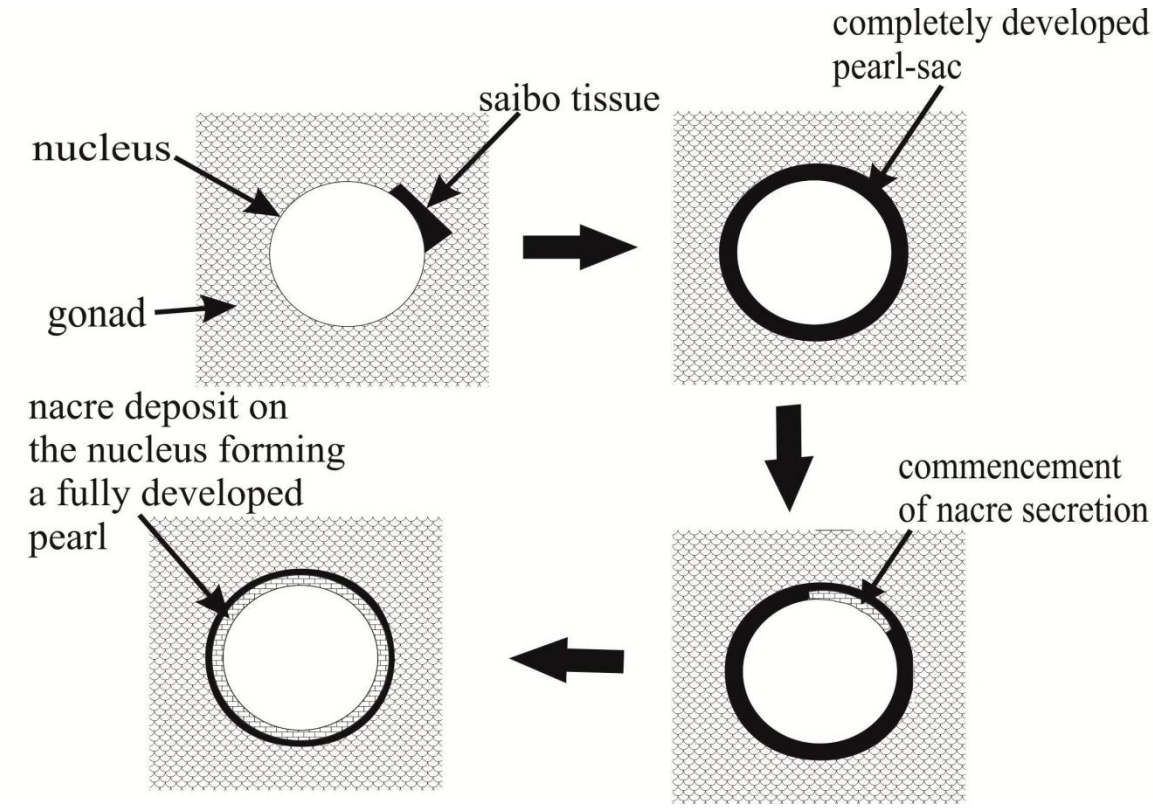

Figure 1.10. Schematic representation of pearl-sac development and nacre deposition after grafting in pearl oysters.

Once the pearl-sac is completely developed, the mantle epithelium begins to secrete a layer of organic matrix (Scoones, 1996; Cochennec-Laureau *et al.*, 2010) made from a network of fine meshes responsible for protein transportation around the nucleus (Strack, 2006; Taylor and Strack, 2008). A prismatic layer made up of radially assembled calcite crystals is next to be secreted (Strack, 2006; Taylor and Strack, 2008) and epithelial cells then begin to secrete nacre. The nacre layer is made up of crystal aragonite platelets with near hexagonal shapes that are arranged on top of each

other, similar to a "brick-mortar" arrangement (Wang *et al.*, 2001; Li and Huang, 2009; Rousseau *et al.*, 2009; Xie *et al.*, 2010). The aragonite platelets represent bricks and the organic matrix the mortar. Aragonite crystals in *P. margaritifera* have an average thickness of 0.5 µm (Taylor and Strack, 2008).

1.6.2. Keshi formation

A 'keshi' pearl is formed when a grafted oyster expels the nucleus before the pearl-sac is completely formed. It can also be formed if the mantle tissue fractures and forms its own pearl-sac without the nucleus (Taylor and Strack, 2008). Nacre secretion then commences and an irregularly shaped keshi is formed. Because keshis are composed of only nacre, their surfaces are generally highly lustrous. However, the production of marine keshi at this stage is insignificant compared to massive production of freshwater keshi which is over 1000 t per annum (Strack, 2006; Taylor and Strack, 2008).

1.7. Factors affecting round pearl quality

1.7.1. Grafting technique

Grafting technique is a critical factor that determines expulsion of nuclei, mortality of grafted oysters and the quality of pearls produced (Scoones, 1996; Cochennec-Laureau *et al.*, 2010). Grafting technicians should be highly skilled and experienced and have the capability to select host and donor oysters for high quality pearl production with maximum nuclei retention. The level of hygiene maintained during the grafting procedure highly influences nucleus retention rates (Scoones, 1996; Mamangkey and Southgate, 2009; Cochennec-Laureau *et al.*, 2010).

1.7.2. Saibo or graft tissue

Saibo is considered to be the most influential factor determining colour and quality of cultured pearls (O'Connor, 1975; Wada and Komaru, 1996; Knauer and Taylor, 2002). The colour of nacre on cultured pearls often resembles the colour of the hypostracum in the inner shell of the saibo donors (Alagarswami, 1970, 1987b; McGinty *et al.*, 2010). This was confirmed by recent studies where xenografts from *P. margaritifera* produced black based pearls in *P. maxima* and xenografts from *P. maxima* produced silver based pearls in *P. margaritifera* (McGinty *et al.*, 2010; McGinty *et al.*, 2011). Furthermore, McGinty et al. (2010) reported that pearl-sacs produced from xenografts of *P. margaritifera* and *P. maxima* showed transcriptionally active donor tissue genes during the biomineralisation process. This further demonstrates the influence of saibo tissues on pearl quality in host oysters. In all cases, young donor oysters are generally selected on the assumption of rapid nacre secretion forming a high quality pearl (Taylor and Strack, 2008).

1.7.3. Pearl development

The growth rates of pearls affect their ultimate quality. Optimum growth rates of pearls provide pearls with a round shape, smooth surface and good lustre (Strack, 2006). Pearls with fast growth rates develop irregular surfaces, often called “hammering” and have very low marketable value due to poor lustre (Ma *et al.*, 2007; Taylor and Strack, 2008). For this reason, pearl farmers harvest pearls during winter in the belief that low water temperature slows the metabolic rate of pearl oysters, nacre deposition rates will be slow resulting in pearls with improved lustre and smooth surfaces (Alagarswami, 1987b; Ruiz-Rubio *et al.*, 2006; Wells and Jernakoff, 2006). This practice was however developed within the Japanese cultured pearl industry which is conducted in temperate

waters where there are relatively large differences between winter and summer water temperatures. How relevant this practice is in tropical waters at much lower latitudes is yet to be demonstrated.

1.8. Pearl grading

Grading determines the overall quality and commercial value of pearls after considering five main characteristics; shape, size, lustre, colour and surface perfection (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008). However, unlike most other gems, there is no internationally recognised criteria to grade pearls (Matlins, 2002; Ruiz-Rubio *et al.*, 2006; Strack, 2006) despite suggestions on grading guidelines from organizations such as Gemological Institute of America, Gem Testing Laboratory and World Pearl Organization (Strack, 2006). Pearl grading is therefore a subjective exercise and the extent to which individual characteristics contribute to a pearl's overall grade may be perceived differently by different pearl graders. Generally, large size, round shape, high lustre, no surface complexity and bright colour are the characteristics of a high quality pearl.

Pearls are normally graded using an AAA-A or A-D system (Matlins, 2002; Strack, 2006). The AAA-A system is normally used to grade Akoya pearls while A-D is considered as the Tahitian grading system for 'black' pearls. In the AAA–A system pearls have decreasing quality from AAA to A, while in the A–D system, the quality decreases from A to D (Matlins, 2002; Strack, 2006).

1.8.1. Shape

Pearls occur in a wide range of shapes (Fig. 1.11). The shape of a cultured round pearl is influenced by its nucleus, which provides the foundation for the production of a round shaped pearl (Strack, 2006). However, production of a perfectly round cultured pearl is rare and a perfectly round shaped pearl is considered of high quality (Matlins, 2002).

Pearls are normally graded into different basic shapes before other grading characteristics are considered (Matlins, 2002; Strack, 2006). The choice of basic shapes varies amongst different pearl graders but the common shape categories into which pearls are usually categorised (Matlins, 2002; Strack, 2006) are:

- **Round**: pearls that occur rarely and are highly desirable due to their perfectly spherical shape.
- **Near round**: these are pearls that appear round at a glance. However, upon closer inspection, some flaws such as slightly flat or elongated shape will be discovered. Nevertheless, near round shaped pearls are often classified as spherical pearls since the flaws on these pearls are very minor.
- **Drop**: these are elongated pearls with teardrop–shapes. The size of the drop varies depending on its proportion to pearl.
- **Button**: Pearls that are flat on one side and round on the other are classified as button shaped pearls or coin shaped pearls.
- **Semi–baroque**: these are pearls with slight irregular shapes compared to button, drop and near round shaped pearls. As a further comparison to pearl shapes mentioned above, semi–baroque pearls are not symmetrical while the above shapes are symmetrical.

- **Baroque**: pearls that are not symmetrical and have irregular shapes.
- **Circle**: circle pearls are symmetrical pearls that have grooves or concentric lines around pearl surface.

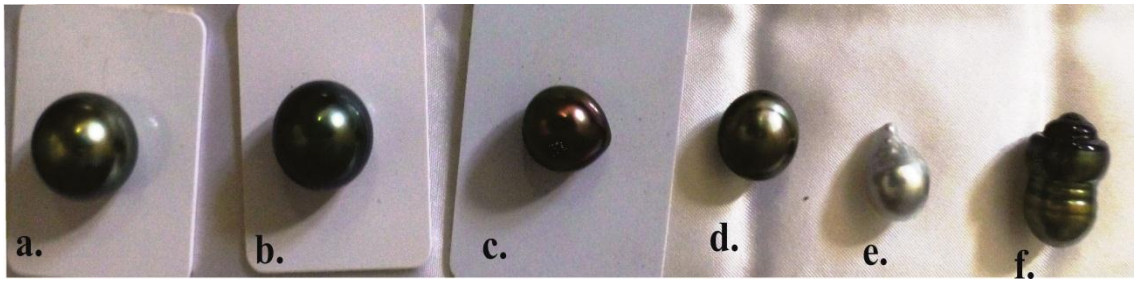

Figure 1.11. 'Black' pearls of different shapes from *Pinctada margaritifera*: a, round pearl; b, near round; c, drop; d, button; e, baroque; f, circle.

1.8.2. Size

The size of a pearl is determined by measuring the diameter at its widest points in *bu* (a Japanese term equal to 3.1 mm) or millimeters (Taylor and Strack, 2008). Pearls less than 7 mm in size are generally regarded as small with much reduced commercial value (Strack, 2006). 'black' pearls from *P. margaritifera* generally range from 9-16 mm in size (Fig. 1.12) (Strack, 2006; Taylor and Strack, 2008), although pearl size can be increased by extending the culture period which will enable more nacre deposition increasing the nacre thickness.

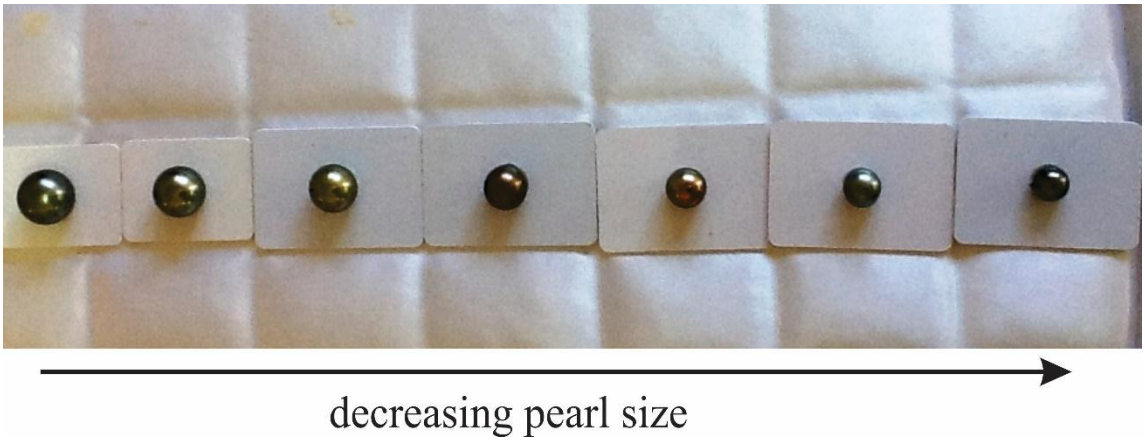

Figure 1.12. 'Black' pearls of different sizes from *Pinctada margaritifera* at J. Hunter Pearls.

1.8.3. Lustre

Lustre is the reflective ability or brilliance of the surface of a pearl. Pearls of high quality will be highly lustrous, shiny (Fig. 1.13) and will reflect lights and images similarly to mirrors (Strack, 2006). In contrast, low quality pearls are dull and chalky with minimum to no reflectivity (Matlins, 2002). The perception of the level of lustre may vary between pearl graders resulting in different appraisals for the same pearl by different graders.

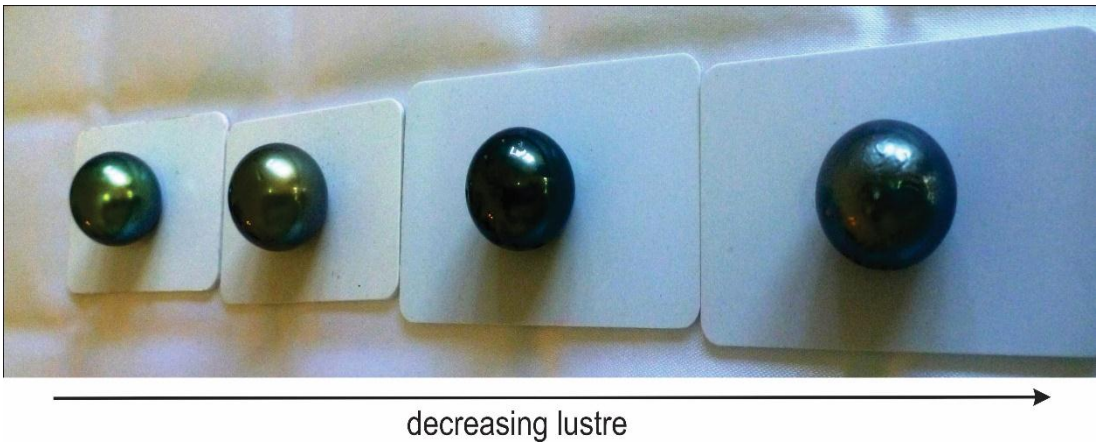

Figure 1.13. 'Black' pearls from *Pinctada margaritifera* with variable lustre at J. Hunter Pearls.

1.8.4 Colour

Pearls appear in assorted colours (Fig. 1.15) and pearls produced by *P. margaritifera* are perhaps the most variable in colour. The different colours of pearls are derived from their reflective and refractive nature and are determined by the individual arrangement of their aragonite platelets and conchiolin or organic matrix layers that are responsible for interference and diffraction of light (Fig. 1.14) (Liu *et al.*, 1999; Snow *et al.*, 2004; Strack, 2006; Taylor and Strack, 2008).

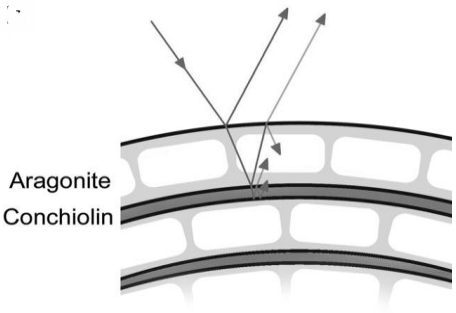

Figure 1.14. Refraction and reflection of light from agragonite and conchiolin layers that gives the pearls their colours. (Source: Taylor and Strack, 2008)

'Black' pearls generally have a deep black and dark silvery body tones with green gleam (Snow *et al.*, 2004). The derivation of body colour in pearls is still not well understood but it is believed that the causes are due to different pigmentation of the material binding the aragonite platelets or optical interference in platelets of nacre (Elen, 2002; Snow *et al.*, 2004). Furthermore, it has been reported that dark body colours in pearls with thin nacre could be due to separate dark layers of conchiolin (Snow *et al.*, 2004). The colour characteristics that increase the value of pearls vary with pearls that are golden, oceanic blue or have overtones of green, pink or purple having the highest ranking amongst 'black' pearls (Strack, 2006).

Figure 1.15. Assorted colours of cultured pearls produced by *Pinctada margaritifera* at J. Hunter Pearls showroom (Source: J. Hunter Pearls)

1.8.5. Surface perfection

The degree of flawing or irregularities, including gaps, chips, lumps, cracks and grooves (Fig. 1.16), on the surface of pearls is taken into account when grading pearls for surface perfection (Matlins, 2002; Taylor and Strack, 2008). With an increasing amount of surface flaws, there is decreasing pearl quality and value. Surface imperfections are common to all types of cultured pearls. However, in 'black' pearls, the presence of concentric surface grooves or circles is a particular concern which decreases the quality and value of pearls significantly (Ito, 2009).

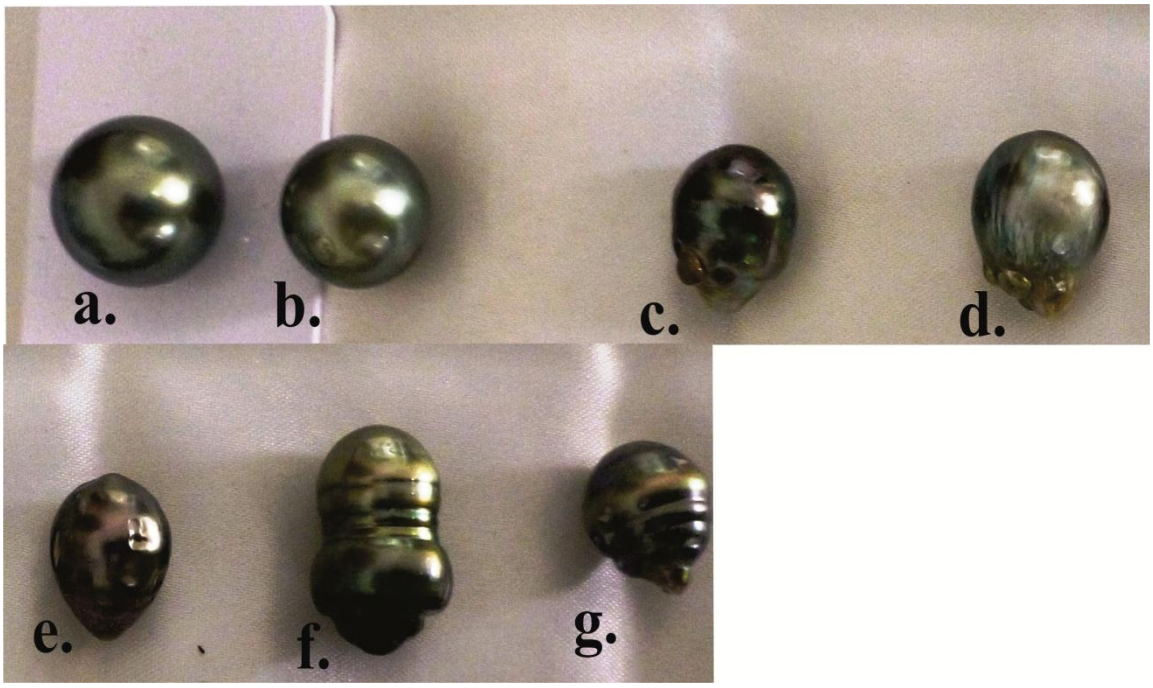

Figure 1.16. 'Black' pearls from *Pinctada margaritifera* with different surface features: a, perfect surface pearl; b, pearl with surface gaps; c and d, pearls with lumps; e, pearl with cracks; f and g, pearls with circles.

1.9. Short falls in the 'black' pearl sector

The cultured 'black' pearl industry is the second largest income generator after tourism in French Polynesia (Southgate, 2007). Pearl production in French Polynesia relies primarily on wild spat recruited on spat collectors. In the year 2000, French Polynesia had the highest recorded production of cultured pearls of 11 t and by 2001, the industry had expanded greatly with the number of authorised marine leases for pearl farming reaching an all-time high of 2500 (Southgate *et al.*, 2008).

However, while there was an increase in pearl production, very little attention was given to pearl quality which declined. The majority of pearls produced were small with nacre thickness of <0.8 mm and poor lustre. Because of this poor quality, global

demand for French Polynesian pearls dropped (Southgate, 2007) and the prices of pearls decreased markedly from US$77 per gram in 1986 to US$13 per gram in 2000 (Lane *et al.*, 2003). In response the government of French Polynesia imposed a series of regulatory measures including x-raying all pearls to determine nacre thickness and those not meeting the required minimum thickness of 0.8 mm (Southgate *et al.*, 2008) to be destroyed. Pearls with high surface irregularities and of low grades are also destroyed while pearls classified as 'D' grade are not permitted for export (Southgate *et al.*, 2008). These regulatory measures resulted in closure of pearl farms that were unable to meet these standards (Southgate *et al.*, 2008) and this in turn resulted in a decrease in the production volumes to around 10 t in 2003 and 8-9 t in 2005 (Southgate *et al.*, 2008; Müller, 2013). As a part of the regulatory measures, the French Polynesian government now regulates the number of marine leases to be issued and provides technical assistance to pearl farmers to improve pearl quality. There were 487 operational pearl farms on 25 atolls and islands that employed around 5000 people in French Polynesia in 2014 (Ky *et al.*, 2015).

In the Cook Islands, 'black' pearl production peaked in the year 2000 earning a total of US$18 m which was 20% of the total domestic product revenue (Müller, 2005, 2009). However, this resulted in pearl farms being overstocked which, coupled with poor farming practices, lead to a major disease outbreak caused by pathogenic bacteria, *Vibrio harveyi,* which resulted in mass mortality of pearl oysters (Heffernan, 2006; Southgate, 2007; Southgate *et al.*, 2008). This caused a decline in income generated from pearl exports to US$2 m in 2005 from 205 pearl farms (Southgate *et al.*, 2008). Pearl revenue declined further over subsequent years and in 2013/14, the value of pearls exported from the Cook Islands was NZ$0.24 m (ca. US$0.18 m) (Anonymous,

2014b). It is predicted that this amount would remain stable for the following 1-2 years, with pearl production generated from 270,000-300,000 grafted oysters producing around 106,000-115,000 saleable pearls (Anonymous, 2014b).

While the two major 'black' pearl producing countries struggled to improve the quality of 'black' pearl production, J. Hunter Pearls was established in Fiji in the year 2000. The company was founded on the basis of an extensive research program lead by the Fisheries department in Fiji that identified Savusavu on the island of Vanua Levu as a site supporting excellent recruitment of *P. margaritifera* spat and high growth rates of pearl oysters resulting from high levels of phytoplankton generated by deep water upwelling. This research first demonstrated the production of cultured pearls with a unique range of colours in Savusavu Bay. J. Hunter Pearls uses Japanese technicians for grafting and produces some of the best 'black' pearls in the world (Anon, 2007) under 'Fiji Pearls' branding. Cultured pearls from Fiji have gained an international reputation for quality and particularly for their unique colour range that differentiates these pearls from Polynesian cultured pearls. A number of other pearl farms have now been established in Fiji (see *section 1.2.*) and, although cultured pearl production is comparatively low to that of French Polynesia and Cook Islands, it is growing and there is considerable international demand for 'Fiji Pearls'.

Current expansion of the cultured pearl industry in Fiji brings with it a risk that similar obstacles to those faced by French Polynesia and Cook Islands could also occur in Fiji if proper protocols are not developed and followed. Fiji currently has an excellent international reputation for high quality cultured pearls, but there is a need to further protect the quality and maintain the current standards. This is likely to be increasingly

difficult as more pearl farms come on line in Fiji. Furthermore, the high quality cultured pearls on which Fiji's reputation is based make up only approximately 3-5 % of the total pearl harvest (Justin Hunter, J. Hunter Pearls, *pers. comm*., 2014) and, like French Polynesia and the Cook Islands, the majority of pearls harvested have surface abnormalities. There will therefore be an increasing volume of lower grade pearl production in Fiji as the industry expands which, should these pearls enter the global pearl market, could threaten the "Fiji Pearl" reputation. Every effort should therefore be made to reduce the production of low quality pearls in Fiji and expansion of the industry should be on the basis of learning from the experiences of the more established cultured pearl industries in French Polynesia and the Cook Islands.

1.10. Research needs

There is extensive scientific literature on the biology and culture of pearls oysters (e.g. Southgate and Lucas, 2008). Much of this focuses on pearl oyster husbandry but surprisingly little addresses the factors that influence pearl quality. As stated above, it is generally assumed that around 3%-5% of a given crop of pearls will generate around 95% of a crop's value (Haws, 2002). On this basis, even a relatively small increase in the proportion of high quality pearls would result in significant economic benefits for farmers and for the industry as a whole. For cultured pearls from *P. margaritifera*, the major characteristic reducing pearl quality and value is the presence of circles (see *section 1.2.*). In the Tuamotu Archipelago of French Polynesia for example, circled pearls were reported to account for 23% of a harvest of 271,000 cultured pearls from *P. margaritifera*, but making up only 6% of the value of the crop (Murzyniec-Laurendeau, 2002). Yet despite such significant economic impacts, the cause(s) of circle formation

in cultured pearls is still not known. The major theme in this study is to generate greater knowledge of the factors affecting pearl quality, including circles. Fundamental to this is a detailed understanding of the formation and structure of the pearl-sac in *P. margaritifera* which, surprisingly, has not previously been reported in detail. Anomalies in pearl-sac structure are likely to impact resulting pearl quality, but what are they and what are their likely impacts?

Section 1.5. outlined the general procedure used for cultured pearl production which was developed in the early 1900s and has changed little since. It uses mantle tissue (saibo) from high quality donor oysters that are usually sacrificed for mantle excision. Around 20-30 pieces of saibo are taken from a single donor oyster (see *section 1.5.2.1.)* for pearl production and, should these be of particularly high quality, the donor cannot be used for pearl production again because it was sacrificed for saibo donation. It is possible however for saibo to be excised from anaesthetised donor oysters without sacrifice (Acosta-Salmón and Southgate, 2005) and the excised mantle tissue will regenerate within few months (Acosta-Salmón and Southgate, 2005; Mamangkey, 2009). This opens up the possibility of using high quality saibo donors on more than one occasion which could result in production of a higher proportion of high quality cultured pearls. Prior research with *P. maxima* has reported that regenerated mantle tissue can be used successfully as saibo (Mamangkey, 2009); it is able to form a functional pearl-sac with nacre secreting ability. However, this possibility has not been previously been investigated for *P. margaritifera* despite the potential advantages which include an increase in the proportion of higher quality pearls and the use of high quality donor oysters for selective breeding. If regenerated mantle tissue can be used

successfully as saibo, does pearl-sac structure differ from those grown from 'normal' saibo and how do nacre deposition rates compare between the two?

In French Polynesia and the Cook Islands, the *P. margaritifera* used for pearl culture readily recruit to spat collectors within atoll lagoons (Cabral *et al.*, 1985; Sims, 1992) In contrast, spat collection in the Fiji is conducted among the 'open reef' systems of high islands which are influenced by currents and other oceanic factors to a much greater degree. Pearl oyster recruitment to spat collectors is consequently lower in Fiji compared to pearl producing areas in Polynesia. Given the relatively restricted availability of oysters to the Fijian cultured pearl industry, every attempt should be made to utilise *P. margaritifera* to their full potential and to maximize production from these oysters. A common practice in the cultured 'black' pearl industry is to discard oysters that produce low quality pearls or pearls with excessive numbers of circles after the first grafting. This is done on the assumption that these oysters will not show significant improvements in pearl quality if grafted for a second time. However, if oysters that produce low quality circled pearls are capable of improved pearl quality after re-grafting, this would provide opportunities for pearl farmers to build farm stock and improve pearl production and income. This possibility has never been tested.

Pearl culture in Polynesia is done predominantly using chaplets (see *section 1.4.1.2.)* which minimizes costs and labour needed for pearl production, but increases the susceptibility of oysters to predation and the impacts of wave agitation. This latter point is probably more important in the 'open reef' waters of Fiji than in the relatively sheltered lagoonal waters of Polynesian atolls. However, there is anecdotal suggestion that this method of culture results in a greater proportion of circled pearls within a

given crop. This is an important consideration because if this is the case then simply changing culture method from chaplets to nets may result in improved pearl quality. Pearl culture in Fiji was established using the husbandry methods developed in French Polynesia, however, given the differences in culture environments between the two countries, this may not be the most appropriate culture method for Fijian conditions. Fundamental to the further development of the Fijian cultured pearl industry is an understanding of the most appropriate culture methods for pearl production and the influence of culture method on pearl production and pearl quality.

1.11. Overall objective

The overall objective of this study is to improve the quality of cultured round pearls produced by *P. margaritifera* in Fiji through a better understanding of the factors influencing pearl quality. This objective will be addressed through a series of experiments with the following aims:

- to describe pearl-sac development in *P. margaritifera* in detail to identify factors that may influence resulting pearl quality (Chapter 2);
- to examine haemocyte responses in *P. margaritifera* during and subsequent to pearl-sac formation identifying factors that may influence resulting pearl quality (Chapter 3);
- to describe pearl-sac development and determine the rate of nacre deposition in *P. margaritifera* grafted for pearl production using regenerated mantle (Chapter 4);
- to determine whether oysters producing circled pearls are able to produce pearls with improved quality after re-grafting (Chapter 5)

- to determine the influence of culture method on byssus production by *P. margaritifera* cultured at sites with dissimilar water currents (Chapter 6); and
- to assess the quality of pearls produced by *P. margaritifera* cultured using different culture methods (Chapter 7).

Chapter 2

[1]A detailed description of pearl-sac development in the black-lip pearl oyster, *Pinctada margaritifera*

2.1. Introduction

A cultured round pearl is produced when a round nucleus and a piece of mantle tissue (called 'saibo') from a donor pearl oyster are inserted into the gonad of a host pearl oyster (Gervis and Sims, 1992; Taylor and Strack, 2008). The nucleus and mantle graft are positioned into the extended antero-ventral portion of the gonad known as the 'pearl pouch' (Scoones, 1996; Fougerouse et al., 2008) and this process is referred to as 'seeding' or 'grafting' (Gervis and Sims, 1992; Taylor and Strack, 2008). The mantle (graft) and the nucleus must have contact with each other to enable the graft tissue to proliferate and form a spherical 'pearl-sac' that envelopes the nucleus. Secretory (epithelial) cells of the pearl-sac, that are only completely formed once the pearl-sac has fully developed (Scoones, 1996), deposit nacre onto the nucleus which will subsequently be harvested as a cultured pearl. For the black-lip pearl oyster, *P. margaritifera*, a culture period of approximately 18 months is required before a commercially acceptable nacre thickness of approximately 0.35 mm (Haws, 2002; Matlins, 2002; Ruiz-Rubio *et al.*, 2006) is achieved and the pearl is ready to be harvested. The functioning of the pearl-sac is crucial in determining key factors of pearl quality such as shape, nacre thickness and surface characteristics. On this basis,

[1] This chapter was published as:
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detailed knowledge of the structure and function of the pearl-sac is an important step towards a better understanding of pearl formation and improving pearl quality.

Scoones (1996) reported that inflammation caused in the pearl-sac of *P. maxima* during the grafting procedure is one of the primary causes of inferior pearl quality. Inflammation in the pearl-sac cavity is the main cause of nucleus rejection following the grafting procedure (Cochennec-Laureau *et al.*, 2010) and formation of irregularly shaped (baroque) pearls (Aoki, 1961; Scoones, 1996; Hänni, 2006; Cuif *et al.*, 2008) is thought to result from large aggregations of haemocytes between the nucleus and epithelial cells during pearl-sac development (Scoones, 1996; Cochennec-Laureau *et al.*, 2010; Awaji and Machii, 2011). Cochennec-Laureau et al. (2010) further suggested that in *P. margaritifera*, if the mantle graft is placed close to the incision site, then development of the pearl-sac is slow and uneven and results in irregularly shaped pearls. However, no such explanation has been provided for the production of 'circled' pearls, which have symmetrical grooves across their surfaces. Circles are considered to be one of the major factors reducing the quality of "black" South Sea Pearls produced by *P. margaritifera* (Cartier *et al.*, 2012).

Most knowledge about pearl-sac development in pearl oysters relates to *P. maxima* (Dix, 1973; Scoones, 1996; Mamangkey, 2009), and *P. fucata* (Kawakami, 1953; Awaji and Machii, 2011). Although there have been a number of studies involving histological analysis of pearl-sac development in *P. margaritifera* (Jabbour-Zahab *et al.*, 1992; Arnaud-Haond *et al.*, 2007; Cuif *et al.*, 2008; Dauphin *et al.*, 2010), no prior study has described development in detail. For example, while the study of Cochennec-Laureau et al. (2010) described pearl-sac development in this species, the emphasis of

their paper was on factors influencing nucleus rejection and retention rates and it did not therefore provide a fine-scale description of pearl-sac development. The sampling protocol used did not allow Cochennec-Laureau et al. (2010) to identify when exactly the pearl-sac was fully formed and, as a result, it was reported that the pearl-sac was "almost complete" 12 days after grafting and "already established" by 30 days after grafting. The objective of this study was therefore to undertake a finer scale description of pearl-sac development in *P.margaritifera* following grafting, and to identify factors that may influence resulting pearl quality.

2.2. Material and methods

This study was carried out at a commercial pearl farm in Savusavu Bay on the island of Vanua Levu in Fiji (16˚47′18.63″S, 179˚18′08.82″E). It is operated by J. Hunter Pearls (Fiji). The oysters considered for this experiment were among those collected as wild juveniles (spat) from spat collectors deployed at various sites within Savusavu Bay. Once removed from spat collectors after cutting string-like byssus threads close to the hinge, oysters were then cultured using standard commercial methods until reaching a size suitable for pearl grafting. The oysters were cleaned before being grafted by one of the three professional and very experienced black-lip pearl oyster grafting technicians. The grafting procedure generally followed that described by Taylor and Strack (2008); briefly, it included careful selection of healthy donor oysters, excision of mantle tissue from each shell valves, stripping the pallial mantle from the excised mantle portion and cutting it into small square pieces to obtain saibo. A nucleus with a piece of saibo was then grafted into the pearl pouch of each recipient oyster. The maximum time between saibo preparation and its use for pearl grafting was less than 25 minutes. A total of 110 oysters with mean (±SE) dorso-ventral measurement of 112.06 ± 0.45 mm and antero-

posterior measurement of 110.82 ± 0.41 mm were randomly selected from the available pool of oysters grafted by an individual technician on a particular day. These oysters were then housed in pocket (panel) nets before being transferred to the farm site (16˚46′13.30″S, 179˚19′20.17″E) and deployed from a long line at a culture depth of 7 m which had a water temperature of 26.1°C.

Beginning two days after grafting, five oysters were sacrificed every second day until 40 days after grafting. Subsequently, five oysters were sacrificed at 4-day interval until 48 days after grafting. Oyster tissues containing the nucleus were preserved in "FAAC" solution composed of 25 ml of glacial acetic acid, 6.5 g of calcium chloride dehydrate and 50 ml of 37% formaldehyde per 500 ml of distilled water (Miller and Stewart, 2013). The tissues were preserved for approximately two months before histological analysis. During this period, glacial acetic acid in the preservative solution dissolved the nuclei present in the pearl-sac samples prior to histological preparation.

The tissues were held in 70% ethanol for 12 hours before the pearl-sacs were sectioned and placed in tissue cassettes prior to dehydration through different stages of ethanol of different concentrations and xylene (Acosta-Salmón and Southgate, 2005; Mamangkey and Southgate, 2009). The tissues were then embedded in paraffin blocks for microtome sectioning to a thickness of 5 μm before specimens were mounted on glass slides and placed in an oven for 24 h at 60 °C to remove paraffin. Specimens were then stained with Haematoxylin and Eosin, a process which approximately took 15 minutes (Acosta-Salmón and Southgate, 2005; Acosta-Salmón and Southgate, 2006; Mamangkey and Southgate, 2009). Specimens were inspected using a photo microscope (Olympus, SZ-CTV) and an optical microscope (Olympus, BX40). The

images were produced using an Olympus DP12 camera and were used to describe the different stages of pearl-sac development within the 48 day period immediately after grafting.

2.3. Results

Of the 110 oysters used in this study, ten rejected the implanted nucleus and another six died during the course of the sampling period. The process of sectioning samples required great attention and care to prevent damage to mantle grafts and host tissues that would subsequently impede morphological description. This is illustrated in Fig. 2.1a which shows torn mantle graft and host tissues. Other figures show that oysters secreted large quantities of haemocytes in response to injury caused when surgical tools were inserted from the base of the foot through the gonad and into the pearl pouch during the grafting procedure (Figs. 2.1b and 2.1c).

2.3.1. Pearl-sac development

Two days after grafting, the entire perimeter of the nucleus cavity showed the presence of haemocytes (Fig. 2.1b). In two samples, parts of the undissolved nucleus were still present in the nucleus cavity closely associated with the graft tissue (Fig. 2.1c) which had retained some cilia (Fig. 2.1c and 2.1d).

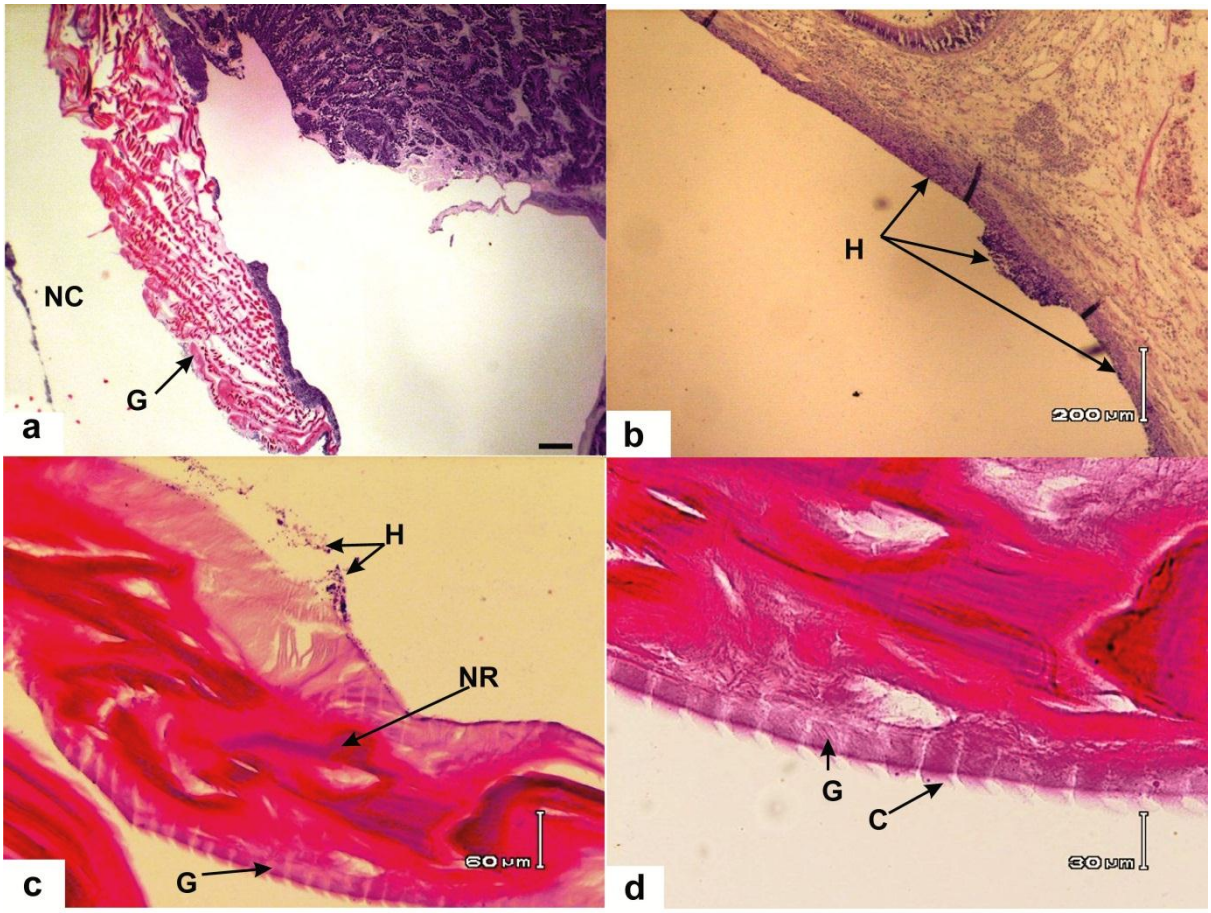

Figure 2.1. Pearl-sac development two days after grafting in *Pinctada margaritifera* showing: a, extension of a section of the torn mantle graft (G) within the nucleus cavity (NC); b, haemocytes (H) accumulating on host tissue; c, undissolved nucleus remnant (NR) and mantle graft (G) in nucleus cavity with haemocytes (H); d, cilia (C) present on the mantle graft (G). Bar scale: 100 µm

A whole view of a nucleus cavity four days after grafting is shown in Fig. 2.2a. It had a continuous layer of haemocytes lining the cavity. Attachment of the mantle graft tissue to host tissues occurred only at its mid-section with gaps visible at the two extending ends (Fig. 2.2b). Gaps arising between the proliferating mantle graft tissue and host tissues were mostly filled with haemocytes (Fig. 2.2c). Development of different

muscle fibers was also evident with their associated connective tissues (Fig. 2.2c). Three samples had a layer of mucus present on the upper surface of the graft (Fig. 2.2d). Grafts were in the process of developing nacre secreting epithelial cells and were extending around the nucleus cavity with acidophilic cells present at their base (Fig. 2.2d).

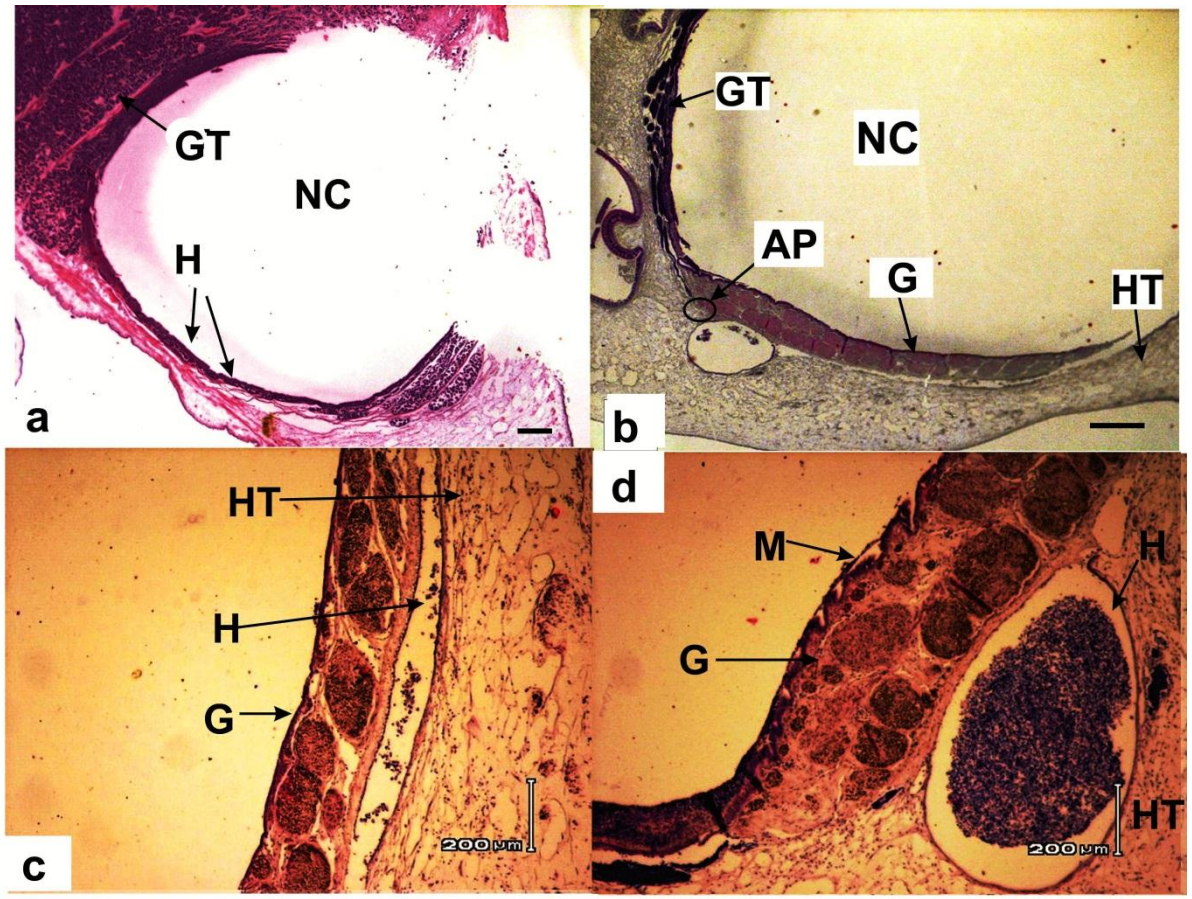

Figure 2.2. Pearl-sac development four days after grafting in *Pinctada margaritifera* showing: a, a low power view of the nucleus cavity (NC) with gametes (GT) and haemocytes (H); b, attachment point (AP) of a mantle graft (G) to host tissues (HT) with gametes (GT); c, haemocytes (H) in gaps between the host tissues (HT) and mantle graft (G); d, aggregated haemocytes (H)

between the host tissue (HT) and mantle graft (G) with a mucus layer (M) on top. A = 200 μm, B = 100 μm

Figure 2.3a shows a low power view of the nucleus cavity, accumulated haemocytes, and the relationship between the nucleus and the gonad tissue six days after grafting. Growth of the mantle graft tissue continued but attachment of the graft tip to host tissues was now apparent at the extending ends. However, in all samples, a gap between graft tissue and host tissue was now evident in the mid-section of the graft (Fig. 2.3b). Gametes were noted between the graft and host tissue (Fig. 2.3c) and outer epithelial cells were seen for the first time within the developing pearl-sac (Fig. 2.3d).

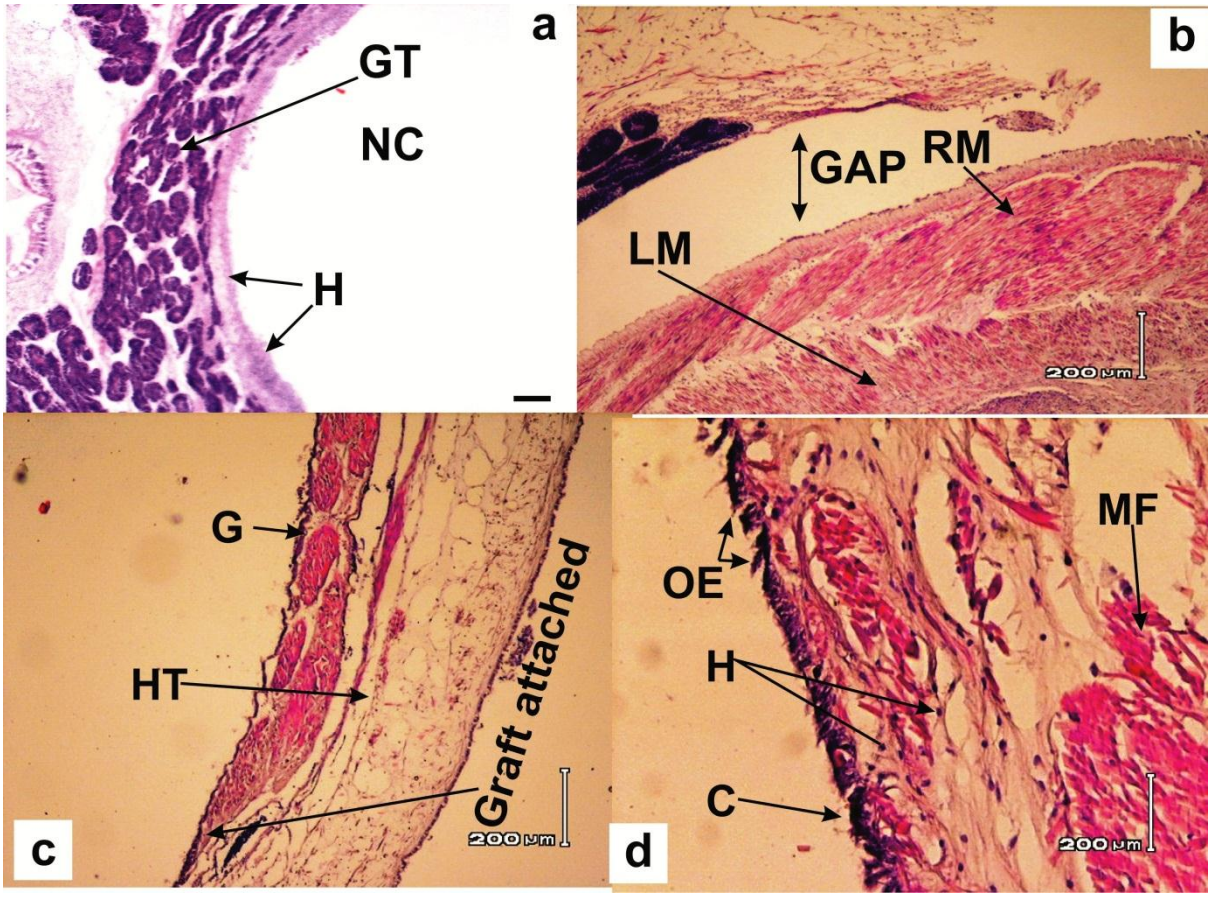

Figure 2.3. Pearl-sac development six days after grafting in *Pinctada margaritifera* showing :a, gametes (GT) and haemocytes (H) along the nucleus cavity (NC) circumference under a low power view; b, longitudinal muscles (LM) and radial muscles (RM) with a gap between the mantle graft and host tissue; c, attachment of the mantle graft (G) to host tissue (HT) at the extending ends of the mantle graft (G); d, haemocytes (H), developing outer epithelial cells (OE), cilia (C) and muscle fibres (MF) in the mantle graft. Bar scale: 100 µm

Gaps between the host tissue and the mantle graft had decreased as a result of continued proliferation of the graft tissue by eight days after grafting (Fig. 2.4a) and muscle fibres were clearly visible within the developing pearl-sac (Fig. 2.4b). Mantle

grafts were still distinguishable from the host tissue and had covered more than half the circumference of the nucleus cavity (Fig. 2.4c). Tissue differentiation was now clearly evident within the developing pearl-sac (Fig. 2.4d). Although variable between samples, there was a general decrease in the quantity of haemocytes present in the nucleus cavity.

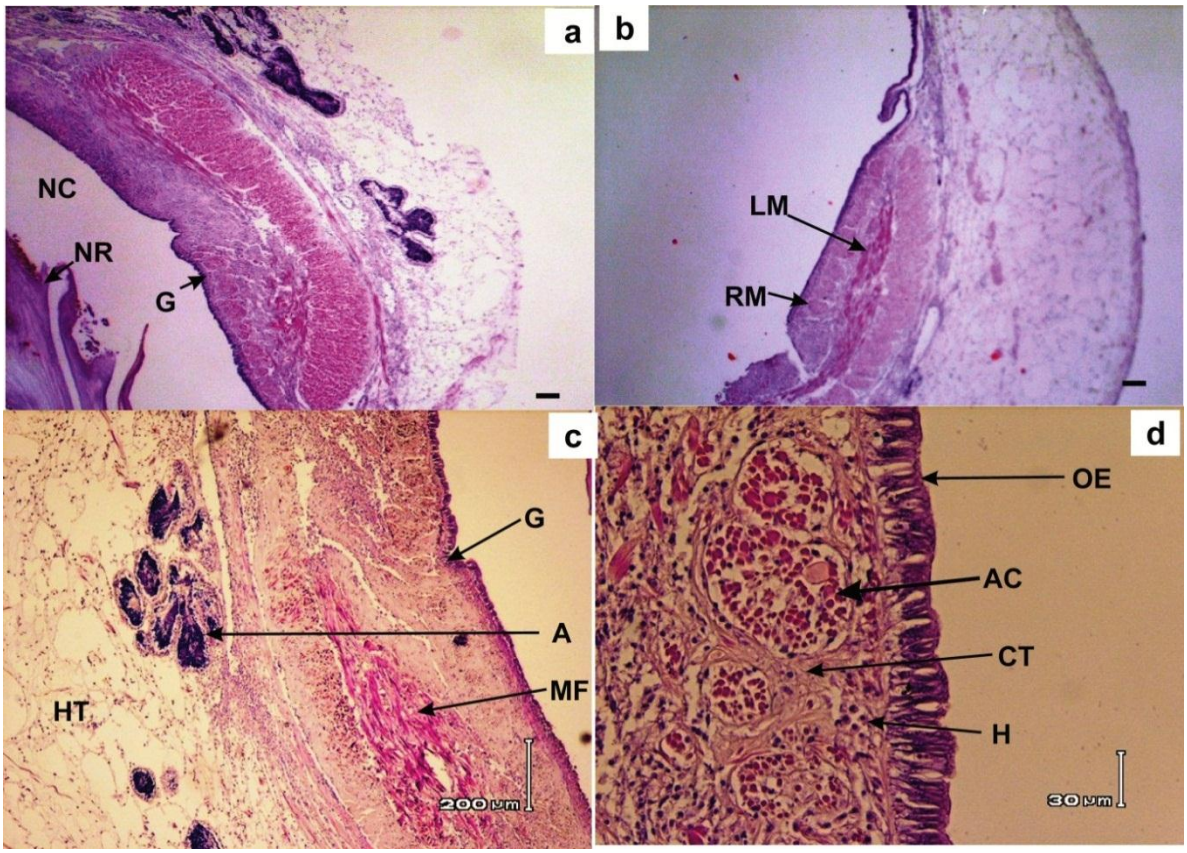

Figure 2.4. Pearl-sac development eight days after grafting in *Pinctada margaritifera* showing: a, proliferating mantle graft (G) and nucleus remnant (NR) in the nucleus cavity (NC); b, further development of longitudinal muscles (LM) and radial muscles (RM) in the mantle graft; c, acnii (A) in the host tissue (HT) and muscles fibres (MF) in the mantle graft (G); d, haemocytes (H), connective tissues (CT) and developing cells that include acidophilic cells

(AC) and outer epithelial cells (OE) in the mantle graft. Bar scale: A, B = 80 µm

Ten days after grafting, a further decrease in the gap between the mantle graft tissue and host tissue was evident (Fig. 2.5a). The graft continued to extend along the circumference of the nucleus cavity in the host tissue (Fig. 2.5b) with further development of epithelial cells and cilia now visible (Fig. 2.5c). Gaps in the pearl-sac shown in Fig. 2.5a are tears in the tissue that occurred during microtome sectioning.

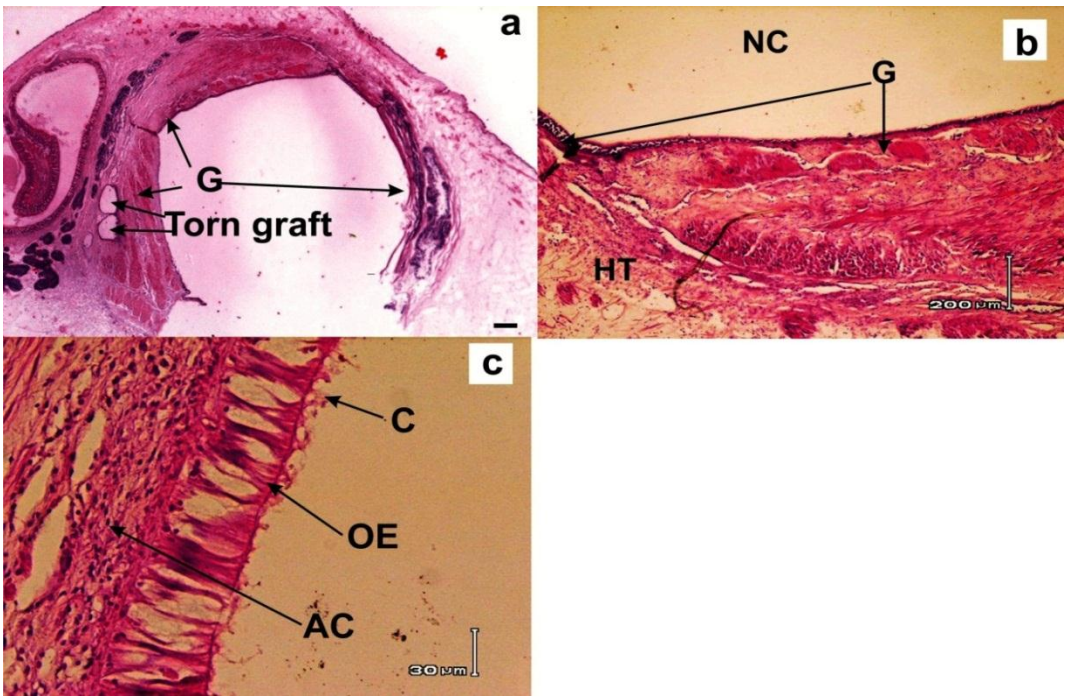

Figure 2.5. Pearl-sac development ten days after grafting in *Pinctada margaritifera* showing: a, decreased gap between the torn mantle graft (G) and host tissue; b, continued extension of the mantle graft (G) along the circumference of the host tissue (HT) cavity; c, further development of the pearl-sac from the mantle graft tissue with clearly distinguishable

acidophilic cells (AC), outer epithelial cells (OE) and cilia (C). Bar scale: 200 µm

By twelve days after grafting, the pearl-sac had completely encapsulated the nucleus cavity (Fig. 2.6a); however, haemocytes and gametes were still present in some remaining gaps between the host tissues and graft tissue even after a completion of the pearl-sac (Fig. 2.6b and 2.6c). A thin layer of organic matrix secreted by the epithelial cells was evident in some samples (Fig. 2.6b and 2.6c).

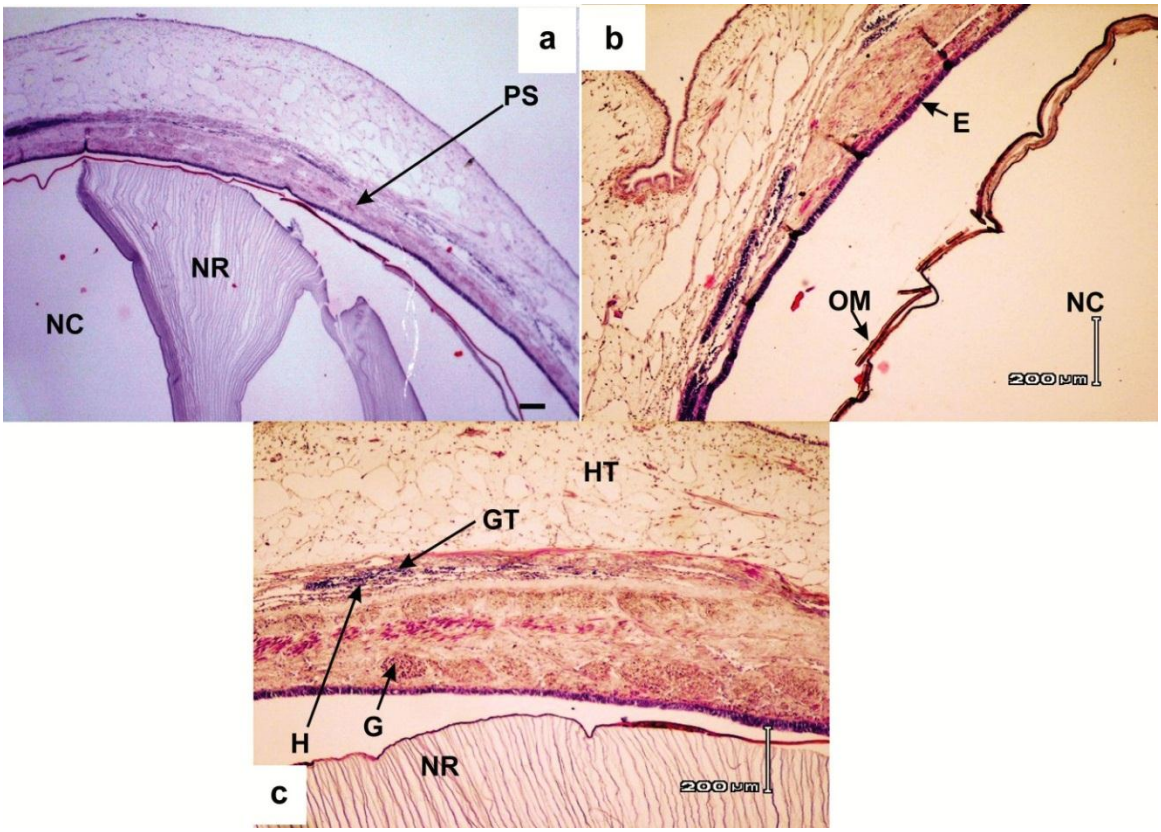

Figure 2.6. Pearl-sac development twelve days after grafting in *Pinctada margaritifera* showing: a. completely encapsulated nucleus cavity (NC) and nucleus remnant (NR) by the pearl-sac (PS); b, organic matrix (OM) within the nucleus cavity (NC) secreted by the epithelial cells (E); c, gametes (GT)

and haemocytes (H) between the mantle graft (G) and host tissue (HT). Bar scale: 100 µm

There was complete attachment of mantle graft tissue to host tissues by 14 days after grafting with no gaps between the two (Fig. 2.7a). This signified completion of the pearl-sac formation process. Although not measured, the thickness of the organic matrix layer secreted by the epithelial cells appeared thicker than those observed on day 12 (Fig. 2.7b).

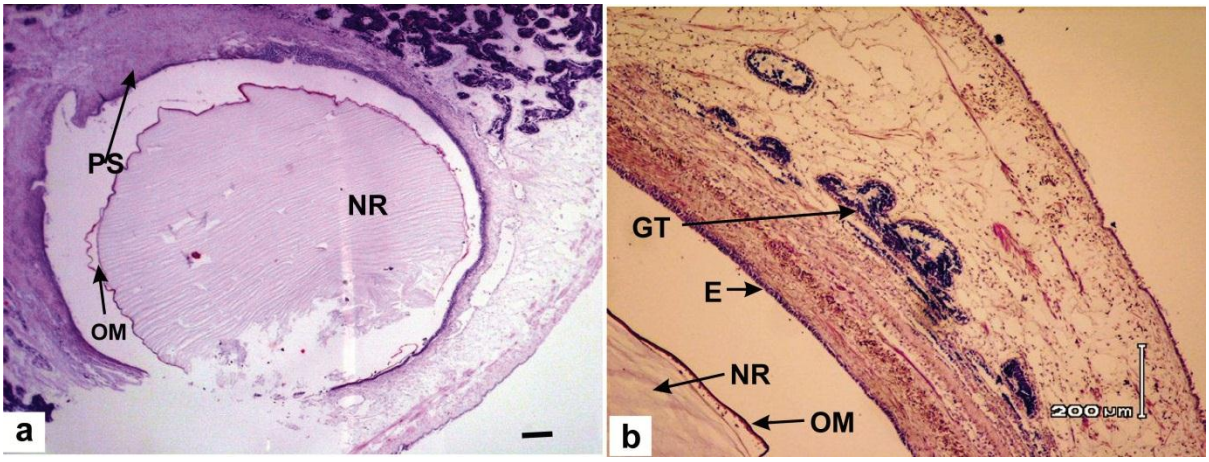

Figure 2.7. Pearl-sac development fourteen days after grafting in *Pinctada margaritifera* showing :a, complete attachment of the pearl-sac (PS) to host tissues (HT) and an extensive organic matrix (OM) surrounding nucleus remnant (NR); b, thicker organic matrix layer (OM) secreted by epithelial cells (E) on nucleus remnant (NR) and gametes (GT) in host tissues. Bar scale: 200 µm

The organic matrix layer secreted by the epithelial cells had thickened by sixteen days after grafting (Fig. 2.8a). The newly developed pearl-sac could barely be distinguished as foreign tissue present in host oysters at this stage (Fig. 2.8b).

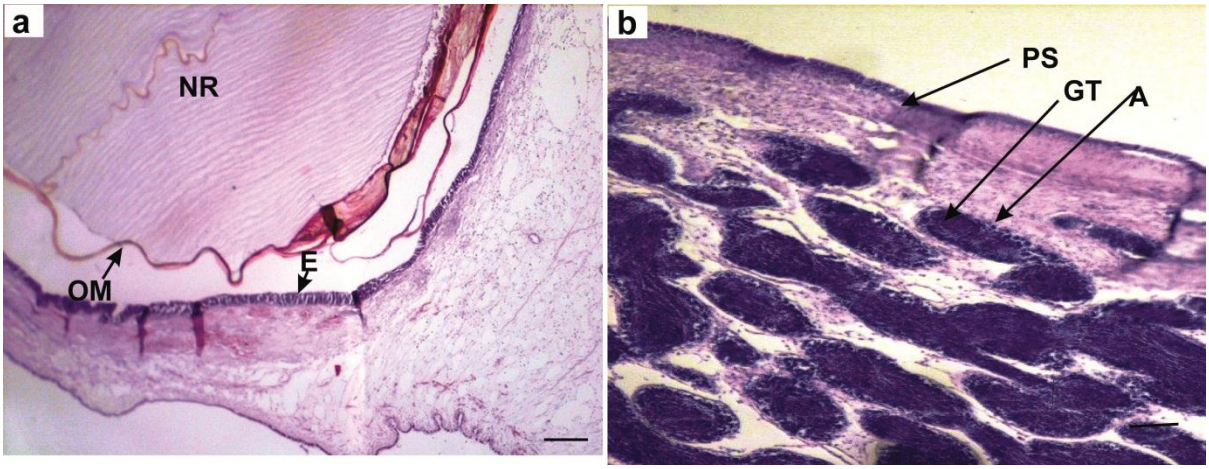

Figure 2.8. Pearl-sac development sixteen days after grafting in *Pinctada margaritifera* showing: a, thicker organic matrix (OM) secreted by the epithelial cells (E) onto the nucleus remnant (NR); b, section of the pearl-sac (PS) showing gametes (GT) in acnii (A). Bar scale: 100 µm

By eighteen days after grafting, the pearl-sac in all samples had attained the visible physical characteristics of the host tissues and could not be differentiated as a foreign tissue (Fig. 2.9a). The epithelial cells were well developed and continued to secrete an increasingly thick organic matrix layer (Fig. 2.9b).

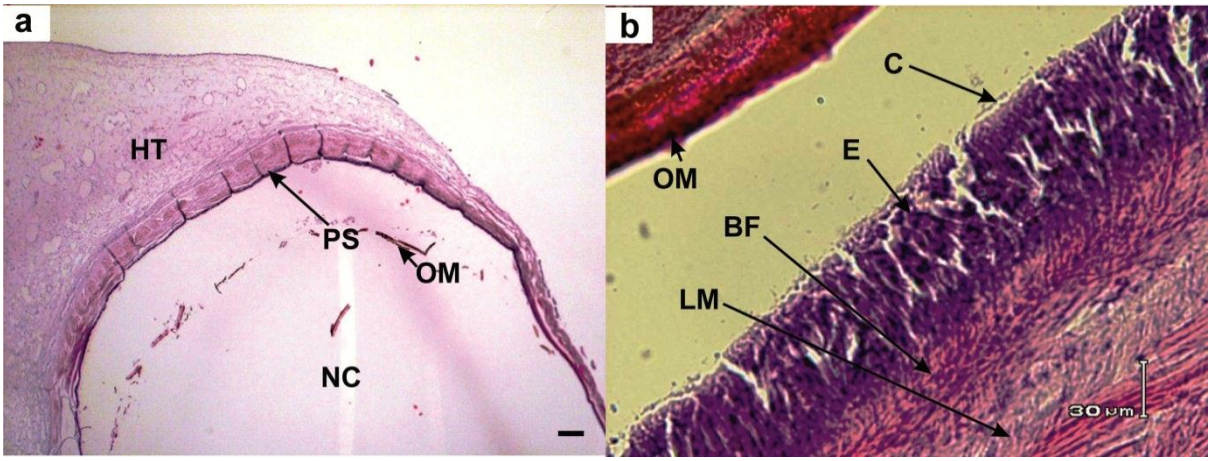

Figure 2.9. Pearl-sac development eighteen days after grafting in *Pinctada margaritifera* showing: a, low power section of pearl-sac (PS) showing host tissues (HT) that has sections of organic matrix (OM) in the nucleus cavity (NC); b, cilia (C), longitudinal muscles (LM), base muscle fibres (BF), and well developed epithelial cells (E) that continued organic matrix (OM) secretion. Bar scale: 100 µm

Secretion of organic matrix by the pearl-sac continued and, by twenty-two days after grafting, sections of it were present in the nucleus cavity (Fig. 2.10a). One of the samples showed the presence of byssal threads in close proximity to the developing pearl-sac (Fig. 2.10b).

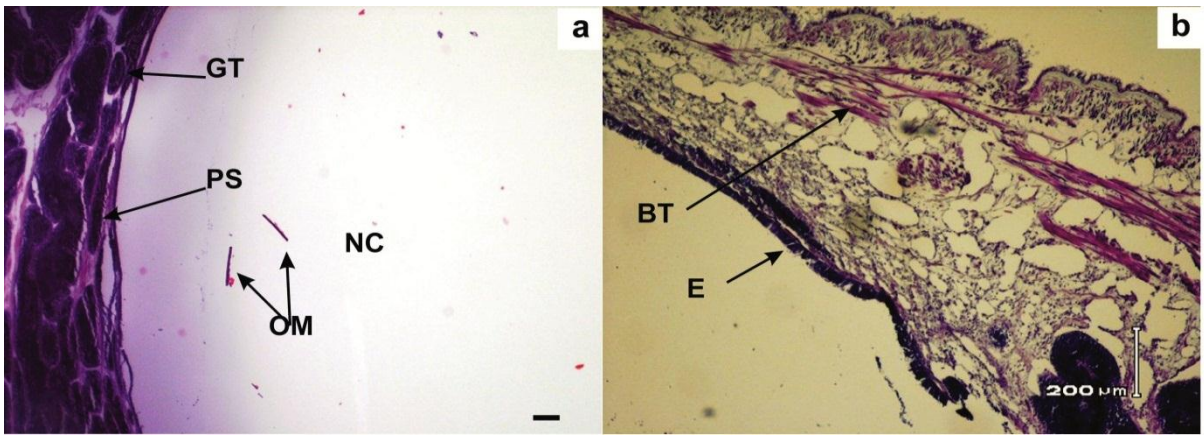

Figure 2.10. Pearl-sac development twenty-two days after grafting in *Pinctada margaritifera* showing: a, gametes (GT) and sections of organic matrix (OM) in the nucleus cavity (NC) within the pearl-sac (PS); b, byssus tissues (BT) close to the pearl-sac with well-developed epithelial cells (E) lining the nucleus cavity. Bar scale: 100 µm

By thirty days after grafting, the organic matrix secreted by the epithelial cells of the pearl-sac (Fig. 2.11a and 2.11b) was now composed of two layers; the inner older layer and the much thicker outer layer (Fig. 2.11c).

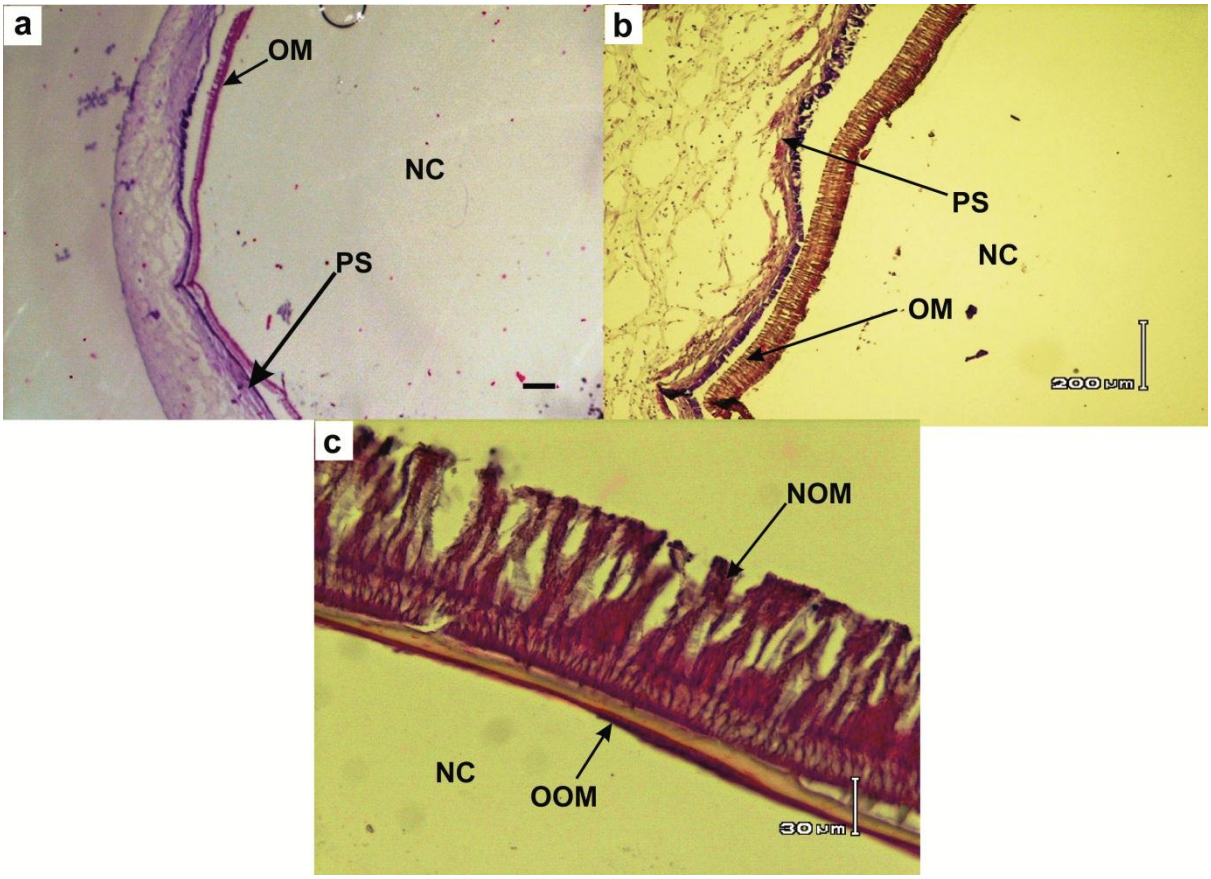

Figure 2.11. Pearl-sac development thirty days after grafting in *Pinctada margaritifera* showing: a, further development of the pearl-sac (PS), organic matrix layer (OM) and nucleus cavity (NC) at low power view; b, thicker organic matrix layer (OM) visible in the nucleus cavity (NC) secreted by the epithelial cells of the pearl-sac (PS); c, the newly secreted organic matrix layer (NOM) and the initially secreted old matrix layer (OOM) in the nucleus cavity (NC). Bar scale: 200 µm

Broken sections of the organic matrix layer resulting from damage sustained while sectioning, were still present in the nucleus cavities of four samples thirty-two days after grafting (Fig. 2.12a). However, traces of secreted nacre were also observed for the first time in one of the samples (Fig. 2.12b) indicating that nacre secretion begins only after the pearl-sac is completely formed. Nacre secretion was recorded in two samples by 34 days after grafting.

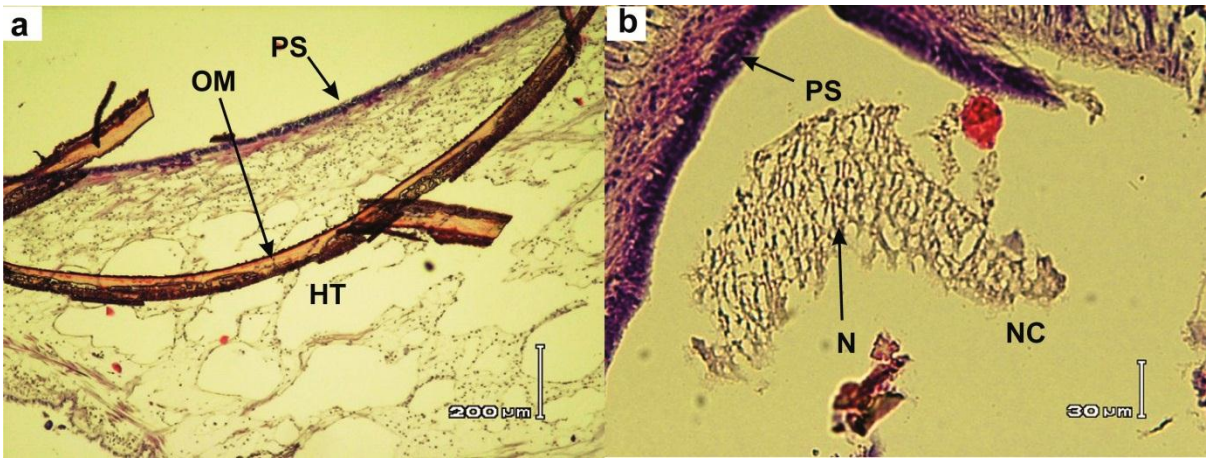

Figure 2.12. Pearl-sac development thirty-two days after grafting in *Pinctada margaritifera* showing: a, sections of broken organic matrix layer (OM) above the host tissue (HT) and pearl-sac (PS); b, nacre (N) deposits in the nucleus cavity (NC) secreted by the epithelial cells of the pearl-sac (PS).

2.3.2. Abnormalities during pearl-sac formation

In addition to normal spherical pearl-sac development, some pearl-sacs developed abnormalities during this study. For example, in one sample from day 4, the host oyster tissues were pushed outwards by a clump of haemocytes that was present between the proliferating mantle graft tissue and the host tissue (Fig. 2.2d). Furthermore, a sample from day 16 had a clump of haemocytes present in the nucleus cavity (Fig. 2.13). In the

presence of the nucleus, this clump would have been situated between the nucleus and the pearl-sac hence causing the pearl-sac to bulge outwards and distorting its usual spherical shape.

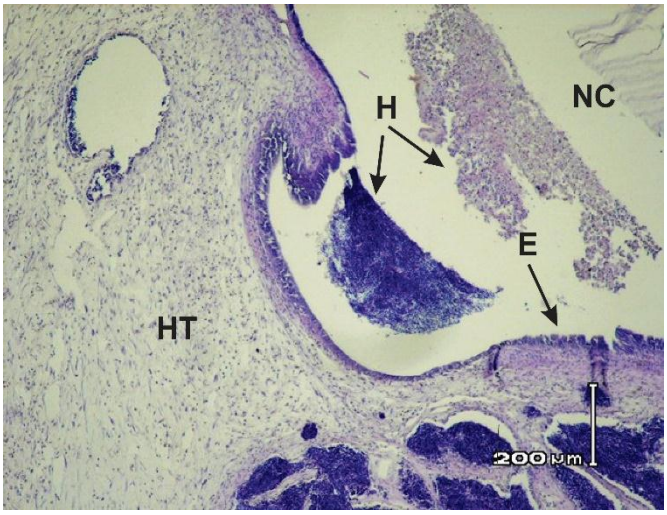

Figure 2.13. Abnormally developed pearl-sac in *Pinctada margaritifera* after four days of development showing host tissue (HT), accumulated haemocytes (H) and epithelial cells (E) in the nucleus cavity (NC).

A similar type of anomaly was also recorded on day 44 where one of the observed pearl-sacs had become distorted after it was pushed outwards by a large clump of haemocytes present in the nucleus cavity (Fig. 2.14a). The resulting cavity had secreted some organic matrix layer and was filled with haemocytes (Fig. 2.14b and 2.14c). Two samples showed gametes to be present between the epithelial cells of the pearl-sac and the nucleus on Day 44 (Fig. 2.14b).

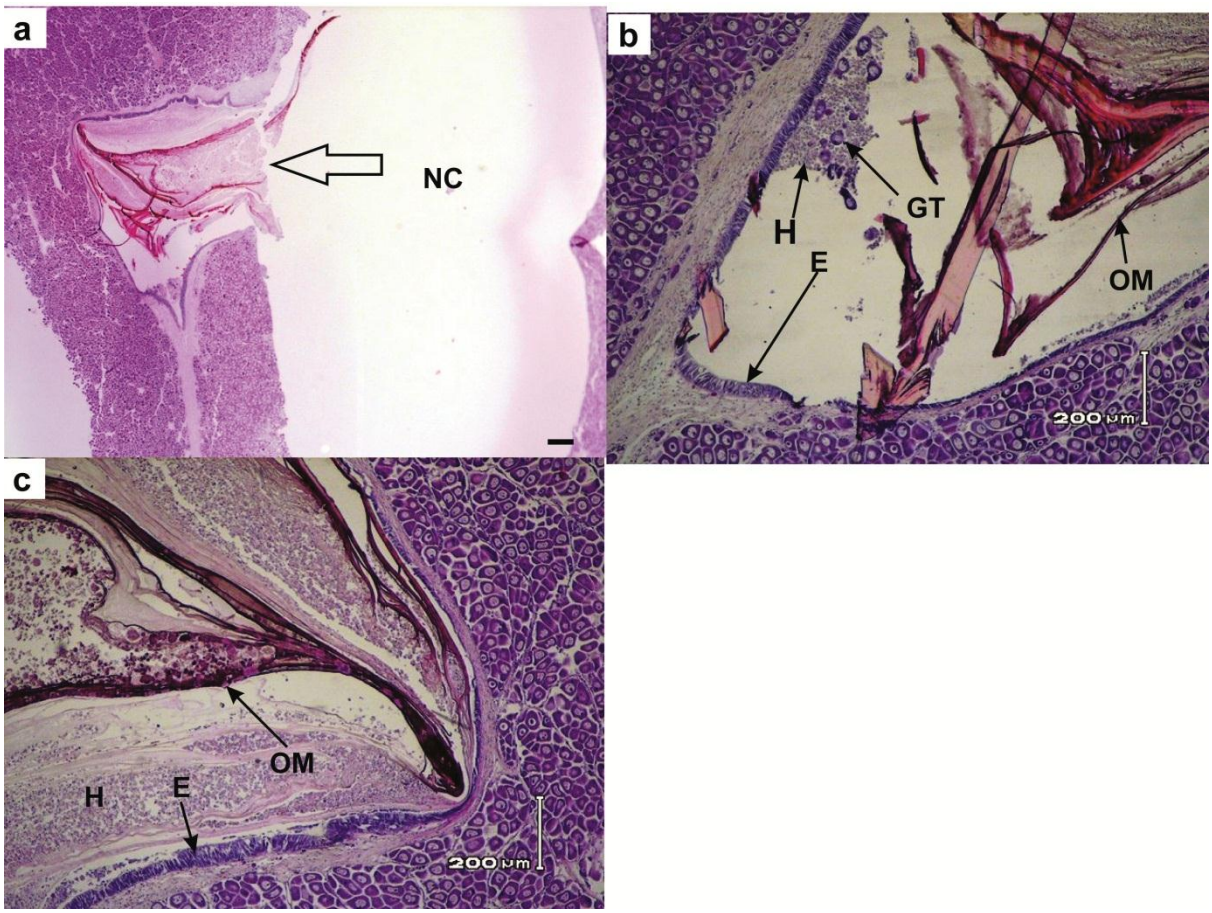

Figure 2.14. Abnormally developed pearl-sac in *Pinctada margaritifera* after forty-four days of development showing: a, pearl-sac pushed outwards along the circumference of the nucleus cavity (NC) under a low power view; b, haemocytes (H) and gametes (GT) with broken sections of organic matrix (OM) secreted by the epithelial cells (E) in the cavity formed by the abnormality; c, accumulation of haemocytes (H) between the organic matrix (OM) layer and epithelial cells (E) in the cavity formed by the abnormality. Bar scale: 200 µm

The major stages of pearl-sac development in *P. margaritifera* and early nacre secretion are summarised chronologically in Table 2.1.

Table 2.1. Timing of significant developmental changes during pearl-sac development in *Pinctada margaritifera.*

Developmental changes	Days after grafting
−No signs of mantle graft disintegration or proliferation were observed. −Mantle graft was not attached to host tissues at any point. −Haemocytes were present in the nucleus cavity.	2
−Attachments of the graft to host tissues were only observed at the mid-section of the graft. −Both ends of the mantle graft had extended and narrowed and showed tissue continuity (i.e. no gap) with the surrounding host tissues; however gaps between graft and host tissues were still visible towards the extremities of growth.	4
−Longitudinal and radial muscles could now be easily distinguished in two of the samples. −The mantle grafts had covered half the nucleus cavity circumference of the host oysters with the outer epithelial cells slowly becoming cuboidal in shape in all samples.	6
−The connective tissues between different muscle fibres and acidophilic cells could now be distinguished in one of the samples. −The epithelial cells were well developed and had attained flat, cuboidal shapes in three of the sampled pearl-sacs.	8
−The mantle grafts proliferated further and covered three quarters of the nucleus cavity circumference of four host oysters. −Cuboidal shaped epithelial cells were now present in all pearl-sac samples.	10
−The extending ends of the mantle grafts came together in three of the pearl-sac samples causing complete encapsulation of nuclei by the mantle graft tissues. −Furthermore, a thin layer of organic matrix was also secreted by the epithelial cells in those samples.	12
−For the first time since grafting, no gaps were evident between the fully developed pearl-sacs and the host tissues indicating complete attachment of the mantle graft to the host tissues in all samples.	14
−The pearl-sac tissues assumed the characteristics of the host tissues and could only be distinguished by close observation in all the samples.	16

– The fully developed pearl-sac could no longer be distinguished from the host tissues. – Base muscles of the pearl-sac were now recognisable between the epithelial layer and the longitudinal muscles in most samples.	18
– In addition to normal tissues and cells mentioned above, byssus tissues were now evident in the gonad, adjacent to the pearl-sac in one of the samples. – The byssus tissues projected in all directions in close proximity to the pearl-sac.	22
– The organic matrix layers were now much thicker and the older matrix layer that was much thinner was easily distinguished from the newly secreted, thicker organic matrix layer.	30
– Nacre deposits were observed for the first time in one of the samples.	32
– Nacre deposits were observed in the nucleus cavity of 80% of samples.	34

2.4. Discussion

Appropriate preparation of saibo tissue for grafting is a vital factor influencing development of the pearl-sac. The pearl grafting method in pearl oysters was developed by the Japanese (Gervis and Sims, 1992; Taylor and Strack, 2008) and these highly specialised skills are closely guarded. However, grafting techniques do vary between technicians. For example, different levels of experience could influence factors such as choice of oysters for saibo donation, method of saibo preparation and details of the grafting procedure, and the choice of oysters as a host for pearl production. A detailed description of saibo preparation is presented by Taylor and Strack (2008) who reported that excised mantle tissue is wiped with a piece of soft cloth to remove debris and any unwanted material; a procedure also followed in this study. During this process, cilia found on mantle epithelial cells (Acosta-Salmón and Southgate, 2005) would also be removed, which explains why cilia presence has never been reported in previous

histological studies of pearl-sac development in *P. fucata* (Nakahara, 1957; Velayudhan *et al.*, 2011) and *P. maxima* (Scoones, 1996). However, in the current study, the initial proliferation of graft tissue is marked by the distinct presence of cilia. It is possible that the technician when wiping the mantle tissue was very gentle to only remove dirt and debris avoiding any damages to the tissues. Hence, cilia present on the mantle tissues were not removed in the process which illustrates the different techniques used by different grafting technicians.

The results obtained from the two-day sampling interval used in this study show that a period of approximately 14 days is required for complete pearl-sac development in *P. margartifera*. This corresponds with similar findings for *P. margaritifera* in French Polynesia where the pearl-sac was reported as "almost complete" after 12 days (Cochennec-Laureau *et al.*, 2010). Data from both studies indicate that pearl-sac development in *P. margaritifera* is completed within two weeks of grafting. This is in contrast with the pearl-sac development periods mentioned for *P. maxima* and *P. fucata* (see *Section 1.6.1.).* Because of the positive relationship between water temperature and metabolic rate in pearl oysters (Yukihira *et al.*, 2000), previous studies on pearl-sac development in *P. fucata* (Dix, 1972) and *P. maxima* (Scoones, 1996) have identified water temperature as a key factor influencing the rate of pearl-sac development.

The pearl grafting process used for all *Pinctada* species involves making an incision at the base of the foot with an operating knife followed by the insertion of the graft tissue and nucleus into the pearl pouch of host oyster (Gervis and Sims, 1992; Taylor and Strack, 2008). Oysters lose haemolyph from wounds caused while grafting and respond by secreting haemocytes to seal the wounds (Suzuki *et al.*, 1991; Acosta-

Salmón and Southgate, 2006). The presence of haemocytes in the early stages of pearl-sac development and in the pearl-sac thereafter is likely to have impacts on the quality of resulting pearls. Prior studies have reported that a few days after grafting, the graft is often encapsulated by haemocytes that prevent normal proliferation of graft tissue and delay formation of the pearl-sac (Dix, 1972; Scoones, 1996; Cochennec-Laureau *et al.*, 2010; Awaji and Machii, 2011). In this study, encapsulation of the graft by haemocytes was not evident at any stage of pearl-sac development, however uneven development of the pearl-sac resulting from haemocyte accumulation was evident, and this has also been reported in prior studies (Scoones, 1996; Cochennec-Laureau *et al.*, 2010). Haemocyte accumulation can cause the pearl-sac to bulge outwards causing deformity to their normally spherical shape. This is likely to result in non-spherical pearls which generally have reduced value. For example, the results of Norton et al. (2000) suggested that the accumulation of "inflammatory cells" in the incision pathway (the wound resulting from nucleus and saibo placement), resulted in the development of calcified "tails" during pearl development. The exact cause of excessive accumulation of haemocytes in some oysters is not known. However, haemocyte production is likely to be positively related to the degree of tissue damage caused during the grafting procedure, which itself is related to the ease with which the nucleus and graft tissue are placed at a desirable position within the host oyster. The more manipulation that is required to achieve this positioning, then the more tissue damage is likely to occur with increased haemocyte response. Sub-optimal hygiene during the grafting procedure has also been implicated with haemocyte production (Scoones, 1996; Norton *et al.*, 2000).

There is anecdotal suggestion that the occurrence of gametes between the nucleus and donor tissue after grafting results in production of pearls that have 'circles', or

concentric surface grooves. The basis for this supposition is that the gametes cause disruption of nacre deposition onto the nucleus. However, no prior histological study of pearl-sac development in pearl oysters has reported the presence of gametes between the epithelial cells and the nucleus and it is possible that the gametes observed in this study entered the pearl-sac when the tissues were sectioned in preparation for histological studies. This study also showed the presence of byssus in close proximity to the developing pearl-sac twenty-two days after grafting. This could possibly result from the introduction of byssus secreting cells into the gonad during the pearl grafting procedure. However, Scoones (1996) also reported that some byssus secreting tissue as well as byssal threads, are often seen in the developing pearl-sacs of *P. maxima*. It is also possible therefore that the presence of byssus close to the pearl-sac could disrupt even nacre deposition on developing pearls.

In summary, this is the first detailed study of pearl-sac formation in *P. margaritifera*. It has confirmed that the pearl-sac in this species is fully formed within 14 days of grafting for pearl production. Results also show that accumulation of haemocytes during pearl-sac development may result in malformation of the pearl-sac which is likely to result in reduced pearl quality. Furthermore, the presence of byssus in close proximity of developing pearl-sac was demonstrated in this study. This factor has the potential to impact pearl-sac formation and resulting pearl quality. Further research is required to test these hypotheses and the fine-scale description of 'normal' pearl-sac development in *P. margaritifera* outlined in this study will provide a benchmark for this.

Chapter 3

[2]Haemocyte persistence after grafting for pearl production in *Pinctada margartifera*

3.1. Introduction

Haemocytes play important roles in the homeostatic functions and defense mechanisms of bivalve molluscs (Ruddell, 1971; Hodgson, 1982; López *et al.*, 1997; Chang *et al.*, 2005; Acosta-Salmón and Southgate, 2006). In pearl oysters, a significant haemocyte response is commonly triggered by the grafting process used for cultured pearl production. This involves making an incision at the base of the foot of a host pearl oyster through which a round nucleus and a piece of mantle tissue (called 'saibo') from a donor pearl oyster are inserted into the antero-ventral portion of the gonad (Gervis and Sims, 1992; Scoones, 1996; Fougerouse *et al.*, 2008; Taylor and Strack, 2008) known as the 'pearl pouch' (Scoones, 1996; Fougerouse *et al.*, 2008).

The nucleus and mantle graft are recognised as foreign materials by the host oyster (Awaji and Suzuki, 1995; Awaji and Machii, 2011) and are surrounded by granular and agranular haemocyte types (Suzuki *et al.*, 1991). In addition to damage caused to host oyster tissues during grafting, Miyashita and Takagi (2011) reported that wounds on the mantle graft itself, resulting from excision, can also be a source of haemocytes which accumulate to seal the damaged area. This process is common to all bivalve molluscs (Ruddell, 1971; Suzuki *et al.*, 1991; Mamangkey and Southgate, 2009;

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Miyashita and Takagi, 2011). Once in place the mantle graft proliferates to form a 'pearl-sac' around the nucleus before secretory (epithelial) cells from fully the developed pearl-sac (Scoones, 1996; Cochennec-Laureau *et al.*, 2010) deposit nacre onto the nucleus to form a cultured pearl over a period of 18-24 months (Gervis and Sims, 1992; Taylor and Strack, 2008). Ideally, the pearl-sac will encapsulate the nucleus to form a spherical shape that improves the probability that it will produce a spherical pearl.

During the course of pearl-sac formation, variation in the degree of haemocyte production and accumulation have been reported in the nucleus cavity of *P. margaritifera* (Norton *et al.*, 2000; Cochennec-Laureau *et al.*, 2010). The occurrence and accumulation of haemocytes in the pearl-sac has been associated with pearl-sacs that develop irregular shapes (Scoones, 1996; Miyashita and Takagi, 2011) that subsequently produces irregularly shaped pearls (Scoones, 1996; Cochennec-Laureau *et al.*, 2010) with reduced value. This may occur for example when haemocytes accumulate between the nucleus and the pearl-sac. However, despite the potential impacts of haemocytes in influencing pearl quality and value, the level of haemocyte production and their persistence in the pearl-sac after grafting has not been specifically reported. In this study, the various haemocyte responses in the black-lip pearl oyster, *P. margaritifera*, during and subsequent to pearl-sac formation was examined.

3.2. Material and methods

One hundred and ten randomly selected healthy adult *P. maragaritifera* with mean (±SE) dorso-ventral measurement of 112.06 ± 0.45 mm and antero-posterior measurement of 110.82 ± 0.41 mm were grafted for pearl production by an experienced

pearl grafting technician (Taylor and Strack, 2008). The surgical tools and other equipment associated with grafting (e.g. saibo cutting board, scalpel and oyster openers) were thoroughly washed in freshwater before being used for grafting. After washing, surgical tools were held in a container of freshwater to which a small volume of proprietary antiseptic solution had been added. Once used to graft an oyster, surgical tools were placed back into the same container before being used on the next oyster. Mantle tissue to be used as saibo was cut from the donor oyster and wiped with a piece of clean cloth to remove any dirt or debris before being further sectioned into small squares on the cutting board for grafting. Grafted oysters were then returned to the ocean with no further treatment and held under normal commercial farming conditions at a depth of 7 m.

Beginning two days after grafting, five oysters were sacrificed every second day until 40 days after grafting. Five oysters were then sacrificed at 4-day intervals until 48 days after grafting. Oyster tissues containing the nucleus were preserved in "FAAC" solution composed of 25 ml of glacial acetic acid, 6.5 g of calcium chloride dehydrate and 50 ml of 37% formaldehyde per 500 ml of distilled water (Miller and Stewart, 2013). The tissues were preserved for approximately two months before histological analysis. During this period, glacial acetic acid in the preservative solution dissolved the nuclei present in the pearl-sac samples prior to histological preparation that was carried out following the method of Acosta-Salmón and Southgate (2006). Specimens were inspected using a photo microscope (Olympus, SZ-CTV) and an optical microscope (Olympus, BX40). Images were produced using an Olympus DP12 camera and those that showed significant haemocyte presence that could potentially influence pearl-sac formation are shown in Figure 3.1.

3.3. Results

Haemocytes were present in all the samples from days two and four (Fig 3.1a & 3.1b). The level of haemocytes in most samples reduced as pearl-sac formation progressed (Fig. 3.1c); however, in some samples, major haemocyte aggregations were observed either in the nucleus cavity (Fig 3.1d & 3.1f) or within the pearl-sacs (Fig 3.1e).

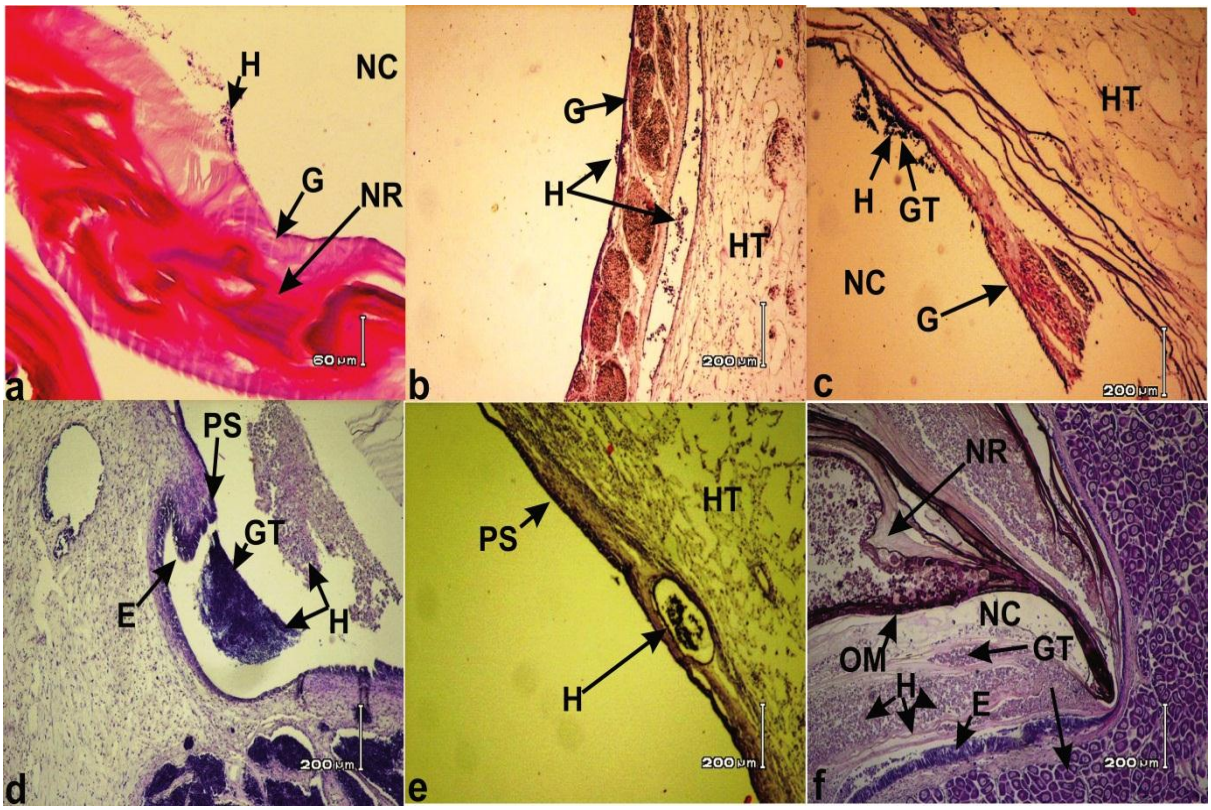

Figure 3.1. Pearl-sac development in *Pinctada margaritifera* showing: a, accumulation of haemocytes (H) on the edges of a torn mantle graft (G) section and undissolved nucleus remnant (NR) in the nucleus cavity (NC) after two days; b, haemocytes (H) trapped in gaps between the host tissues (HT) and mantle graft (G) after four days; c, haemocytes (H) and gametes (GT) accumulating towards the extending end of the mantle graft (G) and host

tissues (HT) after six days; d, haemocyte (H) and gamete (GT) accumulation close to pearl-sac (PS) with developed epithelial cells (E) after 16 days; e, haemocyte (H) accumulation between the pearl-sac (PS) and host tissues (HT) after 26 days; f, abnormally formed pearl-sac with organic matrix (OM), nucleus remnant (NR), haemocytes (H) and gametes (GT) in the nucleus cavity (NC) after 44 days from grafting.

3.4. Discussion

Haemocytes were present in all samples from the second and fourth day after grafting (Fig. 3.1a and 3.1b) reflecting their association with the healing process in host oysters (Suzuki *et al.*, 1991; Awaji and Suzuki, 1995). Reduced haemocyte presence was evident on the sixth day after grafting indicating progression of the healing process (Fig. 3.1c). Reduction in the presence of haemocytes with increasing time from grafting has previously been reported by Cochennec-Laureau et al. (2010). However, not all the samples in this study showed this trend. Major aggregations of haemocytes were present in samples from sixteen, twenty-six and fourty-four days after grafting (Fig. 3.1d, 3.1e and 3.1f). A constant presence of haemocytes during pearl-sac development up to 50 days after grafting has been reported for *P. maxima* (Scoones, 1996) however, this had not been previously reported for *P. margaritifera*. The two-day sampling interval in this study enabled the observation of fine-scale progressive changes in the presence of haemocytes up to 40 days after grafting for the first time in *P. maragritifera*, and report precisely on the influence of this on pearl-sac formation.

The exact cause(s) of varying levels of haemocyte accumulation during pearl-sac development in *P. margaritifera* is not known. However, it is reasonable to assume that

haemocyte production is positively related to the degree of damage caused to host oyster tissues during the grafting procedure. Sub-optimal hygiene during the grafting procedure has also been associated with increased haemocyte production (Scoones, 1996; Norton *et al.*, 2000). Previous studies have also reported a complete surrounding of the graft tissue by haemocytes a few days after grafting which delayed pearl-sac formation (Dix, 1972; Scoones, 1996; Cochennec-Laureau *et al.*, 2010; Awaji and Machii, 2011). However, this was not evident at any stage in this study. Another key influence on the degree of haemocyte production and their persistence is likely to be the skill and experience of the pearl grafting technician which probably influences the degree of wounding to both host and donor tissues.

The presence of aggregated masses of haemocytes (Fig. 3.1d and 3.1f) have been associated with low quality pearl production (Scoones, 1996). The presence of such masses often causes bulges in the pearl-sac, where the pearl-sac grows over the accumulation, resulting in a deformity to what should ideally be a spherical pearl-sac (Chapter 2). Pearls produced within a misshapen pearl-sac may have calcified "tails" (Norton *et al.*, 2000) or be of baroque shapes (Scoones, 1996); both have reduced value. Scoones (1996) suggested that black deposits underlying the nacre of baroque (misshapen) pearls produced by *P. maxima* were the remnants of haemocyte accumulations.

An aggregation of haemocytes within the pearl-sac was also noted in this study (Fig. 3.1e). It is believed that small 'seedless' pearls of no commercial value can be formed from such features. Seedless pearls can also attach to the main pearl and form protuberances which increase surface irregularities and reduce pearl quality. Scoones

(1996) suggested that ‘seedless’ pearls are possibly formed from loosely attached epithelial cells that are separated from the mantle graft during the grafting procedure and form a separate small pearl-sac and resulting pearl.

This study has shown that haemocytes can be present thoughout the entire phase of pearl-sac development in *P. margaritifera.* While haemocytes have an important wound healing role in pearl oysters, excessive haemocyte presence may be detrimental to maximizing pearl quality. For many oysters the haemocyte response is reduced within a few days after grafting reflecting the healing process. It is considered that this process is more likely to support development of a spherical pearl-sac that is more likely to produce a spherical pearl. However, oysters showing persistence of haemocytes within or close to the pearl-sac are more likely to develop a misshapen pearl-sac and produce a lower quality pearl. Further research to provide a greater understanding of the factors influencing the haemocyte response of pearl oysters following grafting, will support development of more effective pearl grafting procedures.

Chapter 4

[3]Development and function of pearl-sacs grown from regenerated mantle graft tissue in the black-lip pearl oyster, *Pinctada margaritifera*

4.1. Introduction

Grafting for cultured round pearl production involves insertion of a piece of mantle tissue (saibo) from a donor pearl oyster and a round inorganic nucleus into the gonad of a host pearl oyster (Gervis and Sims, 1992; Taylor and Strack, 2008). Subsequent proliferation of the saibo forms a pearl-sac, which envelopes the nucleus, and epithelial cells within the pearl-sac secrete nacre onto the nucleus to produce a cultured round pearl (Dix, 1972; Scoones, 1996; Taylor and Strack, 2008; Cochennec-Laureau *et al.*, 2010). The process of pearl-sac formation takes less than two weeks in the black-lip pearl oyster, *P. margaritifera* (Chapter 2) but around 18-24 months is required to produce pearls with a commercially acceptable nacre thickness (Haws, 2002; Taylor and Strack, 2008). Saibo has been shown to have a major influence on the quality of resulting pearls (Alagarswami, 1987a; Wada and Komaru, 1996; Taylor, 2002) with, for example, the colour of a cultured pearl being very similar to that of the nacre of the donor oyster shell (Wada and Komaru, 1996; Taylor, 2002). Recent studies have also reported traces of DNA from saibo tissue in the pearl-sac during pearl formation, further confirming the influence of donor oyster tissue on pearl formation (McGinty *et al.*, 2010; McGinty *et al.*, 2011).

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Obtaining saibo normally involves excision of mantle tissue from donor oysters that are sacrificed (Taylor and Strack, 2008). Tissue from the pallial zone of the ventral part of the mantle is further sectioned (Taylor and Strack, 2008) before it is inserted into the antero-ventral part of the host oyster gonad, known as the 'pearl pouch', during the pearl grafting procedure (Gervis and Sims, 1992; Taylor and Strack, 2008). However, recent studies have reported that mantle tissue can be excised successfully from anaesthetised donor pearl oysters, negating the need for sacrifice (Acosta-Salmón *et al.*, 2004; Acosta-Salmón *et al.*, 2005; Acosta-Salmón and Southgate, 2005; Mamangkey *et al.*, 2009). Furthermore, excised mantle tissue regenerates within a few weeks of surgery and appears to completely regain normal structure and function (Acosta-Salmón *et al.*, 2005; Acosta-Salmón and Southgate, 2005; Mamangkey and Southgate, 2009). The potential advantages of anaesthetizing, rather than sacrificing, pearl oysters to excise mantle tissue for saibo include the subsequent ability to propagate donor oysters that produce high quality pearls, and the potential use of selected oysters as repeat saibo donors using regenerated mantle tissue. While the latter possibility was initially proposed for *P. margaritifera* (Acosta-Salmón *et al.*, 2005), it is yet to be tested with this species.

Key assumptions when proposing multiple saibo donations from a single donor oyster are that regenerated mantle tissue will proliferate to form a normal pearl-sac following grafting, and that the pearl-sac will have normal nacre secreting ability. Complete pearl-sac formation from regenerated mantle graft tissue has been reported after 30 days in *P. maxima* with early nacre secretion noted after 38 days (Mamangkey, 2009). Similar research has not been reported for *P. margaritifera*, however, a recent detailed

description of pearl-sac formation in this species for the first time (Chapter 2) provides a basis on which development, morphology and function of pearl-sacs grown from regenerated saibo tissue can be assessed. This study therefore describes pearl-sac development from regenerated mantle tissue in *P. margaritifera* and subsequent nacre deposition.

4.2. Material and methods

This experiment was carried out at a commercial pearl farm operated by J. Hunter Pearls, at Savusavu Bay, on the island of Vanua Levu in the Fiji (16˚47′17.36″S, 179˚18′09.32″E). The oysters used in this experiment were approximately 10 months old when removed from spat collectors deployed at different sites within the Bay. These oysters were then cultured for a further 12 months under normal commercial conditions. Nine oysters with a mean (±SE) antero-posterior measurement of 102.82 ± 0.41 mm and dorso-ventral measurement of 112.76 ± 0.25 mm were selected by a professional *P. margaritifera* grafting technician as appropriate saibo donors for the experiment. These oysters were anaesthetised for 15 minutes using 2.5 ml L^{-1} of 1-propylene phenoxetol from Sigma-Aldrige (Norton *et al.*, 1996a; Mills *et al.*, 1997; Acosta-Salmón *et al.*, 2005; Acosta-Salmón and Southgate, 2005; Mamangkey *et al.*, 2009; Mamangkey and Southgate, 2009) before sections of mantle were excised for preparation of saibo from the position shown in Figure 4.1. The oysters were then placed in freshly aerated 1 µm filtered seawater for 30 min to aid recovery before being housed in two eight-pocket panel nets (40 x 40 mm mesh size) that were suspended from a long line in Savusavu Bay at a depth of 7 m. The regeneration of new mantle tissues was monitored monthly. After four months, the newly regenerated mantle tissue showed all characteristics of a normal mantle. The regenerated mantle tissue was then

examined by experienced professional pearl grafting technicians and deemed to be suitable for use as saibo for pearl grafting.

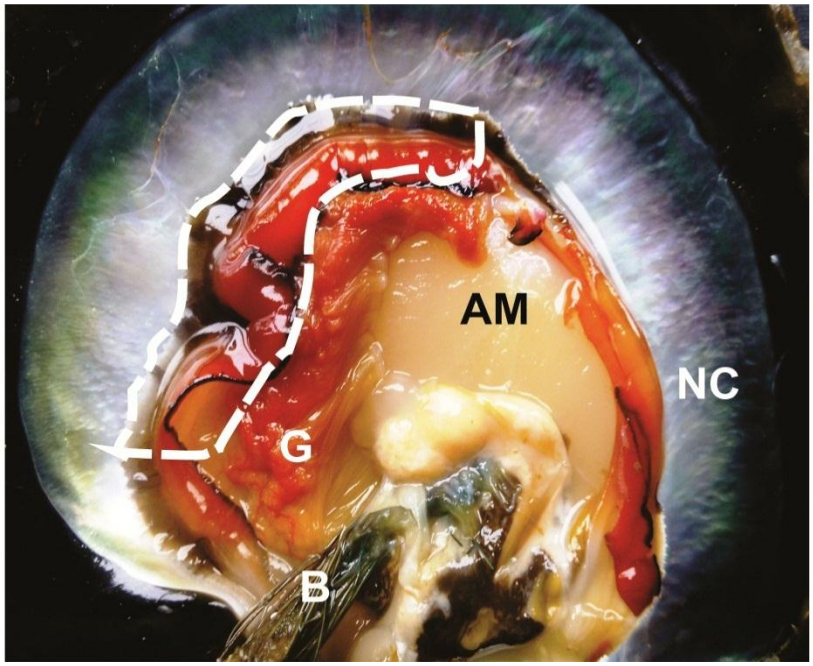

Figure 4.1. An individual *Pinctada margaritifera* with one shell valve removed showing general anatomy: AM, adductor muscle; NC, nacreous zone; G, gills; B, byssus. The dotted line indicates the area of mantle tissue excised following anesthesia.

4.2.1. Pearl-sac development

Two of the oysters with regenerated mantle with a mean (±SE) APM of 104.45 ± 0.33 mm and DVM of 111.72 ± 0.58 mm were chosen by the technicians for this experiment and saibo was removed from these oysters in the usual manner (Taylor and Strack, 2008) (Fig. 4.1). A total of 20 healthy host oysters with a mean (±SE) APM of 118.45 ± 0.63 mm and DVM of 127.63 ± 0.49 mm were selected as host oysters for pearl grafting. These oysters were cleaned before being implanted with nuclei and

regenerated donor mantle tissue in the usual commercial manner (Taylor and Strack, 2008). Nuclei of the same size (2.7 *bu*; ca. 8.2 mm) were used to graft all oysters. Grafted oysters were then placed into four eight-pocket panel nets and suspended from a long line in Savusavu Bay at a depth of 7 m. To monitor pearl-sac formation, six randomly selected nucleated oysters were sacrificed 6, 12, and 16 days after grafting, and preserved in a "FAAC" solution (Miller and Stewart, 2013). Samples were then transferred to the laboratory for histological analysis. Previous research in this laboratory has shown that pearl-sac development in *P. margaritifera* is complete within 14 days of the grafting procedure (Chapter 2).

The gonad containing the pearl-sac was excised from each sample and held in 70% ethanol in the laboratory. This process resulted in dissolution of the nucleus within each pearl-sac through the action of the acetic acid in the "FAAC" solution. Each pearl-sac was then cut into approximately 3 mm sections and placed in tissue cassettes before being dehydrated through different stages of ethanol and xylene (Acosta-Salmón and Southgate, 2005; Mamangkey and Southgate, 2009). The sections were then embedded in paraffin blocks, further sectioned to a thickness of 5 µm and mounted on glass slides before being deparaffinised in an oven for 24 h at 60 °C. Haematoxylin and Eosin stains were used for histological analysis of the specimens (Acosta-Salmón and Southgate, 2005; Acosta-Salmón and Southgate, 2006; Cochennec-Laureau *et al.*, 2010).

4.2.2. Nacre secretion

A further two donor oysters with regenerated mantle tissue and a mean (±SE) APM of 107 ± 0.62 mm and (DVM) of 113.49 ± 0.68 mm were selected and prepared for saibo

donation as described above. Resulting saibo was used to graft a further 22 adult *P. margaritifera* with a mean (±SE) APM of 108.52 ± 0.71 mm and DVM of 118.56 ± 0.53 mm by the same grafting technician used for the research described in section 2.1. Grafted oysters were placed into three eight-pocket panel nets (40 x 40 mm mesh size) and again suspended from a long line in Savusavu Bay at a depth of 7 m. Four nucleated oysters were randomly selected and sacrificed after one month and again after two months to observe nacre deposition on the nuclei and, after a culture period of three months, the remaining eight oysters were sacrificed. The nuclei from these oysters were removed and sectioned into halves to determine whether nacre had been secreted, whether it covered the whole nucleus and to facilitate measurement of nacre thickness after three months. For each nucleus, nacre thickness was measured using a Travelling Microscope (Kingsview Optical Ltd, serial no. 1018) at three randomly selected positions on each sectioned half of the nucleus (i.e. six measurements per nucleus). A further eight 'control' oysters that were grafted using 'normal' mantle tissue, with the same size nuclei and on the same day as oysters grafted with regenerated mantle, were randomly selected from four separate eight-pocket panel nets (two oysters per net) that were held adjacent to experimental oysters on the same long line. The thickness of nacre covering the nuclei excised from control oysters was measured as described above to allow comparison with that from experimental oysters.

4.2.3. Statistical analysis

Data relating to the thickness of nacre recorded on nuclei grafted with normal and regenerated mantle tissue were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). Independent samples T-tests were then used to compare the mean nacre coverage and the rates of nacre deposition between oysters

grafted with regenerated and normal mantle tissue. The results were expressed as mean (±SE) and were considered significant at $p \leq 0.05$.

4.3. Results

Of the 20 oysters that were grafted to assess pearl-sac development, two rejected the nucleus 12 and 16 days after grafting. No oysters died during the course of the experiment and all remained healthy. After four months, excised mantle tissues in the donor oysters had completely regenerated and regained their normal size. The regenerated mantle tissue appeared physically identical to the regions of normal mantle tissues that were not excised for this experiment in the same oyster. However, an anomaly in nacre deposition (uneven nacre surface) had developed on the inner surface of the shell adjacent to the position from which mantle was removed (Fig. 4.2). This was seen in all treated oysters.

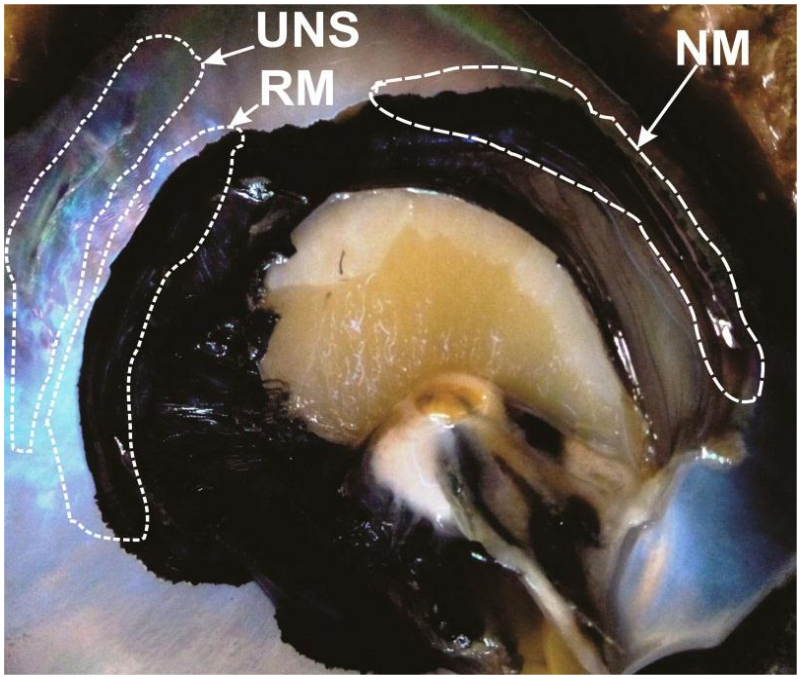

Figure 4.2. A donor *Pinctada margaritifera* with one shell valve removed showing regenerated mantle (RM); uneven nacre surface on the inner shell (UNS); and normal mantle (NM) tissue. This oyster was cultured for a period of four months following mantle excision while anesthetised.

4.3.1. Pearl-sac development

Six days after the grafting procedure, the regenerated graft used as a saibo had proliferated and covered approximately half the circumference of the nucleus cavity in 50% of the oysters sampled. The mantle graft tissue was not fully attached to host tissue as gaps containing haemocytes and gametes were observed between the two tissue types (Fig. 4.3a). The mantle graft tissue had extended and its extremities had become thinner as it extended around the nucleus cavity. Connective tissues and muscle fibers were clearly visible in the graft tissue (Fig. 4.3b). This sample also had haemocytes and gametes present towards the far end of the mantle graft tissue within the nucleus cavity (Fig. 4.3b). However, none of the samples showed any evidence of developing nacre secreting epithelial cells on the inner surface of the graft tissue.

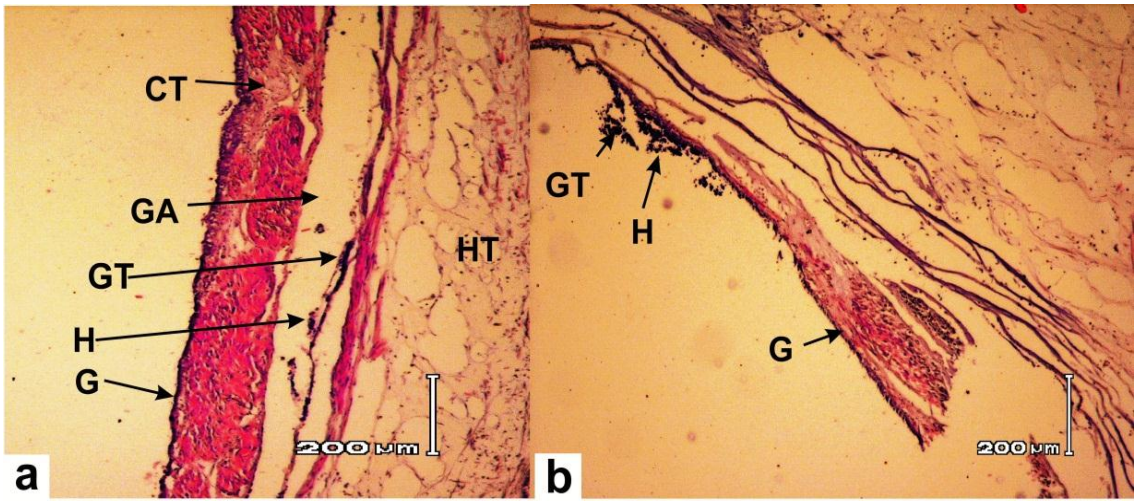

Figure 4.3. Histological sections of the developing pearl-sac in *Pinctada margaritifera* grafted with regenerated mantle tissue six days after grafting showing: a, gap (GA) containing haemocytes (H) and gametes (GT) present between the mantle graft tissue (G) with visible connective tissues (CT) and host tissue (HT); and b, haemocytes (H) and gametes (GT) present towards the extending end of the developing mantle graft tissue (G) in the nucleus cavity (NC).

By 12 days after the grafting procedure, the graft tissue had developed into a complete pearl-sac (Fig. 4.4a), which completely enveloped the nucleus cavity in all (100%) oysters. The mantle graft tissue could barely be differentiated from the host tissues with which it had merged (Fig. 4.4b). Gaps between the pearl-sac and the host tissue observed in Figure 4.4a (arrows) could possibly have occurred during microtome sectioning of the sample. The pearl-sac showed well developed epithelial cells with cilia (Fig. 4.4c) that had begun secreting organic matrix (Fig. 4.4d) that was clearly visible within the nucleus cavity.

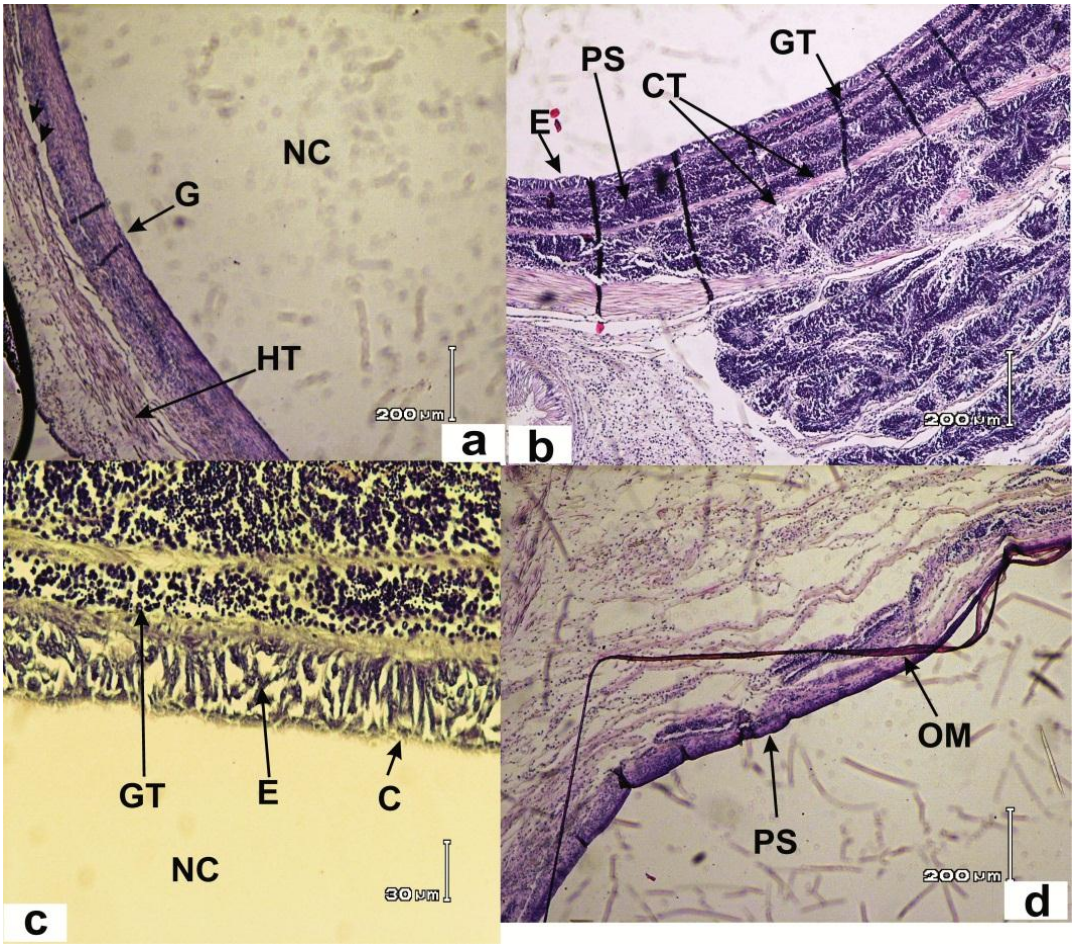

Figure 4.4. Histological sections of the developing pearl-sac in *Pinctada margaritifera* grafted with regenerated mantle tissue twelve days after grafting showing: a, host tissues (HT) and completely developed pearl-sac from the mantle graft tissue (G) encapsulating the nucleus cavity (NC); b, the fully developed pearl-sac (PS) integrated within the host tissues showing epithelial cells (E), connective tissues (CT) and gametes (GT); c, gametes (GT) and well developed epithelial cells (E) with cilia (C) on the inner surface of the pearl-sac lining the nucleus cavity (NC); and d, organic matrix (OM) layer secreted by the epithelial cells of the pearl-sac (PS).

Figure 4.5a shows a low power cross section of a pearl-sac within the gonad 16 days after grafting. The pearl-sac had completely integrated with host tissue and there were no gaps or interruptions between it and host tissue in all oyster samples (Fig. 4.5b). The epithelial cells continued to secrete an organic matrix layer that was now thicker (Fig. 4.5c). In one of the samples, the organic matrix contained haemocytes and gametes were present in the nucleus cavity (Figs. 4.5d and 4.5e). However, there were no signs of nacre deposition in the nucleus cavity of any of the samples.

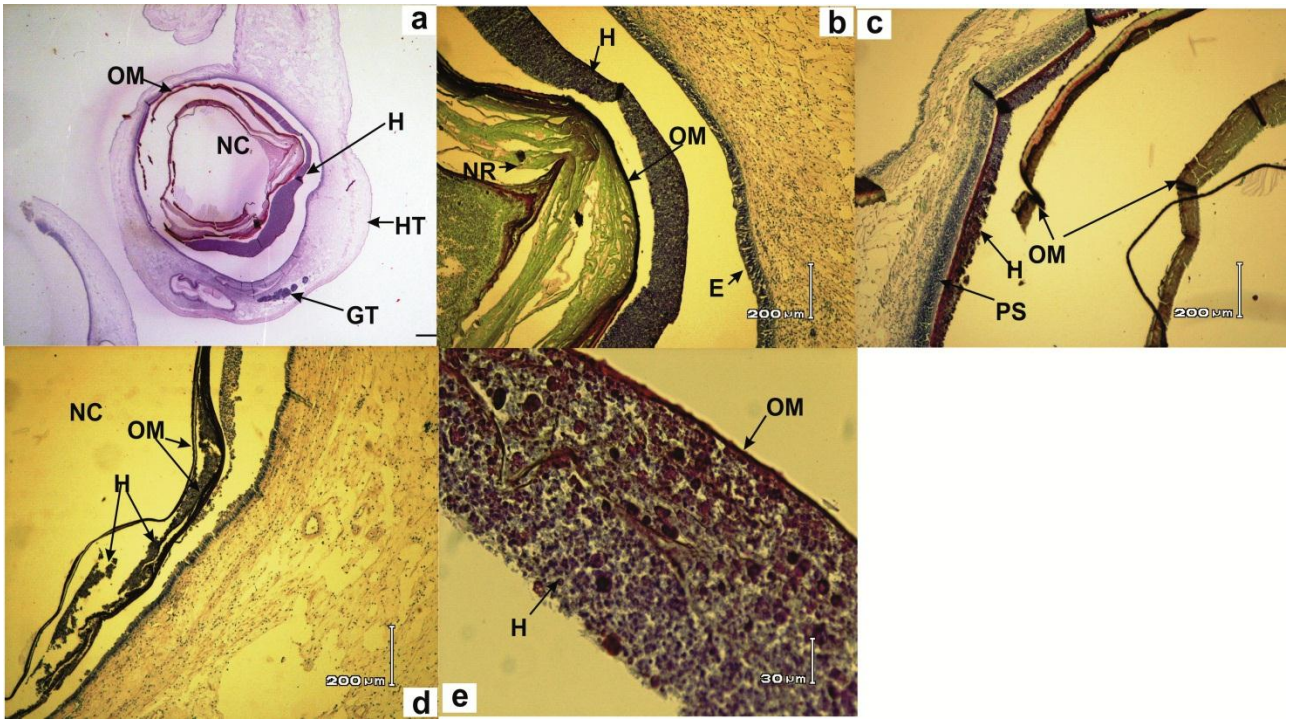

Figure 4.5. Histological sections of the developing pearl-sac in *Pinctada margaritifera* grafted with regenerated mantle tissue sixteen days after grafting showing: a, haemocytes (H), gametes (GT) present in the gonad of the host tissues (HT), and organic matrix (OM) layer in the nucleus cavity (NC) secreted by the epithelial cells of the pearl-sac at a low power view; b, epithelial cells (E),organic matrix (OM) layer present on top of the nucleus remnant

(NR), and a band of haemocytes (H); c, pearl-sac (PS) and layers of organic matrix (OM) with haemocytes (H); d, organic matrix (OM) layers and haemocytes (H) in the nucleus cavity (NC); and e, a close up view of haemocytes (H) mixed with organic matrix (OM). Bar scale 220 µm.

As indicated above, one of the oysters grafted in this trial rejected the nucleus 12 days after grafting. Although the nucleus was rejected, the mantle graft tissue remained within the nucleus cavity of the oyster (Fig. 4.6a). The mantle graft tissue was virtually completely attached to the host tissue and had fully integrated with it (Fig. 4.6b and 5.6c); however, it had not formed a pearl-sac enveloping the nucleus cavity unlike samples where the nucleus was retained (Fig. 4.4a). Muscle fibers were clearly evident within the graft tissue (Fig. 4.6d). Haemocytes were not present within the nucleus cavity and no obvious anomalies that may have caused nucleus rejection were evident in the nucleus cavity or associated tissues.

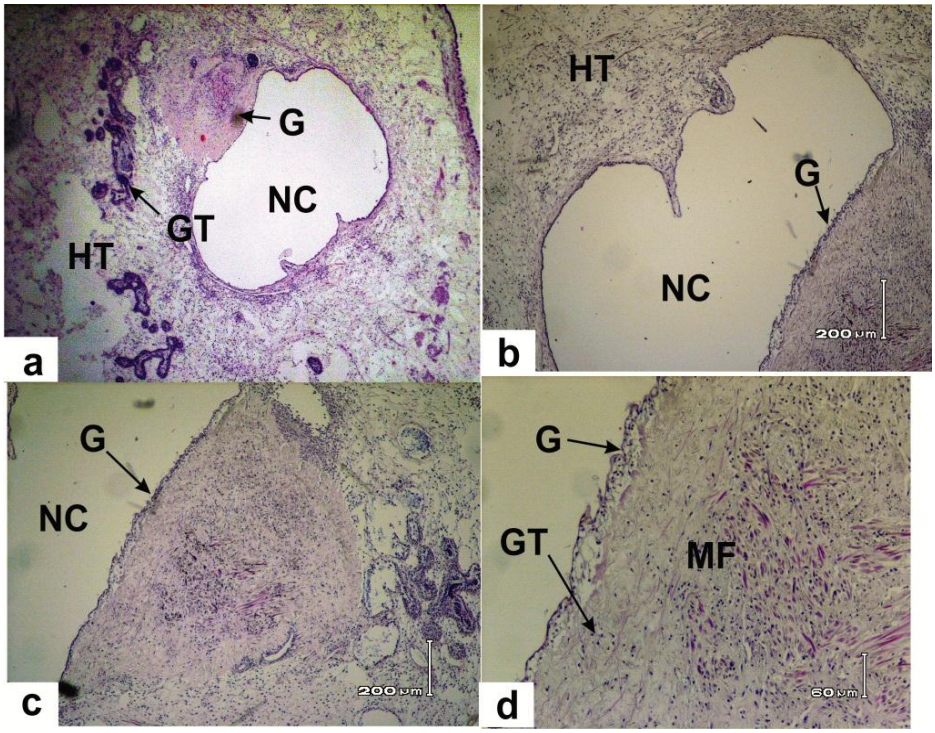

Figure 4.6. Histological sections of a nucleus cavity following nucleus rejection in *Pinctada margaritifera* grafted with regenerated mantle tissue twelve days after grafting showing: a, host tissues (HT), gametes (GT), nucleus cavity (NC), and mantle graft tissue (G) rejection at low power view; b, a close up of the nucleus cavity (NC) and mantle graft tissue (G) within host tissue (HT); c, nucleus cavity (NC) and the attachment of the mantle graft tissue (G) to host tissues; and d, mantle graft tissue (G) with developing muscle fibers (MF) and gametes (GT). Bar scale 230 µm.

4.3.2. Nacre deposition

Of the 22 oysters grafted with regenerated mantle to monitor nacre deposition, two died within one month of grafting while four did not contain a nucleus when brought in for examination or for sampling during the course of the experiment. Two of these rejected their nuclei between two and three months after grafting. After three months of culture,

the nuclei in the remaining eight oysters were covered with nacre (Fig. 4.7). The thickness of nacre on nuclei harvested from oysters grafted with regenerated mantle and normal mantle ranged from 0.65 ± 0.03 to 0.49 ± 0.01 mm and from 0.63 ± 0.01 to 0.49 ± 0.01 mm, respectively. The average (±SE) nacre thickness from regenerated (0.547 ± 0.01 mm, n = 8) and normal (0.532 ± 0.01 mm, n = 8) mantle did not differ significantly ($p > 0.05$; Fig. 4.8).

Figure 4.7. Nacre covered nuclei recovered from pearl-sacs developed in *Pinctada margaritifera* three months after grafting with (a), normal mantle tissue and (b), regenerated mantle tissue.

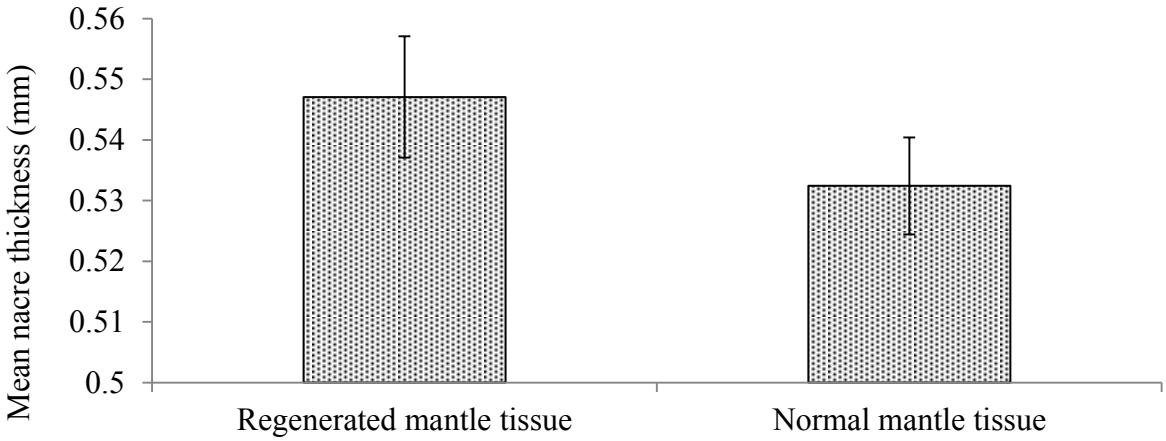

Figure 4.8. Mean (±SE, n=8) thickness of nacre covering nuclei recovered from *P. margartifera* grafted with either regenerated mantle tissue or normal mantle tissue, three months after grafting.

Given that pearl-sac development requires 12 days (see *section 4.3.1*) and that nacre secretion does not begin until the pearl-sac is complete (Chapter 2), the nacre secretion rate, over the 80 day period subsequent to pearl-sac formation were calculated to be 6.84 ± 0.1 µm day^{-1} and 6.66 ± 0.1 µm day^{-1} for oysters grafted with regenerated and normal mantle tissue, respectively. These means were not significantly different (p = 0.258).

4.4. Discussion

Pearl-sac development studies have been carried out with a number of pearl oyster species including *P. margaritifera* (Cochennec-Laureau *et al.*, 2010), *P. maxima* (Scoones, 1996) and *P. fucata* (Gould, 1850) (Kawakami, 1953; Dix, 1972; Velayudhan *et al.*, 2011). Most have reported on development and function of the pearl-sac under 'normal' circumstances where saibo was excised from selected donor

oysters that were sacrificed. The potential of removing mantle tissue from anaesthetised pearl oysters, thus eliminating the need for donor oyster sacrifice, was first proposed for *P. margaritifera* by Acosta-Salmon et al. (2004). However, the only prior assessment of the development and function of regenerated mantle tissue when used as saibo was reported for *P. maxima* (Mamangkey, 2009). This is the first time that regenerated mantle tissue has been used in pearl grafting with *P. margaritifera* and the results from this chapter can be compared with those of a recent description of pearl-sac development in *P. margaritifera* (Chapter 2), enabling direct comparison of pearl-sac structure following grafting with both regenerated and normal mantle tissue grafts. Regenerated mantle graft tissue proliferated within the nucleus cavity in a similar fashion to normal mantle tissue and formed a complete pearl-sac with secretory function within two weeks of grafting, similar to normal mantle tissue. Furthermore, the histological structures of pearl-sacs developed from regenerated mantle tissue could not be differentiated from those developed from normal mantle tissue (Chapter 2).

Pearl-sacs that developed from regenerated mantle tissue had completely formed and had fully developed epithelial cells within 12 days of the grafting procedure (Table 4.1). The time taken for complete pearl-sac formation in this study is very similar to that reported in Chapter 2 and by Cochennec-Laureau et al. (2010) (12-14 days) where normal non-regenerated mantle tissue was used for pearl grafting in *P. margaritifera*. The major developmental and functional stages of pearl-sacs grown from normal and regenerated mantle tissue are shown in Table 4.1.

Table 4.1. The different developmental stages of pearl-sacs grown from normal and regenerated mantle tissue in *Pinctada margaritifera.* Data for normal pearl-sac development in *P. margaritifera* is from Chapter 2.

Days	Pearl-sac development from normal mantle	Pearl-sac development from regenerated mantle
6	– mantle grafts covered half the nucleus cavity circumference of the host oysters – longitudinal and radial muscles could now be easily distinguished	– half of the nucleus cavity circumference covered by extending regenerated mantle. – connective tissues and fibers (longitudinal and radial muscles) now visible
12	– -pearl-sac formation completed – epithelial cells with columnar shapes – secretion of organic matrix commenced by epithelial cells	– complete pearl-sac formation – pearl-sac can be barely differentiated from host tissue – epithelial cells with columnar shapes – organic matrix secretion commenced by the epithelial cells
16	– pearl-sac can hardly be differentiated from host tissues – organic matrix layer thickened	– pearl-sac can no longer be differentiated from host oyster tissue – organic matrix layer thickened

The thickness of nacre deposited on nuclei in the pearl-sacs of oysters grafted with the normal and regenerated mantle tissue was very similar and did not differ significantly. This indicates that secretion of nacre would have begun at a similar time after grafting in the respective pearl-sacs and further confirms that the development rate of pearl-sacs and their secretory cells were similar regardless of whether normal or regenerated mantle tissue was used as saibo. There is limited information relating to the rate of nacre deposition in *P. margaritifera* and how it might vary during the pearl culture period. In French Polynesia, pearls must have a minimum nacre thickness of 0.8 mm, after a culture period of 18-24 months, to be permitted for export (Southgate *et al.*, 2008). The thickness of nacre deposited by pearl-sacs that developed from both normal

and regenerated mantle tissue in this study (up to 0.65 mm) was surprising over the relatively short culture period of three months. At the Fijian farm site used in this study, the usual culture period for round pearls is 18 months and this generates a nacre thickness of around 1.3 mm. Data in this chapter indicate that nacre secretion rates vary over the pearl culture period, perhaps with relatively rapid deposition rates immediately after pearl-sac development and a slowing of nacre deposition as the cultured pearl matures. Variation in nacre deposition rates was reported for mabè (half) pearl nuclei implanted into *Pteria sterna* where maximum deposition occurred within the first few months and decreased thereafter (Ruiz-Rubio *et al.*, 2006). However, more research is required in this field and into the influence of environmental conditions, such as water temperature and seasonality, on nacre deposition rates in *P. margaritifera.*

Mamangkey (2009) conducted a similar study to this where *P. maxima* were grafted with regenerated mantle tissue. He reported relatively low nucleus retention rates but the pearls produced from regenerated mantle tissue were of similar or better quality than those produced using normal mantle tissue (Mamangkey, 2009). Given the success demonstrated in the present study regarding nucleus retention and survival of oysters grafted with regenerated mantle tissue, and considering the findings of Mamangkey (2009), it is likely that commercially viable pearl yields and pearl quality could be obtained using regenerated mantle tissue in *P. margaritifera*.

Our results clearly show that regenerated mantle tissue can function successfully as saibo for pearl production in *P. margaritifera*. This finding could provide significant benefits to pearl farmers and a basis for further development of current pearl grafting practices. The two major potential benefits to pearl farmers from this development are

the ability to maintain high quality pearl oysters as broodstock to build the quality of farm stock, and the potential to use high quality saibo donors for pearl production on more than one occasion, using regenerated mantle tissue. Both activities are likely to result in improvements to pearl quality. Further advantages relate to potential economic gains for pearl farmers through continued commercial pearl production from oysters that would normally be discarded after the first pearl harvest. This is likely to result in increased pearl production and considerable cost savings relating to farm labour and infrastructure.

Chapter 5

[4]Does the quality of cultured pearls from the black-lip pearl oyster, *Pinctada margaritifera*, improve after the second graft?

5.1. Introduction

Pearls are produced when a piece of mantle tissue (saibo) from a foreign (donor) pearl oyster and a round inorganic nucleus are inserted into the gonad of a host pearl oyster (Gervis and Sims, 1992; Taylor and Strack, 2008); a process known as 'seeding' or 'grafting'. Pearl grafting is usually performed by skilled technicians that commonly work only with a single species of pearl oyster. After the grafting operation, saibo tissue proliferates to form a pearl-sac, which envelopes the nucleus, and nacre secreting epithelial cells from the pearl-sac begin to deposit nacre onto the nucleus (Dix, 1972; Scoones, 1996; Taylor and Strack, 2008; Cochennec-Laureau *et al.*, 2010). For the black-lip pearl oyster, *P. margaritifera,* a culture period of approximately 18 months is required before a commercially acceptable nacre thickness of 0.35-0.8 mm (Haws, 2002; Matlins, 2002; Ruiz-Rubio *et al.*, 2006) is achieved and the resulting cultured pearl is ready to be harvested.

Pearl oysters that produce good quality pearls are generally considered for further nucleus implantation(s) to produce a second, third or even fourth cultured pearl, depending on their health and continued ability to produce good quality pearls (Fong *et al.*, 2005; Taylor and Strack, 2008). These 're-seed' of 're-graft' operations are called

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'surgreffe' in French Polynesia and involve insertion of a nucleus into the existing pearl-sac formed during the previous graft (Taylor and Strack, 2008). The size of the nucleus is increased each time to produce pearls that are larger than those from the previous harvest(s) (Haws, 2002). However, the value of a cultured pearl not only depends on size but also accounts for lustre, shape, surface perfection and colour (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008). Round pearls generally command higher prices than those with other shapes and pearls that are perfectly round, have high lustre and are flawless in all other regards are considered of to be of the highest quality (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008). However, such pearls are rare making up only around 3% (Ky *et al.*, 2015) to 5% (Haws, 2002) of the total harvest . It is generally considered that the top 5% of pearls generate around 95% of pearl farm revenue (Haws, 2002). Thus, even a small increase in the proportion of high quality pearls produced by pearl farms would generate significant increase in revenue.

The quality of cultured pearls is influenced by environmental factors such as water quality parameters at the culture site and food availability (Wada and Komaru, 1996; Lucas, 2008), as well as non-environmental factors such as grafting techniques (Ky *et al.*, 2014b; Ky *et al.*, 2015), host oyster condition (Taylor and Strack, 2008), saibo influences (McGinty *et al.*, 2011) and husbandry techniques (Taylor and Strack, 2008). A significant proportion of cultured pearls harvested from *P. margaritifera* have symmetrical grooves across their surfaces, commonly called 'circles' (Ky *et al.*, 2015) . Pearls with circles have considerably lower value than round pearls and a crop with a high proportion of circled pearls has significantly reduced value. In the Tuamotu Archipelago of French Polynesia for example, circled pearls were reported to account

for 23% of a harvest of 271,000 cultured pearls from *P. margaritifera*, but making up only 6% of the value of the crop (Murzyniec-Laurendeau, 2002). Ky et al. (2015) more recently reported that circled pearls made up 53% of 42,575 cultured pearls harvested from six separate experiments involving 17 different pearl grafters in French Polynesia. A number of factors have been suggested as a cause of circles in pearls. These include the skills of grafting technicians, environment conditions at pearl culture sites and grafting season (Ky *et al*., 2015), interruption in the supply or changes in concentration of material in the peal-sac (Cartwright *et al.*, 2013) and the presence of byssus close to the pearl-sac during pearl formation that could disrupt even nacre deposition on developing pearls (Chapter 2).

It is widely assumed that oysters producing pearls with circles are unlikely to produce pearls with improved quality if grafted again, and for this reason these oysters are often discarded. This is a reasonable assumption if circles are caused by an anomaly in the pearl-sac that is likely to remain after re-grafting. However, very little research had been done in this field. If oysters that produce low quality circled pearls are capable of producing pearls with improved quality after re-grafting, then this would provide opportunities for pearl farmers to build farm stock and improve income. The aim of this study was therefore to determine whether oysters producing circled pearls are able to produce pearls with improved quality after re-grafting. The results of this chapter allow further discussion of the factors that influence the formation of circles on pearls produced by *P. margaritifera*.

5.2. Material and methods

This study was carried out at a commercial pearl farm operated by J. Hunter Pearls (Fiji) in Savusavu Bay on the island of Vanua Levu in the Fiji.

The oysters considered for this experiment were among those collected as wild juveniles (spat) obtained from spat collectors deployed at various sites within Savusavu Bay. Once removed from spat collectors, oysters were cultured using standard commercial methods until reaching a size suitable for pearl grafting. Oysters selected for the first graft in November 2012 (early summer) had mean dorso-ventral and antero-posterior measurements of 114.48 ± 0.61 mm and 104.42 ± 0.52 mm, respectively. These oysters were cleaned before being grafted by one of the three professional and experienced black-lip pearl oyster grafting technicians. The grafting procedure generally followed that described by Taylor and Strack (2008). Briefly, it included careful selection of healthy donor oysters, excision of mantle tissue from each shell valves, stripping the pallial mantle from the excised mantle portion and cutting it into small square pieces to obtain saibo. A nucleus of 2.7-3.0 *bu* (8.5-9.44 mm diameter) and a piece of saibo were then grafted into the pearl pouch of each recipient oyster. The maximum time between saibo preparation and its use for pearl grafting was less than 25 minutes. Grafted oysters were housed on their sides in lantern nets at a depth of 7 m for six weeks as part of the standard culture practice at J. Hunter Pearls. These oysters were turned through 180^{o} to rest on their other side after three weeks (Taylor and Strack, 2008) and after the six week convalescent period, grafted oysters were transferred to panel nets (40 x 40 mm mesh pore size) that were deployed vertically from a long line at the farm site (16˚46′13.30″S, 179˚19′20.17″E) at a culture

depth of 7 m. The oysters were inspected and cleaned every two months according to normal farm procedures.

Oysters were cultured for 18 months before resulting pearls were harvested by the technician that undertook the initial grafting of a given oyster. Of the oysters that produced pearls with circles and would normally be discarded, 100 were randomly selected for re-grafting in this experiment. These oysters were among 1,100 oysters brought in for harvesting on the same day, of which 50-55% produced pearls with circles. The number of circles on the pearls produced by these oysters varied between one and ten in the following proportions: one circle (2 oysters), two circles (12 oysters), three circles (41 oysters), four circles (15 oysters), five circles (18 oysters), six circles (5 oysters), seven circles (5 oysters) and ten circles (2 oysters) (n = 100). Pearls produced by all 100 selected oysters had circles although five of these pearls (5%) were classed within the 'baroque' shape category (Table 5.1) because of their asymmetrical and distinctly irregular shapes.

The second graft was conducted in May 2013 and each oyster was individually tagged after being re-grafted by carving a number on a square piece (30 mm X 30 mm) of plastic cut from plastic container which was then tied to the pocket of the panel net holding the respective oyster. This allowed direct comparison of the first and second graft pearls produced by the same oyster. Re-grafted oysters were transferred directly to panel nets and held at the farm site under conditions described above. After a further culture period of 18 months, second graft pearls were harvested and the quality of successive pearls produced by individual oysters was compared in terms of shape, size, lustre, colour and surface perfection. Assessment of pearl quality involved inspection

and grading of individual pearl characteristics by J. Hunter Pearls' professional pearl grader.

5.2.1. Pearl shape

Pearls harvested from *P. margaritifera* are normally classified into five categories of shape; round, semi-round, circles, baroque and keshi (Table 5.1). The pearls produced from the second graft were graded according to these categories. The number of circles on these pearls was also counted and compared with the number of circles on the pearl produced by the same oyster from the first graft.

Table 5.1. The different categories of pearl shape used to grade pearls produced by *Pinctada margaritifera* in this study (modified from Strack, 2006; Taylor and Strack, 2008).

Shape	**Description**
Round (R)	Pearl completely spherical with virtually no variation present on the surface.
Semi Round (SR)	Pearl not completely spherical but appears spherical when viewed from a particular angle. Slightly flattened or elongated shape is only visible when observed very closely.
Circle (C)	Pearls have symmetrical lines or 'grooves' on their surface. Pearls of all above shapes but with grooves were covered by this category.
Baroque (B)	Pearls are asymmetrical and appear distinctly irregular.
Keshi	Non-nucleated pearls with unique shapes resulting from nucleus rejection.

5.2.2. Pearl size

Pearl size was determined as the maximum diameter at the widest point of each pearl (Taylor and Strack, 2008) and was measured to the nearest 0.05 mm using a micrometer. Pearls (excluding 'keshi') were then classed into five size categories; 9-10 mm, 10-11 mm, 11-12 mm, 12-13 mm and 13-14 mm. The sizes of pearls harvested after the first and second graft were then compared.

5.2.3. Pearl lustre

Lustre is described as the sharpness and intensity of light reflected from the surface of a pearl where greater and clearer reflection generates higher lustre (Matlins, 2002; Strack, 2006). The lustre of pearls produced after the first and second graft from an individual oyster was visually assessed by the pearl grader and each pearl was classed into one of the three categories; 'high lustre', 'medium lustre' and 'dull'. The influence of lustre on the overall quality of cultured pearls is reflected in their overall grading (Table 5.2).

5.2.4. Surface perfection

When assessing the surface perfection of pearls, irregularities such as tiny spots, blemishes, circles, cracks, scratches, small bumps and blisters are considered (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008). This characteristic is not assessed quantitatively for pearls produced in Fiji, but generally the quality of pearls decreases with increasing irregularities. Pearl surfaces of both first and second grafted pearls were examined by the pearl grader and the influence of irregularities was reflected in the overall grade assigned to a pearl (Table 5.2).

5.2.5. Pearl colour

Cultured pearls produced by *P. margaritifera* are appreciated for their broad range of colours (Taylor and Strack, 2008; Cartier *et al.*, 2012). Fiji pearls have a particularly broad range of colours (Anon, 2007) many of which are unique. For marketing purposes, Fiji pearls are generally assigned to one of four major colour categories, each of which may incorporate pearls with a range of colours. 'Fiji Pastel' includes pearls that are light in colour (e.g. white, silver and grey), 'Fiji Bright' includes pearls with bright colours (e.g. bright blue, bright green and bright gold), 'Fiji Rare' includes unique pearls with different coloured overtones (e.g. green, blue, gold) and colour combinations, and pearls with relatively dark colours (mainly black) are assigned to the 'Fiji Traditional' category. Pearls harvested after the first and second grafts were classified into these categories by the pearl grader.

5.2.6. Overall grading

Pearls from *P. margaritifera* are normally graded into four categories that account for both quantitatively (i.e. size) and qualitatively (i.e. lustre, surface perfection and colour) assessed characteristics as outlined in Table 5.2. Overall proportions of pearls from the first and second graft within the different grades were then compared.

Table 5.2. Overall grading of pearls produced by *Pinctada margaritifera* incorporating assessment within five grading criteria (shape, lustre, size, surface perfection and colour) (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008).

Grade	Shape	Lustre	Size	Surface perfection	Colour
A	Round	Very high lustre	Often of larger size than other grades.	Very minor or no imperfection. Often less than 5% of the total surface.	Very bright with attractive colour.
B	Round to semi-round	High lustre	Variable sizes but generally of larger size then grades C and D.	Minor surface imperfections. Usually not more 30% of the total surface.	Brightly coloured
C	Baroque Most pearls in this category have circles.	Variable (medium) lustre. Pearls with high lustre are classed as 'C' grade if these pearls have circles or baroque shape.	Variable sizes	Has notable surface imperfections that include blemishes, dents, bulges and circles.	Variable
D	Mostly uneven shapes. Presence of circles.	Dull	Variable sizes	Major surface imperfections covering >60% of surface.	Variable

5.2.7. Statistical analysis

Categories within four pearl quality assessment criteria (shape, size, lustre and overall grade) were assigned different scores where '1' represented the lowest grade, and increasingly higher scores were assigned to superior grades within each criterion, depending on the number of levels within each. For example, 'shape' was assessed from the lowest grade of '1' to a high of '5', 'size' was assessed from '1' to '5', 'lustre' from '1' to '3' and overall pearl grade from '1' to '4'. Normality tests were carried out to confirm that data were not normally distributed and Chi-square tests for Independence were used to determine if there were significant differences in the number of pearls in each assessment criterion (shape, size, lustre and overall grade) after first and second graft. The same test was also used to detect whether the number of pearls with circles differed significantly after first and second graft. The tests were carried out using the IBM SPSS Ver. 20 statistical software and values were considered significant when $p < 0.05$.

5.3. Results

Of the 100 *P. margaritifera* chosen for the second graft, only 65 of these oysters produced pearls. Fifteen oysters died during the 18 month culture period and were discarded from the culture units during regular oyster cleaning, while 20 of these oysters did not have pearls when harvested.

5.3.1. Pearl shape

The percentages of first and second graft pearls allocated to the various shape categories are shown in Figure 5.1. The highest number of pearls from the first graft (95%) and second graft (n = 31, 48%) were classified within the 'circle' category.

There was an increase in the proportion of pearls in the 'baroque' category after the second graft (n = 22, 34%) compared to the first graft (5%). Of particular note is the production of pearls within the 'semi-round' category after the second graft (18%), given that pearls from this superior shape category were absent after the first graft. There was a significant difference ($p = 0.04$) in the number of pearls belonging to different shape categories after first and second graft.

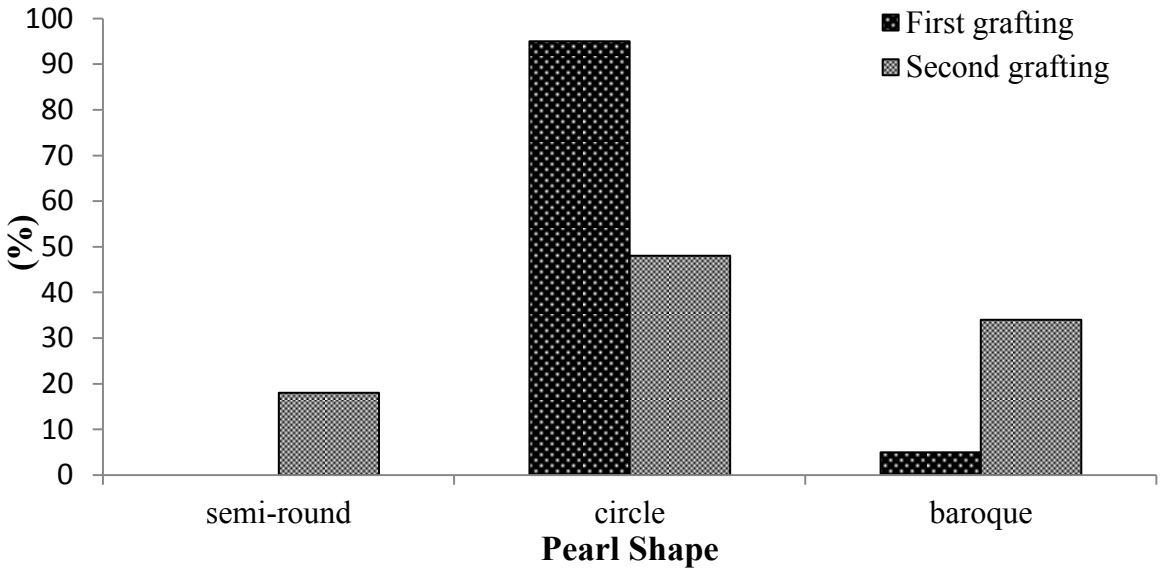

Figure 5.1. Percentages of pearls allocated to different shape categories produced after first and second graft from the same *Pinctada margaritifera*.

5.3.1.1. Number of circles

The proportions of pearls with different numbers of circles after the first and second graft are shown in Figure 5.2. Pearls with three circles made up the largest component after the first graft but there was a 23% reduction in the number of pearls with three circles after the second graft. Pearl from the second graft had a lower proportion of pearls with two, three, four, five and seven circles (Fig. 5.2). Furthermore, some pearls

from the first graft had 6 and 10 circles (5% and 2%, respectively) but similar pearls were not present after the second graft. Importantly, 50% of the pearls from the second graft did not have any circles compared to 100% of pearls with circles after the first graft. In general, with the exception of pearls with a single circle, pearls from the second graft had fewer circles than those form the first graft and this difference was significant ($p = 0.03$).

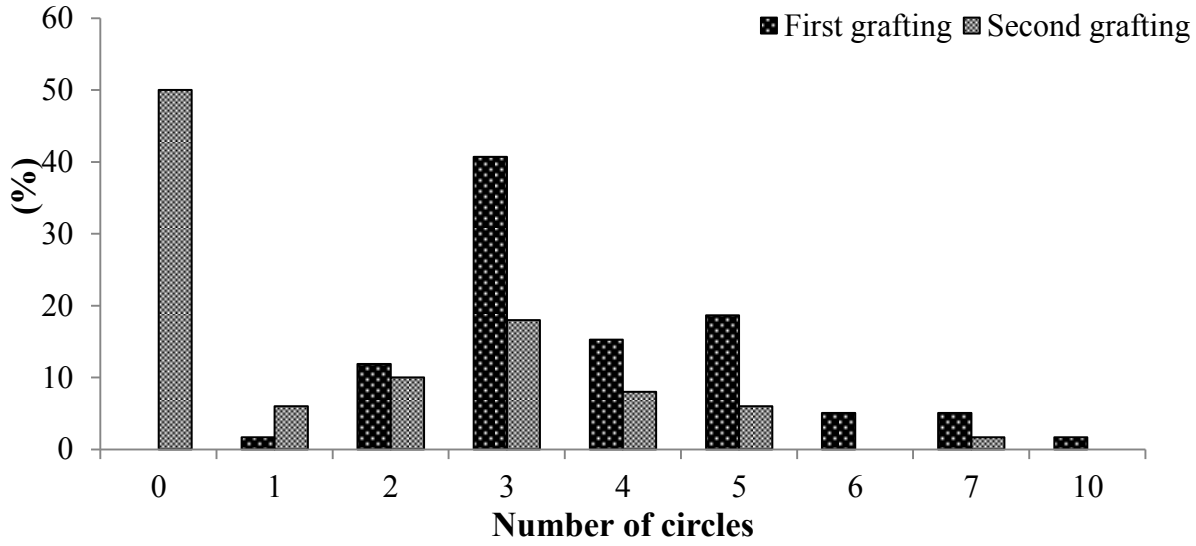

Figure 5.2. Percentages of pearls with varying numbers of circles after first and second graft from the same *Pinctada margaritifera.*

5.3.2. Pearl size

The proportions of pearls from the first and second graft within each of the five size categories used in this study are shown in Figure 5.3. The majority of pearls from the first graft (63%) were 10-11 mm in size, while most of those from the second graft (51%) were in the larger 11-12 mm size category. There were higher proportions of pearls in the 12-13 mm and 13-14 mm size classes from the second graft than from the first graft (Fig. 5.3); however, these pearls were of very poor quality with irregular

shapes often having ‘tails’ (Norton et al., 2000). The sizes of pearls from the second graft were generally bigger than those from the first graft and this difference was significant ($p = 0.04$).

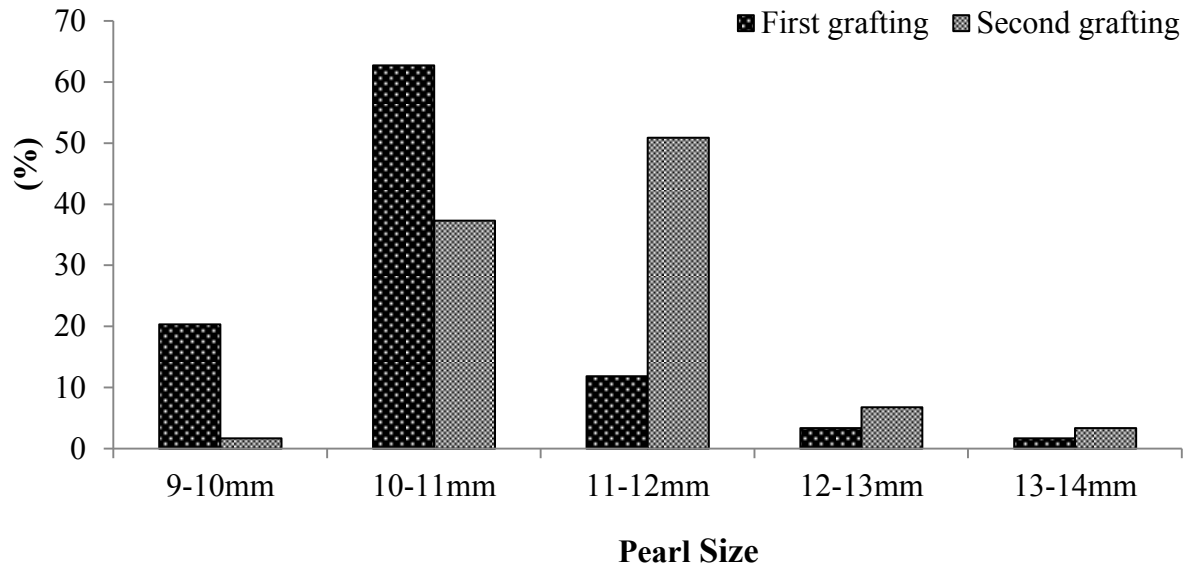

Figure 5.3. Percentages of pearls belonging to different size categories harvested after the first and second graft from the same *Pinctada margaritifera.*

5.3.3. Pearl lustre

The relative proportions of pearls from the first and second grafts allocated to each of the four lustre categories used in this study are shown in Figure 5.4. A high proportion of oysters (64%) used in this experiment produced pearls that were dull after the first graft. However, the proportion of dull pearls increased to 73% in second graft pearls. A greater proportion of first graft pearls (34%) than second graft pearls (27%) were categorised into the medium lustre category while only pearls from the first graft (2%) were present in the ‘high’ lustre category. Overall, second graft pearls had poorer lustre than first graft pearls with a higher proportion of dull pearls, a lower proportion of

medium lustre pearls and no pearls with high lustre. Despite this, the number of pearls belonging to different lustre categories after the first and second graft did not differ significantly ($p = 0.07$).

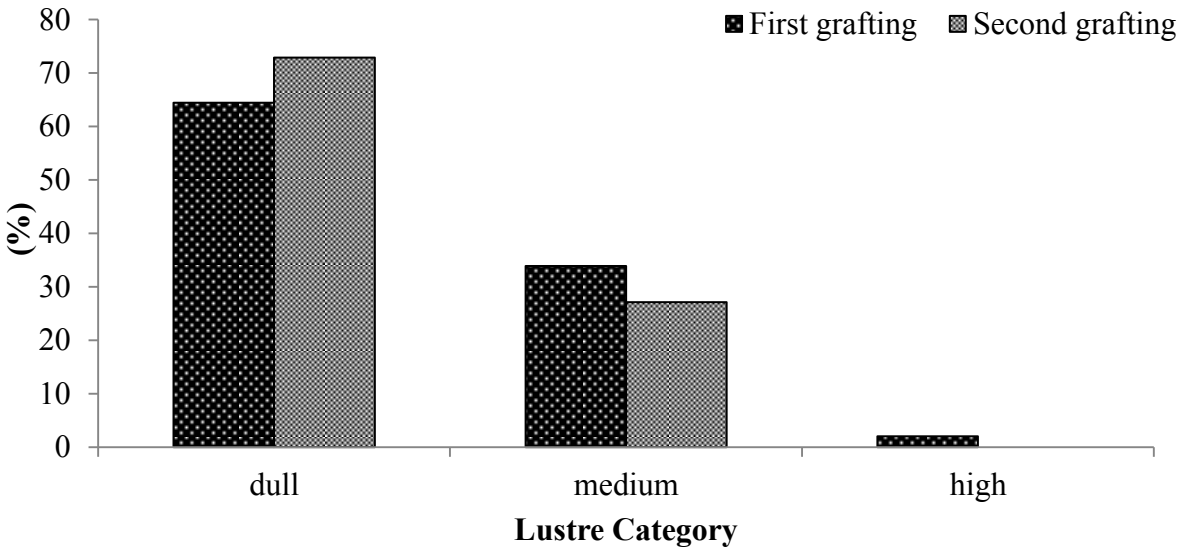

Figure 5.4. Percentages of pearls belonging to different lustre categories harvested after first and second graft from the same *Pinctada margaritifera.*

5.3.4. Colour

Pearls produced after both the first and second graft had a wide array of colours. Pearls in the 'Fiji Pastel' category made up the majority of those from first graft (34%) and second graft (36%). This was followed by the pearls assigned to the 'Fiji Traditional' category which made up 25% of pearls from the first graft and 23% from the second graft. Pearls in the 'Fiji Bright' category made up 12% of pearls from the first graft and 14% from the second graft, while pearls in the 'Fiji Rare' category made up only 3% of the pearls from both the first and second grafts. Some pearls from both batches in chocolate, peacock, pistachio and lavender tones did not fall into any of the above

colour categories although the numbers of these pearls were very low. Interestingly, an oyster that produced a certain colour of pearl after the first graft did not necessary produced the same colour pearl after the second graft. Only 14% of oysters produced pearls that were the same colour after the first and second grafts.

5.3.5. Overall grade

The proportions of pearls from the first and second grafts within each of the overall grades used in this study are shown in Figure 5.5. Most first graft pearls (83%) were assessed as 'C' grade with 17% categorised as 'D' grade. Similarly, most second graft pearls (78%) were assessed as 'C' grade and 20% as 'D' grade (Fig. 5.5); however, 2% of second graft pearls were assessed as 'B' grade which were not present in first graft pearls. Despite pearls from the second graft having improved shape, size, and a reduced number of circles compared to first graft pearls, there was no significant improvement in overall quality of second graft pearls ($p = 0.08$).

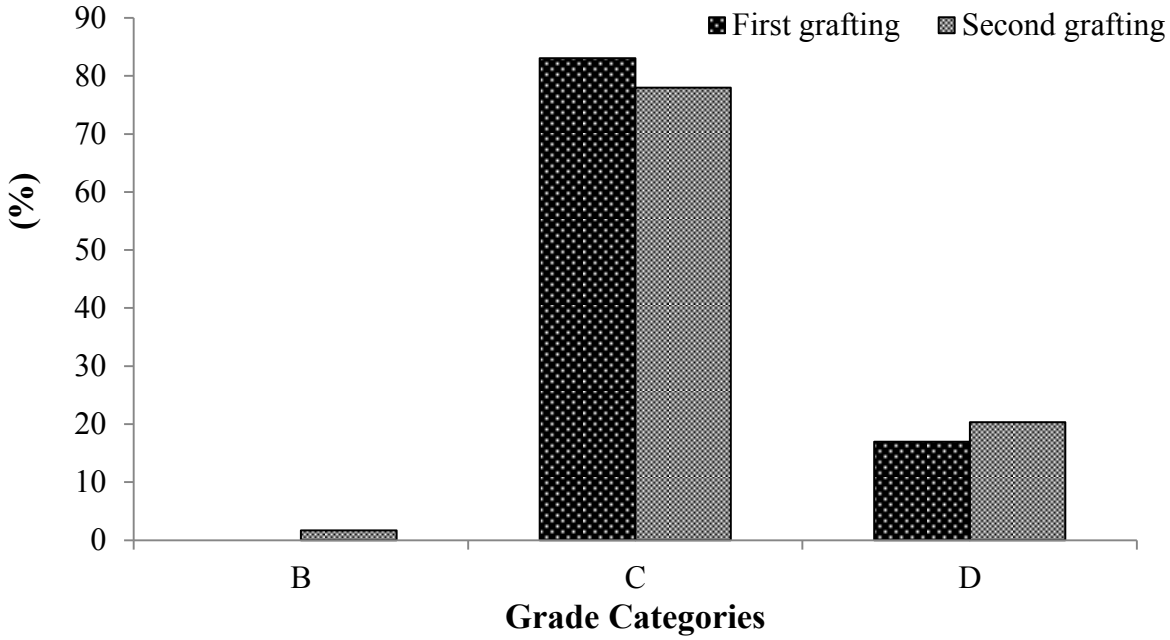

Figure 5.5. Percentages of pearls allocated to different grade categories following first and second graft from the same *Pinctada margaritifera.*

5.4. Discussion

Pearls from the second graft were, as expected, larger than those from the first graft because of the larger sized nuclei used in re-graft operations (Haws, 2002). The results also showed that there was an improvement in the shape of pearls between the first and second grafts. Most of the circled pearls harvested after the first graft were classed into the 'circle' shape category (95%) with some in the 'baroque' category (5%) but none in the 'semi-round' category. The shape composition of these first graft pearls is to be expected because the oysters chosen for the second graft were those that produced circled pearls after first graft. Despite this, 18% of these oysters produced pearls of 'semi-round' shape after the second graft demonstrating improvement in pearl shape. There was also a major reduction in the number of pearls with circles after the second graft. All pearls from the first graft had varying numbers of circles and 86% of these

pearls had three or more circles. In contrast, 50% of the pearls produced from the second graft did not have any circles. The data in this chapter show for the first time that that production of circled pearls after second graft is not obligatory for *P. margaritifera* that produced circled pearls after the first graft.

Prior research in this laboratory involved histological analysis of developing and mature pearl-sacs in an effort to identify whether anomalies in pearl-sac structure were related to the production of circled pearls (Chapters 2 and 3). While no obvious structural anomalies in the pearl-sac were identified, close proximity of byssus threads to the pearl-sac in some samples indicated that perhaps pressure applied to the pearl-sac by byssus or associated muscles may be involved in circle formation (Chapter 2). Accepting that the nucleus or developing pearl rotates within the pearl-sac (Cartwright *et al.*, 2013), then pressure applied at a single point on the pearl-sac could result in a concentric anomaly on the pearl. However, if such a mechanism was responsible for circle formation on developing pearls, a similar proportion of circled pearls would be expected to result from first and second grafts given that byssus and their associated muscles would be present in the same or similar position during development of both first graft and second graft pearls. Furthermore, because re-grafting involves implanting a larger (than the original) nucleus and that the second pearl is likely to be larger than the first, it is reasonable to assume that anomalies resulting from pressure applied to the pearl-sac during pearl formation could be more evident in second graft pearls.

An important factor that is likely to vary between first and second grafts is the tightness and rigidity of the pearl-sac around the nucleus following the second graft. Harvesting

pearls involves an incision made into the pearl-sac to remove the mature pearl. A second larger nucleus (generally of similar size to the harvested pearl) is then inserted into the pearl-sac through the incision that will subsequently heal. The pearl-sac that develops from the initial graft grows around the nucleus (Chapter 2) and subsequent and continual deposition of nacre onto the nucleus is likely to result in increasing tension within the pearl-sac. There is no doubt that cutting into the pearl-sac to remove first graft pearls and subsequent insertion of a second nucleus will result in changes in tension within the pearl-sac. Given that this study has shown a reduction in the proportion of circled pearls following second graft, it is likely that changes in pearl-sac tension resulting from the second graft operation reduces or eliminates the factor(s) that cause circles. If tension within the pearl-sac is a contributing factor in circle formation, it would be interesting for future research to examine the influence of nucleus size at second graft, which could be used to manipulate pearl-sac tension, on the proportion of circled pearls produced.

We are unaware of any prior studies that have reported the relative proportions of circled pearls produced for *P. margaritifera* following first and second grafts. But a number have hypothesised the cause(s) of circles which include the skills of grafting technicians, environment conditions at pearl culture sites and grafting season (Ky *et al*., 2015), interruption in the supply or changes in concentration of material(s) in the peal-sac (Cartwright *et al.,* 2013) and the presence of byssus close to the pearl-sac during pearl formation (Chapter 2), as discussed above. Higher levels of suspended particulate matter present in island lagoons compared to atoll lagoons has been suggested as the cause of differences in the proportion of pearls with circles produced in these two environments (Ky *et al.*, 2015), and a higher proportion of circled pearls are produced

by oysters held on chaplets compared to those held in panel (pocket) nets in Fiji (Chapter 7). It is likely that a number of factors influence the formation of circles on cultured pearls produced by *P. margaritifera* and further research is required.

This is the first study to report on differences in the overall quality of first and second graft pearls produced by the same oysters (*P. margaritifera*). Despite pearls from the second graft having improved shape, size, and a reduced number of circles compared to first graft pearls, there was no significant improvement in overall quality of second graft pearls. However, of particularly importance is that results of this chapter show that marketable pearls can be produced from oysters that are normally discarded after first pearl harvest and this has potential to generate increased revenue for pearl farmers in Fiji and other Pacific islands. Pearl farmers would need to consider the labour and financial inputs required to replenish oysters discarded after the first pearl harvest versus the potential revenue generated from re-grafting these oysters. A detailed economic analysis of the two options would greatly benefit pearl farmers.

Chapter 6

[5]The effects of different culture units and current velocity on byssus production by the black-lip pearl oyster, *Pinctada margaritifera.*

6.1. Introduction

Byssal threads, or byssus, are secreted by pearl oysters to anchor oysters to various substrates (Wada and Tëmkin, 2008) and the duration of byssal attachment varies between species. *P. margaritifera* and *P. fucata* , for example, maintain byssus secretion throughout their lives (Gervis and Sims, 1992), while *Pinctada maxima* has been reported to cease byssal secretion once large and heavy enough to withstand agitation or dislodgement caused by ocean currents (Taylor *et al.*, 1997). In natural habitats, the byssus of pearl oysters attaches pearl oysters to corals (live or dead), boulders, rocks or artificial substrates (Gervis and Sims, 1992), while on pearl farms the oysters generally attach directly to the culture units used to house these oysters.

Situated at the mid zone of the retractor muscle and proximal end of the foot, the double lobed byssus–producing byssal gland, is comprised of two bundles of grooves that end externally as byssal threads (Fougerouse *et al.*, 2008). Byssal threads are composed of three sections; the root, the stem and the distal disk. The root is made up of whitish fibres that are embedded at the base of the foot (Dharmaraj *et al.*, 1987b), while the stem is a strong, green thread-like structure that joins multiple roots converging at the base of the foot, to distal disks (Dharmaraj *et al.*, 1987b; Gervis and

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Sims, 1992). The flattened distal end of byssal threads forms the distal disc which attach a pearl oyster to the substrate (Haws and Ellis, 2000; Fougerouse *et al.*, 2008).

Production of byssus in pearl oysters is influenced by many factors including water temperature, pH (Welladsen *et al.*, 2011), salinity (O'Connor and Lawler, 2004) and current (Taylor *et al.*, 1997). The latter has been extensively studied in mussels and scallops, and there has been much deliberation as to whether this is a factor that greatly determines the secretion rate, thickness and tensile strength of byssus (Babarro *et al.*, 2008; Babarro and Reiriz, 2010; Babarro and Carrington, 2013). The only study on the influence of current strengths on byssus secretion by pearl oysters showed that when *P. maxima* was cultured at sites with strong currents, the oysters secreted more byssus over time compared to oysters cultured at mild current sites (Taylor *et al.*, 1997). Similar results have been shown for the mussel, *Mytilus edulis*, which showed increased byssus secretion rates and greater tensile strength of byssus in high wave areas (Price, 1982; Witman and Suchanek, 1984; Young, 1985; Lee *et al.*, 1990; Bell and Gosline, 1996; Hunt and Scheibling, 2001; Carrington, 2002; Babarro and Carrington, 2013). However, there is study which contrastingly report that the zebra mussel, *Driessena polymorpha*, showed highest byssus secretion rates when cultured in still water (Clarke and McMahon, 1996).

Pearl oysters are cultured using a variety of culture units (Southgate, 2008) that expose pearl oysters to different levels of agitation as a result of varying current intensities at dissimilar sites or depths. The two commonly used culture methods for the black-lip pearl oyster, *P. margaritifera*, are ‘ear-hanging’ and culture in panel nets (Gervis and Sims, 1992; Southgate, 2008). Ear-hanging involves drilling a small hole close to the

hinge in the antero–dorsal region of the oyster shell through which a monofilament line is inserted to tie the oyster to a single rope, which is itself suspended from a longline or raft (Gervis and Sims, 1992; Haws, 2002; Southgate, 2008). Multiple oysters are attached to each rope forming a 'chaplet' (Chapter 7). Culture of pearl oysters using chaplets minimizes costs and labour needed for pearl production, but increases the susceptibility of oysters to predation and the impacts of wave agitation. Panel nets are composed of plastic coated or galvanised metal frames covered with mesh that is sewn to form pockets to hold the oysters (Gervis and Sims, 1992; Southgate, 2008). Oysters are generally cultured singly in pockets and pocket size and mesh size are increased with increasing oyster size (Chapter 7). Panel nets are suspended from a raft or long line and provide some protection from predation and greater stability to cultured oysters.

To produce a cultured round pearl, a skilled technician must implant a nucleus into the gonad of a recipient oyster, together with a piece of mantle tissue from a sacrificed donor oyster (Taylor and Strack, 2008); a procedure known as grafting or seeding. The graft is placed into the narrowed antero-ventral portion of the gonad often referred to as the 'pearl pouch' (Scoones, 1996; Fougerouse *et al.*, 2008). Subsequent proliferation of the donor mantle tissue forms the 'pearl-sac' around the nucleus, and continued deposition of nacre onto the nucleus from secretory cells in the pearl-sac forms a cultured pearl over a period of about two years. The thin root threads of byssus that are embedded in oyster tissues are found in close proximity to the pearl-sac (Scoones, 1996). On this basis there has been anecdotal consideration of the possibility that production of byssus may impact development or function of the pearl-sac which, in turn, may negatively affect resulting pearl quality. If this is the case, then a greater

understanding of the factors influencing byssus production and, in particular, the influence of culture method on byssus production, would greatly assist the cultured pearl industry to optimise husbandry practices for cultured pearl oysters.

6.2. Material and methods

This study was conducted at two commercial pearl culture sites operated by J. Hunter Pearls (JHP) of Fiji. The two sites, Nawi (16˚46′13.28″S, 179˚19′20.50″E) and Raviravi (16˚47′19.83″S, 179˚18′10.60″E) are located in Savusavu Bay on the island of Vanua Levu. Nawi is regarded as a site with mild water currents and is used primarily to hold oysters during the recovery stage following the pearl grafting procedure. The second study site, Raviravi, has relatively high water current and is used primarily to hold oysters during grow-out and for pearl production. To confirm differences in the current rates between sites, an Acoustic Depth Profiler (ADP SONTEK) was deployed at each of these sites for the duration of the experiment. Nawi was indeed a mild current site with a current rate of 2.8 to 3.2 cm/s during the study period while Raviravi had a higher current speed of 4.5 to 4.8 cm/s over the study period. Recordings of water quality parameters (turbidity, salinity, water temperature) at the two culture sites used in this study have occurred over a number of years as part of the regular commercial farm activity and there are no significant variations between different pearl culture sites in Savusavu Bay (J. Hunter, unpublished data).

The *P. margaritifera* used in this study were nine months old and were obtained as wild spat from spat collectors deployed at various sites in Savusavu Bay. A total of 400 oysters with a mean (±SE) dorso-ventral measurement of 91.56 ± 0.45 mm and an antero-posterior measurement of 86.06 ± 0.83 mm were randomly selected for this

trial. Fouling organisms were cleaned from the oysters before their byssus were severed at the byssal notch using a scalpel. Half (200) of the oysters were ear–hung to chaplets and the remaining oysters were placed into panel nets with a 30 x 30 mm mesh size. Each chaplet and panel net held 10 and 20 oysters, respectively. At both Nawi and Raviravi, a total of 200 oysters (100 ear–hung oysters on chaplets and 100 oysters housed in panel nets) were suspended from a longline at a depth of 7 m. Oysters were deployed to sites on the same day that their byssus were severed and the number of byssus secreted by each oyster was counted 5, 10, 15 and 20 days after deployment.

At the end of the experiment (20 days after byssus were severed), 40 randomly selected byssal threads from oysters cultured in the two different types of culture units at each site were cut to investigate any differences in their tensile strengths and thicknesses. Tensile strength was measured using a Shimadzu Material Tester/Rheometer Software that projected tensile strength graphs from which the break points of byssus were determined. The stems of the byssus were measured using a Moving Microscope to determine their thickness.

6.2.1. Statistical analysis

Data relating to the number of byssus produced on different sampling days, their tensile strengths and thicknesses of byssus, were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). Because a number of oysters did not produce byssus on some recording days, there were a number of zero counts in the dataset requiring arcsinh transformation (Fowler *et al.*, 1998) before two-way ANOVA was carried out to determine the effects of culture method on byssus production over time. Possible differences in tensile strengths and thicknesses of byssus between the

different types culture units at each site were determined using one–way ANOVA. Tukey's test was used post hoc and all analyses were performed using SPSS statistics software version 16.

6.3. Results

Table 6.1 shows the proportion of oysters that had secreted byssus at each sampling day and in each treatment at both sites. The proportion of oysters that produced byssus in panel nets at the Raviravi site was slightly more on sampling days 5 and 20 than those cultured in panel nets at the Nawi site. However, a much higher proportion of ear-hung oysters secreted byssus at the Raviravi site compared to those at the Nawi site on any particular sampling day. The sizes of the different sections of byssus (root, stem, distal disks) varied between oysters held in the two types culture units used. Although not measured, byssus from ear–hung oysters had obviously broader surface areas at the distal disks and thicker stems compared to byssus produced by oysters housed in panel nets.

Table 6.1. The proportion of oysters that secreted byssus on different sampling days at two sites (Nawi; low current site and Raviravi; high current site) when ear-hung or housed in panel nets.

Sampling day	Nawi		Raviravi	
	Ear-hung	Panel nets	Ear-hung	Panel nets
5	39%	40%	43%	41%
10	46%	49%	49%	48%
15	51%	54%	56%	53%
20	57%	59%	65%	62%

There was no significant difference between the rate of byssus secretion of ear-hung and panel net cultured oysters at Nawi on any of the four sampling days; however, ear-hung oysters produced significantly more byssus than oysters housed in panel nets at the Raviravi site overall (Fig. 6.1). The number of byssus produced by ear-hung and panel net cultured oyster was not significantly different after five days at either site (Nawi, $p = 0.325$; Raviravi, $p = 0.312$) and this situation remained the same on day 10 (Nawi, $p = 0.159$; Raviravi, $p = 0.288$) (Fig. 6.1). However, while no significant difference in byssus production between the two types of culture units was recorded at Nawi after 15 days ($p = 0.285$), ear-hung oysters had produced significantly more byssus then panel net cultured oysters at the Raviravi site ($p = 0.01$) on the same sampling day (Fig. 6.1). Similarly, after 20 days, byssus production by ear-hung oysters was significantly greater at Raviravi ($p = 0.01$) while there was still no significant difference in byssus production between the two types of culture units at Nawi ($p = 0.092$) (Fig. 6.1).

Despite variation in current velocity at the two culture sites, oysters housed in panel nets at Raviravi (higher current site) did not produce significantly more byssus ($p = 0.251$) than those in panel nets at the Nawi site by the end of the experiment (Fig. 6.1). In contrast, ear-hung oysters at Raviravi produced significantly more byssus ($p = 0.02$) than ear-hung oysters at Nawi over the same culture period.

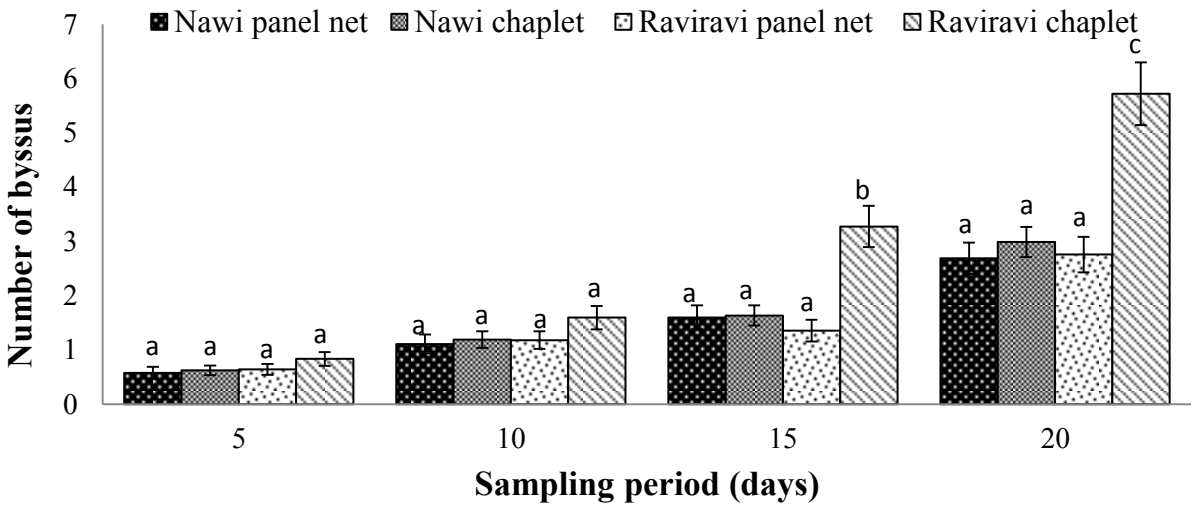

Figure 6.1. Mean (± SE) number of byssus produced by ear-hung (chaplet) and panel net cultured *P. margaritifera* at a low water current site (Nawi) and a high water current site (Raviravi) sampled at different periods (5, 10, 15 and 20 days) following severing of byssus. Means with different superscript are significantly different ($p < 0.05$).

The impact of different current rates on tensile strengths and thicknesses of byssus produced by oysters held in the two types of culture units was profound at both culture sites. Byssus from ear-hung oysters were significantly stronger and thicker than the byssus produced by oysters housed in panel nets at both culture sites (Table 6.2). However, there was no significant difference between the sites (Table 6.2). Furthermore, observations made when diving on every second day during the experiment showed that ear-hung oysters were agitated individually in the current whereas oysters housed in panel nets remained stable within the nets because the net responded as a unit to the prevailing current. Often during the course of this experiment, it was observed that the thicker byssus secreted by ear-hung oysters were

shorter and did not attach to the chaplet ropes from which the oysters were suspended. Instead, these oysters attached to the outer shell surface of the oysters secreting byssus which provided a larger surface area for attachment. In contrast, byssus produced by oysters housed in panel nets were thinner and longer and became entangled in the mesh of the net.

Table 6.2. Mean (±SE) tensile strengths (N) and thicknesses (mm) of byssus produced by ear-hung and panel net cultured *P. margaritifera* at Nawi and Raviravi. Means with different superscripts across each row for each parameter are significantly different (p = 0.01).

Sites	Tensile strength		Thickness	
	Ear-hung	Panel nets	Ear-hung	Panel nets
Nawi	3.45 ± 0.1^{a}	1.50 ± 0.3^{b}	0.53 ± 0.2^{C}	0.31 ± 0.1^{D}
Raviravi	3.39 ± 0.1^{a}	1.63 ± 0.3^{b}	0.52 ± 0.2^{C}	0.32 ± 0.1^{D}

6.4. Discussion

In general, a higher proportion of oysters produced byssus when subject to the higher current velocity at Raviravi compared to lower current at Nawi, regardless of the types of culture units used. This trend was observed throughout the course of the experiment indicating that higher currents cause more agitation among pearl oysters and that this stimulates greater production of byssus for added stability.

Production of byssus by oysters in panel nets was similar at both culture sites. This suggests that despite differences in the current rates between sites, oysters in panel nets

at the higher current site (Raviravi) were exposed to similar current rate impacts to those at the lower current site (Nawi). This is probably because oysters housed in panel nets were enclosed by the surrounding mesh frames that provide a broad surface area which is likely to have reduced direct agitation impacts on the oysters within the nets. The panel nets are likely to 'dampen' the effect of water current by absorbing current energy across a broader surface area. In contrast, ear-hung oysters are exposed to more direct wave and current generated agitation.

Although exposed to greater direct impact of currents, ear-hung oysters had not produced significantly more byssus than oysters housed in panel nets at both the culture sites when sampled 5 and 10 days after deployment. This finding is in contrast to that of Taylor et al. (1997) who reported that *P. maxima* housed in panel nets, and subject to low water currents, produced significantly more byssus than oysters in high current areas after only five days. Similar byssus production by ear-hung *P. margaritifera* to those in panel nets could be due to the fact that oysters in both types of culture units initially secreted byssus at a similar, perhaps maximal rate, to attain stability in response to complete removal of their byssus at the start of the experiment. While oysters housed in panel nets apparently achieved adequate stability from the quantity of byssus secreted after 10 days (indicated by a subsequent decrease in the secretion rate), ear-hung oysters apparently could not achieve adequate stability with the quantity of byssus secreted over the same period. This led to a significantly greater quantity of byssus being produced by ear-hung oysters after 15 days compared to those in panel nets. However, a significant difference in byssus production between the two types of culture units was only evident at the high current site (Raviravi).

The influence of current strength was also profound between the two sites with respect to ear-hung oysters on chaplets. In response to higher currents, ear-hung oysters at Raviravi produced more byssus than ear-hung oysters at the Nawi site. Similar correlation between byssus production and wave strength has been reported in mussels, *Mytilus galloprovincialis* and *Perna perna* (Bell and Gosline, 1996; Carrington, 2002; Zardi *et al.*, 2007). However, a recent study with *Mytilus edulis* reported that attachment strength was weaker in a more turbulent open sea environment than in a lagoonal environment despite mussels in the open sea producing larger and stronger byssal threads than those in the lagoon (Seguin-Heine *et al.*, 2014). The lower attachment strength of mussels in the open sea was related to reduced production of byssal threads which the authors proposed was related to lower water temperature in the open sea (Seguin-Heine *et al.*, 2014)

Previous studies on mussels have reported that the high tensile strengths of the byssus provide prevention from being dislodged in high wave action areas (Dolmer and Svane, 1994; Steffani and Branch, 2003; Moeser *et al.*, 2006). Seguin-Heine et al. (2014) recently confirmed that mussels do indeed produce larger and stronger byssal threads when cultured in more turbulent areas compared to those in more sheltered environments. In contrast, the results of this chapter show that for *P. margaritifera* there was no significant difference in the tensile strengths and thicknesses of byssus between high current and low current sites with respect to any one type of culture unit. Instead, improved attachment of oysters at the higher current site was provided through secretion of more byssus as demonstrated by ear-hung oysters at Raviravi. Nevertheless, exposure of ear-hung oysters to greater agitation at the Raviravi site stimulated secretion of thicker byssus that had higher tensile strengths compared to

those of oysters housed in panel nets. Although not measured in the current study, the surface area of the distal disks (i.e. area of attachment) of ear-hung oysters was clearly larger than those of oysters housed in panel nets. Apart from current strength, other factors shown to influence byssus production in bivalves include increase in metabolic rate at higher water temperature, which is positively related to byssus production (Lee *et al.*, 1990; Selin and Vekhova, 2004), and gamete production (Hawkins and Bayne, 1985), reduction in salinity from an optimum level (Price, 1982) and nutrient deficiency (Carrington, 2002) which are inversely related to byssus production rates. However, these factors are likely to have had minimal effects on the byssus secretion rates recorded in this study since there was no major differences in water quality parameters between culture sites that are approximately 1.3 km apart.

Byssus roots have thin individual root threads that are buried within pearl oyster tissues. These tissues include the gonad (Scoones, 1996) which is used as a site for nucleus grafting for cultured pearl production (Taylor and Strack, 2008). Although the root threads were not measured to determine the relative thicknesses of those from oysters in the two different types of culture units in this study, it is reasonable to assume that the root threads of ear-hung oysters that produced significantly thicker byssus were thicker than those of oysters housed in panel nets. Given the proximity of byssus roots to the pearl producing site in the gonad (Chapter 2), it is possible that individual byssus root threads may influence the quality of pearls formed by *P. margaritifera*. A thick and rigid byssus root thread could, for example, physically impinge on the pearl-sac causing disruption of normal nacre deposition. If this is the case then the results of this study suggest that culture method may influence resulting pearl quality.

Chapter 7

[6]The effect of different culture methods on the quality of round pearls produced by the black-lip pearl oyster *Pinctada margaritifera*

7.1. Introduction

Cultured pearl production is initiated when a piece of mantle tissue (saibo) from a donor pearl oyster and a round inorganic nucleus are inserted into the gonad of a host pearl oyster (Gervis and Sims, 1992; Taylor and Strack, 2008). This process is commonly called 'seeding' or 'grafting'. Subsequent proliferation of the donor mantle tissue forms a 'pearl-sac' around the nucleus (Chapters 2 and 4) and continued deposition of nacre from secretory cells in the pearl-sac onto the nucleus eventually forms a cultured pearl over a period of about two years (Dix, 1972; Scoones, 1996; Taylor and Strack, 2008; Cochennec-Laureau *et al.*, 2010).

A range of culture units and husbandry methods may be used to hold pearl oysters during culture (Gervis and Sims, 1992; Southgate and Beer, 2000; Southgate, 2008). These vary from enclosed units such as plastic mesh trays, mesh cages, and various types of nets (Gervis and Sims, 1992; Southgate and Beer, 1997; Friedman and Southgate, 1999; Southgate and Beer, 2000) to 'ear-hanging' which does not involve a culture unit *per se*. In Australia and south-east Asian countries, pearl oysters are predominantly cultured using panel nets that are made from strong steel or galvanised frames covered by mesh that is sewn to form pockets to hold the oysters (Southgate,

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2008). Pocket size and mesh size are increased with increasing oyster size. In French Polynesia and the western Pacific however, 'ear-hanging' is the major culture method used for juvenile and adult *P. margaritifera* (Ellis and Haws, 1999; Haws and Ellis, 2000; Haws, 2002; Southgate, 2008). Ear-hanging involves drilling a small hole close to the hinge in the antero–dorsal region of the oyster shell through which a monofilament line is inserted to tie the oyster to a single rope, which is itself suspended from a long line or raft (Gervis and Sims, 1992; Haws, 2002; Southgate, 2008). Multiple oysters are attached to each rope forming a 'chaplet'. Culture of pearl oysters using chaplets minimizes costs and labour needed for pearl production (Southgate 2008), but increases the susceptibility of oysters to predation and the impacts of wave agitation (Chapter 6).

Many studies have considered the influence of various culture units on the growth rates and survival of pearl oysters (Southgate, 2008) and much of this research has focused on the black-lip pearl oyster *P. margaritifera* (Coeroli *et al.*, 1984; Southgate and Beer, 1997; Friedman and Southgate, 1999; Southgate and Beer, 2000). However, there has been no prior study into the effects of culture method on the quality of resulting round pearls. Lack of research in this area is surprising given that only around 5% of the total harvest of cultured pearls from *P. margaritifera* are of the highest quality and these generate approximately 95% of farm profits (Haws, 2002). On this basis, only a small increase in the proportion of the highest quality pearls could result in substantial economic benefits to pearl farmers.

The major characteristic affecting the quality of pearls produced by *P. margaritifera* is the presence of 'circles' or concentric depressions or grooves on their surfaces (Ito,

2009). There is anecdotal suggestion that oysters cultured on chaplets produce a higher proportion of pearls with circles than those held in nets. Chapter 6 showed that ear-hung *P. margaritifera* produced greater numbers of byssus that were thicker and had greater tensile strength than those produced by oysters held in panel nets. It was speculated that these factors may negatively impact the development or function of the pearl-sac which, in turn, may affect resulting pearl quality (Chapter 2). If this is the case, then a greater understanding of the influence of culture method on pearl quality would greatly assist the cultured pearl industry to optimise husbandry practices for cultured pearl oysters. This study therefore assessed the quality of pearls produced by *P. margaritifera* held in panel nets and on chaplets during the pearl development period.

7.2. Material and methods

This study was conducted at three commercial pearl culture sites owned by J. Hunter Pearls (JHP) of Fiji. The three sites, Nawi (16˚46′12.14″S, 179˚19′15.48″E), Raviravi (16˚47′19.44″S, 179˚18′10.55″E) and Cousteau (16˚47′18.83″S, 179˚18’12.65″E) are located in Savusavu Bay on the island of Vanua Levu.

The oysters considered for this experiment were among those collected as wild juveniles (spat) obtained from spat collectors deployed at various sites within Savusavu Bay. Once removed from spat collectors, oysters were cultured using standard commercial methods until reaching a size suitable for pearl grafting. A total of 600 oysters with mean (±SE) antero-posterior measurement (APM) of 100.78 ± 0.21 mm and dorso-ventral measurement (DVM) of 112.41 ± 0.43 mm were randomly selected from the available pool of oysters for grafting. The oysters had not previously been

used for pearl production. Oysters were cleaned before being grafted by one of the three professional and experienced *P. margaritifera* grafting technicians. The grafting procedure generally followed that described by Taylor and Strack (2008). Briefly, it included careful selection of healthy donor oysters, excision of mantle tissue from each shell valve, stripping the pallial mantle from the excised mantle portion and cutting it into small square pieces to obtain saibo. A 2.7 *bu* nucleus (ca. 8.2 mm diameter) together with a single piece of saibo was then grafted into the gonad of each recipient oyster. The maximum time between saibo preparation and its use for pearl grafting was less than 25 minutes. Grafted oysters were then housed in lantern nets at one of the three culture sites with each site hosting 200 oysters at a depth of 7 m. Grafted oysters were held in lantern nets for a period of three weeks and were not turned during this convalescent period. Oysters were then transferred to either eight-pocket panel nets (40 x 40 mm mesh pore) or chaplets. One hundred grafted oysters were held using each culture method at each of the culture sites (i.e. a total of 600 oysters were used in the experiment). Grafted oysters were maintained according the normal commercial husbandry procedures at J. Hunter Pearls, which included inspection and cleaning every two months for a period of 18 months before the pearls were harvested and graded.

7.2.1. Pearl grading

The five main characteristics used to determine pearl quality are shape, size, lustre, colour and surface perfection (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008). These characteristics are used cumulatively to determine a pearl's overall grade and value. Pearls are normally graded using an AAA-A or A-D (Tahitian Grading) system (Matlins, 2002; Strack, 2006), where quality decreases from AAA to A or from A to D

(Matlins, 2002). However, this is a subjective exercise and the extent to which individual characteristics contribute to a pearl's overall grade may be perceived differently by different pearl graders. Pearls produced in this study were graded using an A-D grading system by an experienced, professional grader of *P. margaritifera* pearls at J. Hunter Pearls.

7.2.1.1. Pearl shape

Pearls harvested from *P. margaritifera* in this study were classified into one of five shape categories; round, semi-round, circles, baroque and keshi, described in Table 7.1. The proportions of pearls in each of these categories produced by oysters cultured in panel nets or on chaplets were then compared.

Table 7.1. The different categories of pearl shape used to grade pearls produced by *Pinctada margaritifera* in this study (Modified from Strack, 2006; Taylor and Strack, 2008).

Shape	Description
Round (R)	Pearl completely spherical with virtually no variation present on the surface.
Semi Round (SR)	Pearl not completely spherical but may appear spherical when viewed from a particular angle. Slightly flattened or elongated shape is only visible when observed very closely. These pearls appear nearly round to the naked eye and were grouped with round pearls in this study.
Circles (C)	Pearls have symmetrical lines or 'grooves' on their surface. Pearls of all above shapes but with grooves were classed in this category.
Baroque (B)	Pearls are asymmetrical and appear distinctly irregular.
Keshi	Non-nucleated pearls with unique shapes produced following nucleus rejection.

7.2.1.2. Pearl size

Pearl size was determined as the maximum diameter at the widest point of each pearl (Taylor and Strack, 2008) and was measured to the nearest 0.05 mm using a micrometer. Pearls (excluding 'keshi' pearls) were then classed into five size categories; 9-10 mm, 10-11 mm, 11-12 mm, 12-13 mm and 13-14 mm. The

proportions of pearls in each of these categories produced by oysters cultured in panel nets or on chaplets were then compared.

7.2.1.3. Pearl lustre

Lustre is described as the sharpness and intensity of light reflected from the surface of a pearl where greater and clearer reflection generates higher lustre. Pearl lustre was visually assessed by the pearl grader and each pearl was placed into one of four categories; very high lustre, high luster, medium luster and dull. The influence of luster on the overall quality of cultured pearls is reflected in their overall grading as outlined in Table 7.2.

7.2.1.4. Surface perfection

When assessing the surface perfection of pearls, irregularities such as tiny spots, blemishes, circles, cracks, scratches, small bumps and blisters are considered (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008). This characteristic is not assessed quantitatively for pearls produced in Fiji, but generally the quality of pearls decreases with increasing irregularities. The surfaces of pearls harvested from oysters cultured in panel nets and on chaplets were examined by the pearl grader and the influence of irregularities was reflected in the overall grade assigned to a pearl (Table 7.2).

7.2.1.5. Pearl colour

Cultured pearls produced by *P. margaritifera* are appreciated for their broad range of colours (Taylor and Strack, 2008; Cartier *et al.*, 2012). Fiji pearls have a particularly broad range of colours (Anon, 2007) many of which are unique. For marketing purposes, Fiji pearls are generally assigned to one of four major colour categories, each

of which may incorporate pearls with a range of colours. ‘Fiji Pastel’ includes pearls that are light in colour (e.g. white, silver and grey), ‘Fiji Bright’ includes pearls with bright colours (e.g. bright blue, bright green and bright gold), ‘Fiji Rare’ includes unique pearls with different coloured overtones (e.g. green, blue, gold) and colour combinations, and pearls with relatively dark colours (mainly black) are assigned to the ‘Fiji Traditional’ category. Some pearls with rare colours did not fall into any of the above categories; these included those in lavender, peacock, pistachio, aqua-blue, chocolate, champagne and cranberry tones. Pearls harvested from oysters cultured in panel nets or on chaplets were classified into these colour categories by the pearl grader. Final grading of each pearl accounted for the rarity of its colour and the general relationship between pearl colour and overall pearl grading is summarized in Table 7.2.

7.2.1.6. Overall grading

Pearls from *P. margaritifera* are normally graded into four categories that account for both quantitatively (i.e. size) and qualitatively (i.e. lustre, surface perfection and colour) assessed characteristics as outlined in Table 7.2. Overall proportions of pearls within each of these grades produced by oysters cultured in panel nets and on chaplets were then compared.

Table 7.2. Overall grading of round pearls produced by *Pinctada margaritifera* incorporating assessment within five grading categories (shape, lustre, size, surface perfection and colour) (Matlins, 2002; Strack, 2006; Taylor and Strack, 2008).

Grades	Shapes	Lustre	Size	Surface perfection	Colour
A	Round	Very high lustre	Often larger than pearls in lower grades	Very minor or no imperfection; often less than 5% of the total surface.	Very bright and attractive colour
B	Round to semi-round	High lustre	Variable but generally larger than pearls in C/D grade	Minor surface imperfections. Usually not more 30% of the total surface.	Brightly coloured
C	Baroque; the majority of pearls in this category have circles on the surface.	Variable (medium) lustre, but even pearls with high lustre were classed as 'C' grade if circles were present or if these pearls had baroque shapes.	Variable	Notable surface imperfections that may include blemishes, dents, bulges and circles.	Variable
D	Mostly uneven shapes. Presence of circles.	Dull	Variable	Major surface imperfections. More than 60%.	Variable

7.2.2. Statistical analysis

Differences in oyster mortality between the two culture methods at the three different culture sites were determined using the Kruskal-Wallis test. The same test was also used to identify differences in nucleus rejection rates between culture methods at the three sites. Mann-Whitney tests were then applied to determine the highest significant difference between sites. Categories within each of four pearl quality assessment criteria (shape, size, lustre and overall grade) were assigned different scores where '1' represented the lowest grade and increasingly higher scores were assigned to superior grades within each criterion, depending on the number of levels within each. For example, 'shape' was assessed from the lowest grade of '1' to a high of '5', 'size' was assessed from '1' to '5', 'lustre' from '1' to '4' and overall pearl grade from '1' to '4'. Normality tests (Levene's Test) were then carried out that determined that the data from the different categories from each variable was not normally distributed. Chi-square tests were used to identify significant linear associations between culture units/sites and the four assessment criteria (shape, size, lustre and overall grade). Mann-Whitney tests were carried out to determine if the two types of culture units had any impact on the total number of pearls produced in each of the different grading categories.

The influence of culture method on each category in the four assessment criteria between the three sites was determined using a Kruskal-Wallis test. The highest differences between different categories and sites were determined using Mann-Whitney tests. Statistical analyses were carried out using IBM SPSS Ver. 20 statistical software.

7.3. Results

The total number of pearls produced by *P. margaritifera* held in panel nets or on chaplets at the three different culture sites is shown in Table 7.3. The total number of pearls produced in this experiment was 536 from which, oysters in panel nets produced 273 while those on chaplets produced 263. Oysters in from panel nets and chaplets at the Raviravi site produced both the highest (92) and lowest (87) number of pearls, respectively.

Table 7.3. The total number of pearls produced by *Pinctada margaritifera* cultured using panel nets and chaplets at three culture sites (Raviravi, Nawi and Cousteau).

Raviravi		**Nawi**		**Cousteau**	
Panel net	**Chaplet**	**Panel net**	**Chaplet**	**Panel net**	**Chaplet**
92	87	91	88	90	88

7.3.1. Mortality

Mortality of oysters held in panel nets and chaplets at the three culture sites during this study is shown in Figure 7.1. Highest mortality (8%) was recorded for oysters held in panel nets at the Raviravi site and the lowest (6%) was recorded for oysters held in panel nets at both the Cousteau and Nawi sites, and for oysters held on chaplets at Raviravi (Fig. 7.1). Highest mortality in all culture units and across all culture sites was recorded when oysters were first cleaned two months after grafting. That is, most mortality occurred soon after grafting and decreased thereafter. Overall, oyster mortality did not differ significantly ($p = 0.279$) between culture methods across all culture sites in this study.

7.3.2. Nucleus rejection.

Nucleus rejection was determined when pearls were harvested; oysters that did not produce pearls or produced non-nucleated 'keshi' pearls instead, were assumed to have rejected the nucleus at some stage of pearl development. Nucleus rejection by *P. margartifera* housed in panel nets and held on chaplets is shown in Fig. 7. 1. Oysters held on chaplets rejected more nuclei than those in panel nets at each of the three culture sites. Highest nucleus rejection (6%) was recorded for oysters held on chaplets at the Nawi and Raviravi sites. In contrast, oysters held in panel nets at the Cousteau and Raviravi sites had the lowest nucleus rejection rates (2% per site). Nucleus rejection by oysters held in panel nets and those on chaplets differed significantly (p = 0.036) overall with the highest significant difference (p = 0.041) between the two culture methods recorded at the Raviravi site. When comparing overall nucleus rejection rates between different culture sites, a significant difference (p = 0.032) was only evident between the Raviravi and Nawi sites.

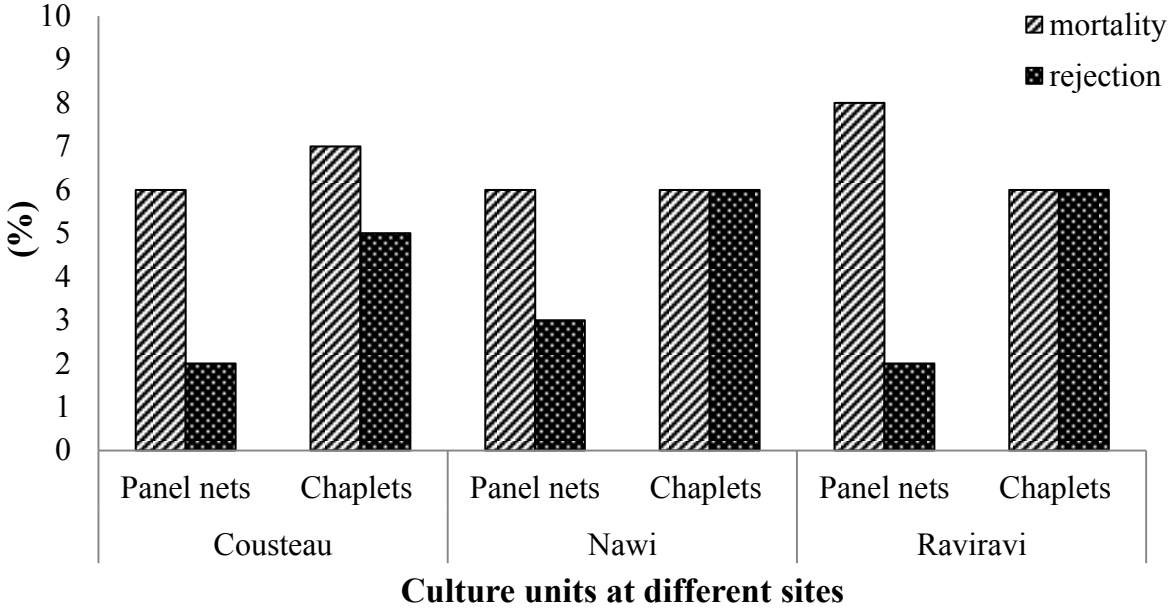


Figure 7.1. Percentage of dead oysters and oysters that rejected the nucleus when held using two culture methods (panel nets and chaplets) at three culture sites.

7.3.3. Pearl shape

The varying proportions of pearls assigned to the different shape categories produced by *P. margaritifera* housed in panel nets and held on chaplets are shown in Figure 7.2. The highest proportion of pearls produced at each of the three culture sites by oysters held by the two culture methods belonged to the 'circle' shape category (Fig. 7.2). Oysters at Raviravi recorded the highest (60%) and also the lowest (43%) proportions of these pearls from oysters held on chaplets and housed in panel nets, respectively. The proportion of pearls produced by oysters in panel nets that were assigned to the 'round' and 'semi-round' shape categories were greater than those produced by oysters held on chaplets at all three culture sites (Fig. 7.2). Oysters in panel nets from the Cousteau site produced the highest number (9%) of pearls in the 'round' shape

category while the lowest number (4%) was produced by oysters held on chaplets at the Nawi and Raviravi sites. Similarly, panel net cultured oysters at the Cousteau site produced the highest proportion of 'semi-round' pearls (25%) whereas oysters held on chaplets at the Raviravi site produced the lowest proportion of pearls (12%) in this category. While oysters held in panel nets at the Cousteau and Raviravi sites also produced higher proportions of pearls in the 'baroque' shape category (20% and 29%, respectively), those at the Nawi site produced 1% fewer 'baroque' shaped pearls than oysters held on chaplets. Overall, oysters held on chaplets produced more 'circle' shape pearls and less 'round' and 'semi-round' pearls compared to those housed in panel nets. Culture method had a significant impact ($p = 0.031$) on pearl shapes overall. The highest significant difference ($p = 0.024$) between the two culture methods was shown for baroque shaped pearls, followed by pearls with circles ($p = 0.026$). Pearl shapes were also significantly influenced ($p = 0.041$) by culture site with the highest significant difference ($p = 0.026$) recorded between Raviravi and Nawi sites.

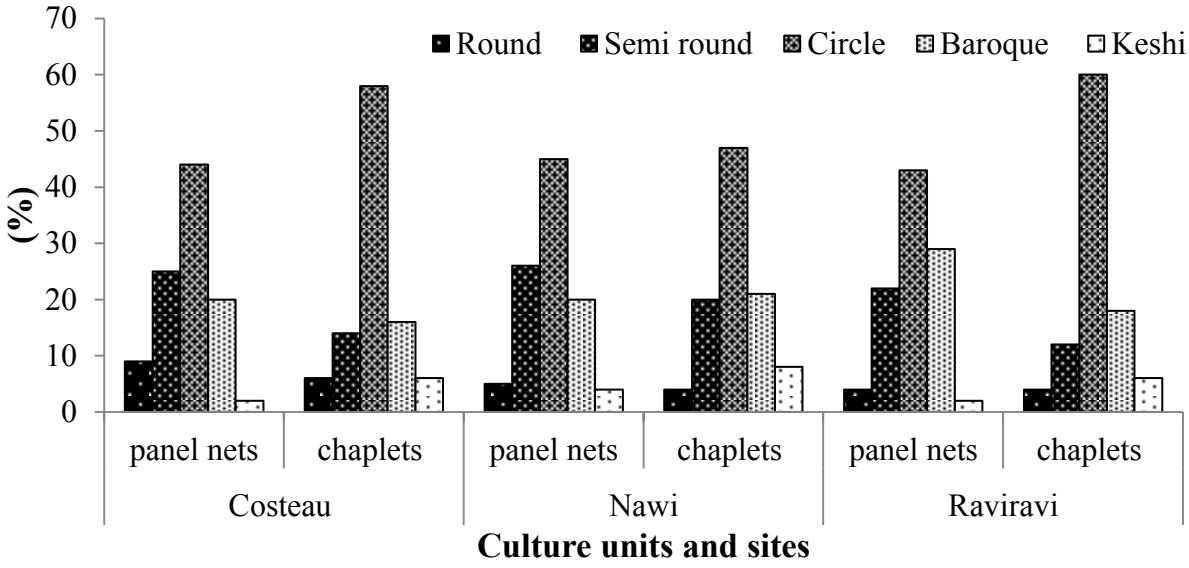


Figure 7.2. Percentages of pearls within five shape categories produced from *Pinctada margaritifera* when held using two culture methods (panel nets and chaplets) at three culture sites.

7.3.4. Pearl size

The proportions of pearls within five size categories produced by *P. margaritifera* held in panel nets or on chaplets at three culture sites are shown in Figure 7.3. The highest proportion of pearls produced in all treatments was in the 10-11 mm size category (37%-54%) Overall, more pearls were produced in the 10-11 mm size category, followed by those in the 11-12 mm, 9-10 mm and 12-13 mm size categories, respectively (Fig. 7.3). The least number of pearls were produced in the 13-14 mm size category (Fig. 7.3). However, neither culture method (p = 0.211) or culture site (p = 0.323) significantly influenced the sizes of pearls produced.

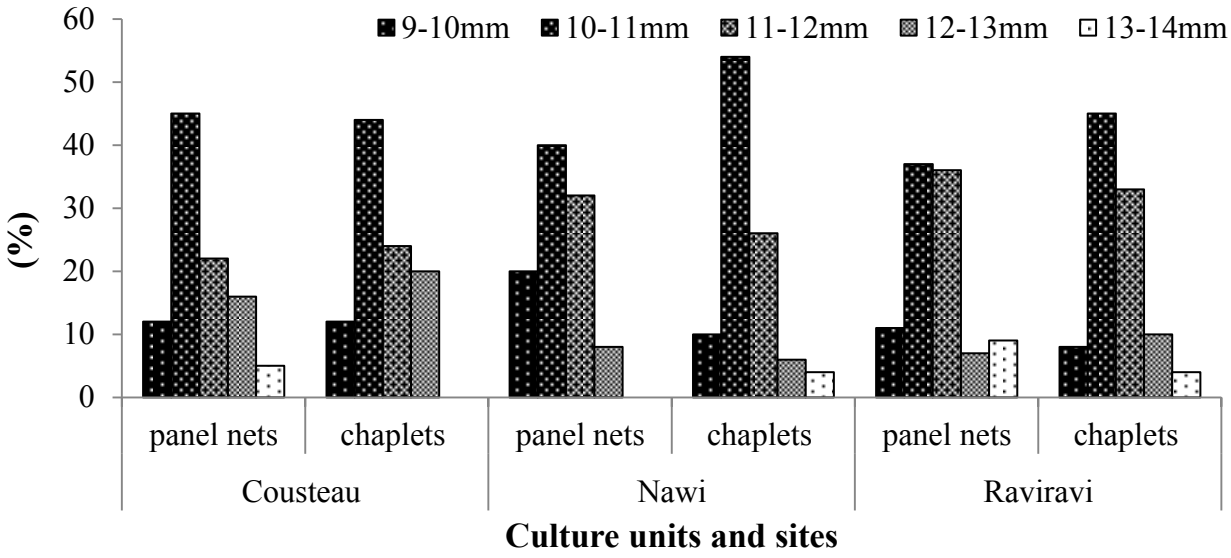


Figure 7.3. Percentages of pearls within five size categories produced from *Pinctada margaritifera* cultured using two methods at three culture sites.

7.3.5. Pearl lustre

The varying proportions of pearls classified within the different lustre categories produced by *P. margaritifera* cultured using panel nets and chaplets at the three different sites are shown in Figure 7.4. Higher proportions of pearls were produced in the 'very high' and 'high' lustre categories by oysters held in panel nets compared to those held on chaplets at each of the three culture sites. The highest proportion of pearls in the 'very high' lustre category were from oysters held in panel nets at the Cousteau site (12%) while the lowest (1%) was from oysters held on chaplets at the Raviravi site (Fig. 7.4). The highest proportion of pearls (52%) in the 'high' lustre category were produced by oysters held in panel nets at the Nawi site while the lowest (17%) was again from oysters cultured on chaplets at the Raviravi site. Pearls assigned to the 'medium' and 'dull' lustre categories were dominated by those produced by oysters held on chaplets at the Raviravi (63%) and Nawi (29%) sites, respectively.

Overall, oysters cultured using panel nets produced a higher proportion of pearls within the 'very high' and 'high' lustre categories while oysters held on chaplets produced a higher proportion of pearls in the 'medium' and 'dull' lustre categories (Fig. 7.4). However, the overall impact of culture method on pearl lustre was not significant (p = 0.100), despite being significantly influenced (p = 0.012) by culture site where with greatest difference (p = 0.031) was between pearls from the Raviravi and Nawi sites.

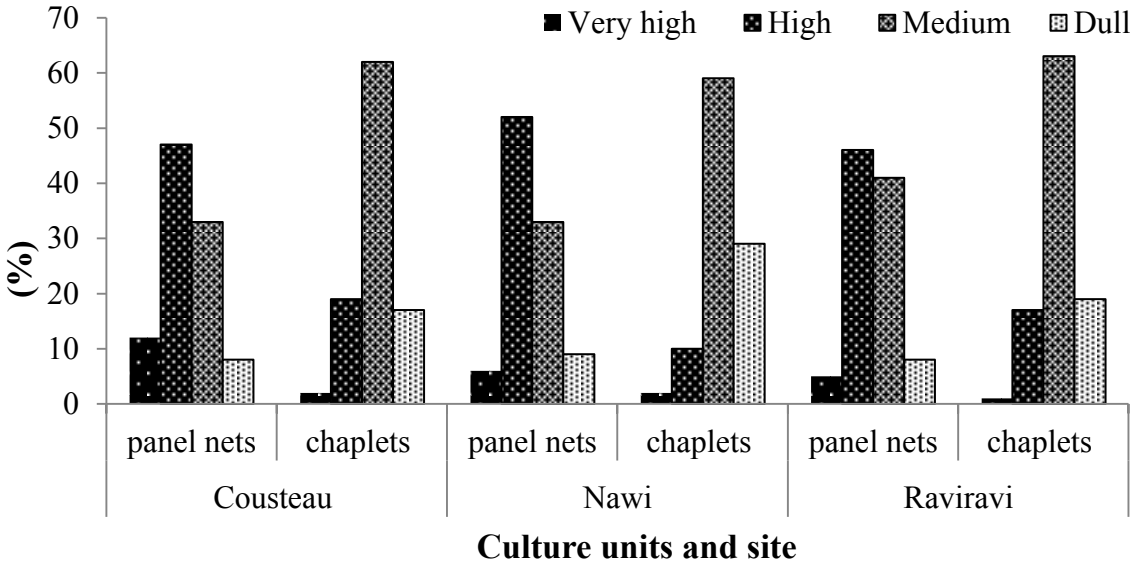


Figure 7.4. Percentages of pearls within four lustre categories produced from *Pinctada margaritifera* held using two culture methods (panel nets and chaplets) at three culture sites.

7.3.6. Pearl colour

The relative proportions of pearls belonging to different colour categories produced by oysters cultured in panel nets or on chaplets at three sites are shown in Table 7.4.

Table 7.4. Percentages of pearls produced by *Pinctada margaritifera* assigned to the various colour categories used for 'Fiji Pearls' following culture using panel net and chaplet at three culture sites (Cousteau, Nawi, Raviravi).

	Cousteau		**Nawi**		**Raviravi**	
	Panel net	Chaplet	Panel net	Chaplet	Panel net	Chaplet
Fiji Pastel	48	25	41	56	50	35
Fiji Bright	24	45	36	21	36	36
Fiji Traditional	12	12	9	18	7	9
Fiji Rare	5	6	8	2	2	6
Chocolate	4	-	-	-	-	-
Pistachio	4	-	-	-	-	2
Aqua-blue	3	-	-	-	-	-
Peacock	-	8	-	-	-	4
Lavender	-	4	-	-	3	-
Gold	-	-	3		-	-
Cranberry	-	-	3	-	-	2
Green	-	-	-	4	-	4
Champagne	-	-	-	-	-	2
Blue	-	-	-	-	2	-

With the exception of pearls produced by oysters held on chaplets at the Cousteau and Raviravi sites, the majority of pearls were classified within the 'Fiji Pastel' category, followed by 'Fiji Bright', 'Fiji Traditional' and 'Fiji Rare' categories, respectively (Table 7.4). Some pearls from all treatments were also categorised into rarer colour

categories including chocolate, champagne, peacock, pistachio, lavender, cranberry, green, blue and aqua blue.. However, the number of pearls with these rare colours was low and inconsistent across treatments, and oysters at some sites did not produce any pearls within these categories (Table 7.4).

7.3.7. Overall grading

The proportions of pearls produced by *P. margaritifera* cultured using two culture methods at three sites within the four overall grades used in this study are shown in Figure 7.5. At all three sites, higher proportions of pearls assigned to grades 'A' and 'B' were produced by oysters cultured in panel nets compared to those held on chaplets. Oysters at the Cousteau site held in panel nets and on chaplets produced both the highest (10%) and lowest (2%) proportions of grade 'A' pearls, respectively. Highest production of grade 'B' pearls (47%) was also from oysters held in panel nets at the Raviravi site while lowest (28%) was from oysters held on chaplets at the Cousteau site (Fig. 7.5). Oysters held on chaplets produced higher proportions of grade 'C' and grade 'D' pearls than those in panel nets at all three culture sites. The grades of pearls produced in this study were significantly influenced ($p = 0.035$) by culture methods with the highest significant difference ($p = 0.026$) observed for grade 'C' pearls. However, culture site did not significantly influence ($p = 0.755$) the overall grades of resulting pearls.

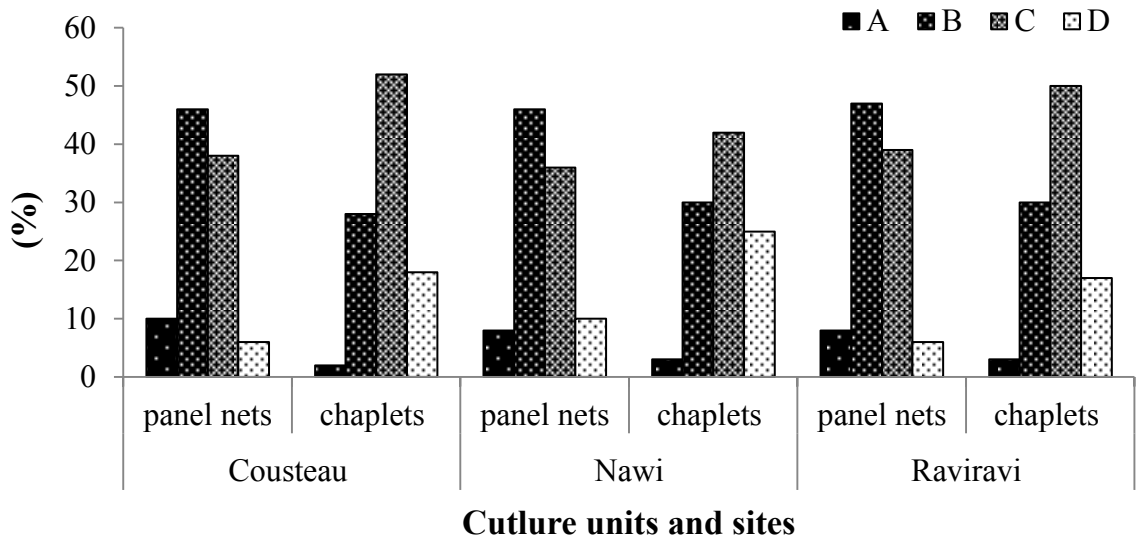


Figure 7.5. Percentages of pearls assigned to four grade categories following production from *Pinctada margaritifera* cultured using two methods at three culture sites.

7.4. Discussion

Although there was no difference in oyster morality between the two culture methods used in this study, there was a significant difference in nucleus rejection rate which was much lower for oysters held in panel nets than for those held on chaplets at each of the three culture sites. The reason for this is unclear, but it is likely that oysters in panel nets were more stable than those on chaplets and subject to less agitation (Chapter 6). The mesh surrounding oysters in panel nets provides stability and is likely to reduce the direct impact of wave action and currents on oysters. In contrast, oysters held on chaplets are likely to be continually agitated as a result of wave and current energy and this may potentially cause stress resulting in increased rates of nucleus rejection. Importantly in this study, grafted *P. margaritifera* were placed into lantern nets for three weeks immediately after grafting to reduce stress and facilitate wound healing and development of the pearl-sac; this period of convalescence is standard practice at

the collaborating pearl farm. Had this step have been omitted, it is likely that higher levels of mortality and nucleus rejection may have been recorded. The orientation of grafted *P. margaritifera* when held on chaplets is different to that of oysters in panel nets. When housed in panel nets, oysters rest dorsally on their hinges and their ventral side, with finger-like growth projections, faces upwards. In contrast, oysters are tied to chaplets at the dorsal-posterior end of the shells and the growth projections on the ventral edge of the shell face downwards (Southgate and Beer, 2000; Haws, 2002). This could be another factor that influenced the nucleus rejection rates recorded in this study which, from both panels nets (2-3%) and chaplets (5-6%), compare well to seasonably variable rejection rates of 7.7% to 12.6% reported from French Polynesia (Ky *et al.*, 2014b).

Numerous studies have been conducted to determine the effects of different culture units and culture methods on the growth rates and/or survival of pearl oysters (Saucedo and Southgate, 2008), but this is the first to assess the effects of different culture methods on the quality of cultured round pearls. There were no major differences between the size ranges of the pearls produced by oysters from the two culture methods in this study. Past records show that the majority of the pearls harvested after first graft in Savusavu Bay, Fiji, are typically in the 9-12 mm size range, similar to those produced in this study. Of the characteristics used to determine pearl quality, only shape differed between pearls produced by oysters held in panel nets and those held on chaplets. This difference related particularly to the presence of circles which is a major problem for pearl production from *P. margaritifera* (Ito, 1996; Ky *et al.*, 2015). Pearls produced by oysters held on chaplets had significantly more circles than those from oysters housed in panel nets, and this resulted in a significant reduction in their quality

with the majority of the pearls with circles being classified as 'C' grade. No significant differences between pearls produced using the two culture methods were found for any other pearl quality determining criterion (size, luster or colour). In the only prior study to have investigated the influence of culture method on pearl quality, Ruiz-Rubio et al. (2006) reported that the quality of half-pearls (mabè) produced by *Pteria sterna* did not differ between those cultured in panel nets or plastic cages.

The results of this study show that *P. margaritifera* housed in panel nets produced a greater proportion of higher quality pearls than those held on chaplets. In Chapter 6, it is reported that exposure of *P. margaritifera* held on chaplets to greater agitation at a site with relatively high current, stimulated secretion of thicker byssus with higher tensile strengths than those of oysters housed in panel nets. The high current (Raviravi) and low current (Nawi) sites used in that study were also used in the present work. It is postulated that enhanced byssus production under such circumstances, may influence resulting pearl quality, specifically the presence of circles (Chapter 6), because of the proximity of byssus to the pearl-sac in *P. margaritifera* (Chapter 2). The results of the present study confirm that circled pearls made up a greater proportion of the pearls produced by chaplet-held oysters at Raviravi than by oysters held in panel nets at the same site, or by chaplet-held oysters at Nawi. Furthermore, this resulted in higher proportions of better quality pearls produced by panel net held oysters compared to chaplet held oysters at Raviravi.

The use of panel nets to hold oysters during pearl production results in higher pearl quality and provides better potential returns for farmers compared to chaplet-based culture. This has major implications for pearl culture in Fiji. The major commercial

collaborator for this study (J. Hunter Pearls, Fiji) has applied these results to commercial culture practices and changed from chaplet-based to panel net-based culture at all farm sites operated by the company. This change in culture practice has resulted in an approximate 30% increase in the value of pearl production by this company (Justin Hunter, J. Hunter Pearls, *pers. comm*., 2014). However, the choice of oyster culture method used by pearl farmers depends on many factors both practical and economic. Chaplets are relatively simple to make from readily available materials; a source of rope and fishing line or wire to tie oysters to it (Southgate and Beer, 2000). However panel nets are normally purchased from commercial suppliers at a cost of around US$6.50-10.00 each. Each net holds 6-8 oysters so even a relatively small pearl farm would have considerable financial outlay to change to a panel net based culture regime. From a practical perspective the use of panel nets is also likely to require greater labour input than chaplets. Oysters in panel nets in this study, for example, were cleaned *in situ* on a two-monthly basis but were still observed to be more fouled than those held on chaplets at cleaning. Fouling can have major impacts on the health and productivity of pearl oysters and control of fouling contributes significantly to the running costs of pearl farms (de Nys and Ison, 2008). The results of this study clearly show the benefits of pearl production using panel nets compared to the more traditional chaplet-based oyster culture system used by the majority of pearl farmers in Fiji and throughout the Pacific. Pearls produced using panel nets will provide better returns with higher profit margins but greater outlays for infrastructure and labour may be beyond the scope of most pearl farmers in Fiji and the Pacific. A detailed cost-benefit analysis of the two husbandry options would be beneficial to pearl farmers.

Chapter 8

General Discussion

8.1. Introduction

This project addressed factors affecting the quality of cultured pearls produced by the black-lip pearl oyster, *P. margaritifera* in Fiji. The overall objective of this study was to improve the quality of cultured round pearls produced by *P. margaritifera* in Fiji through a better understanding of the factors influencing pearl quality**.** This was addressed through a number of experiments that assessed the impacts of both developmental and biological factors (e.g. pearl-sac development and function, oyster response to culture method and culture environment) as well as husbandry and culture conditions (e.g. culture method and current strength) on pearl production and pearl quality. The major outputs of this study and their relevance and potential application are summarised in Fig. 8.1 and described below.

8.2. Pearl-sac development

Perhaps surprisingly given the commercial value of *P. margaritifera*, no prior study has provided a detailed description of pearl-sac development in this species covering major developmental and functional steps. Appropriate development of the pearl-sac in pearl oysters is an important factor influencing the quality of cultured pearls, and an understanding of this process was a fundamental requirement of this study. The results of Chapter 2 showed that graft tissue proliferated and differentiated to form a complete pearl-sac within 14 days of grafting when the epithelial cells responsible for nacre secretion were fully developed. First nacre secretion onto the nucleus however was not observed until 32 days after grafting. The grafting process used for pearl production

triggers a significant haemocyte response and this was further described in Chapter 3. The level of haemocytes present in the pearl-sac was variable but decreased overtime with the greatest presence of haemocytes recorded two days after grafting. The exact cause(s) of varying levels of haemocyte accumulation during pearl-sac development in *P. margaritifera* is not known. However, it is reasonable to assume that haemocyte production is positively related to the degree of damage caused to host oyster tissues during the grafting procedure.

Two factors that could potentially affect resulting pearl quality were identified in Chapter 2: (1) clumps of haemocytes present between the pearl-sac and nucleus caused distension of the pearl-sac from an ideally spherical shape; and (2) the presence of byssus in close proximity of developing pearl-sac may cause pressure points on the pearl-sac affecting even nacre deposition. The haemocyte response was further described in Chapter 3 while the potential for byssus and byssus production to influence pearl quality was investigated as a function of culture method in Chapters 6 and 7.

8.3. Pearl-sac development from regenerated mantle.

Current pearl grafting techniques were developed in the early 1900s (Strack and Taylor, 2008) and have changed little since. The technique involves the sacrifice of donor pearl oysters to provide graft tissue (saibo) that is implanted into host oysters. Acosta-Salmon et al. (2005) was the first to show that saibo could be removed from anaesthetised pearl oysters (*P. margaritifera*) without the need for sacrifice and that excised tissue regenerates within a few weeks of excision. Mamangkey and Southgate (2009) further showed that regenerated mantle tissue, when used as saibo, had the

capacity to proliferate to form a functional pearl-sac in *P. maxima*. Chapter 4 assessed the feasibility of using regenerated graft tissue for pearl production from *P. margaritifera*. Similar to findings with *P. maxima*, regenerated mantle saibo quickly proliferated to form a pearl-sac by 12 days after grafting in *P. margaritifera* which was completely integrated with host tissue by 16 days after grafting. After three months of culture, nuclei in oysters grafted with regenerated mantle tissue were completely covered with nacre demonstrating clearly that regenerated mantle tissue can function successfully as saibo for pearl production in *P. margaritifera*. This finding could provide a basis for further development of current pearl grafting practices. Saibo donors producing high quality pearls could potentially be used as broodstock to improve the quality of culture stock. Saibo can be used for multiple saibo donations potentially improving the proportion of high quality pearls (Fig. 8.1).

8.4. Should oyster producing circled pearls be regrafted?

Perhaps the major characteristic reducing the quality and value of pearls from *P. margaritifera* is the presence of concentric surface grooves or circles. This is a particular problem for pearls produced by this species (Ky *et al.*, 2014a) that can affect a high proportion of a pearl crop. Oysters producing such pearls are often discarded on the assumption that these oysters are unlikely to produce pearls with improved quality if grafted again for pearl production. Chapter 5 of this study determined whether oysters producing circled pearls are able to produce pearls with improved quality if re-grafted. Results showed that pearls from the second graft had improved shape, size, and a reduced number of circles compared to first graft pearls. However, there was no significant improvement in overall quality of second graft pearls compared to first graft pearls. The results show however that marketable pearls can be produced from oysters

that are normally discarded after first pearl harvest and this has potential to generate increased revenue for pearl farmers in Fiji and other Pacific islands (Fig. 8.1). Clearly, pearl farmers would need to consider the labour and financial inputs required to replenish oysters discarded after first pearl harvest versus the potential revenue generated from re-grafting these oysters before deciding a strategy, and a detailed economic analysis of the two options would be beneficial to pearl farmers.

8.5. Culture methods and their influence on pearl quality

The two most commonly used culture methods for *P. margaritifera* are 'ear-hanging' to chaplets and enclosure within pocket-nets. The former is the 'traditional' method used for pearl culture in Polynesia and is associated with anecdotal consideration that this culture method may facilitate formation of circles on resulting pearls. The use of pocket (panel) nets for pearl oyster culture is common in Australian and SE Asia but is less suited to Polynesia because of the costs associated with the purchase of nets. The results of Chapter 6 showed that oysters held on chaplets produced greater numbers of byssal threads than oysters held in panel nets at high current sites. Furthermore, byssal threads produced by chaplet-held oysters were thicker and had greater tensile strength than byssus produced by oysters held in panel nets (Fig. 8.1). It is likely that secretion of an increased number of thicker and stronger byssal threads by ear-hung oysters is a response to a greater degree of agitation than those held in panel nets. Chapter 2 of this study identified that byssus can be found in close proximity to the pearl-sac in *P. margaritifera* (section 8.2) and it was hypothesised that pressure applied to the pearl-sac by byssus during pearl formation may influence resulting pearl quality and possibly contribute to the formation of circles. This possibility was investigated in Chapter 7 of

this study which examined the influence of culture method ('ear-hanging' on chaplets versus culture in pocket nets) on resulting pearl quality.

The results of Chapter 7 indicated that there were no significant effects of culture method on pearl size or lustre, but there was a significant effect of culture method on pearl shape, with pearls produced by oysters in panel nets having superior shape. Importantly, overall grades of pearls were significantly influenced by the different culture methods with oysters in panel nets producing more A/B grade pearls than those on chaplets which produced more C/D grade pearls. This finding has major implications for pearl culture in Fiji. The use of panel nets to hold oysters during pearl production results in higher pearl quality and provides better returns for farmers compared to chaplet-based culture. The major commercial collaborator for this study (J. Hunter Pearls, Fiji) has applied these results to commercial culture practices and changed from chaplet-based to panel net-based culture at all farm sites operated by the company. This change resulted in an approximate 30% increase in the value of pearl production from this company (Justin Hunter, J. Hunter Pearls, *pers. comm*., 2014). However, switching from chaplets to panel nets requires significant capital input which may be beyond the scope of most pearl farmers in Fiji and the Pacific. Ear-hanging is relatively cheap and requires only a source of rope and material to tie oysters to it. Panel nets however are normally purchased from commercial suppliers at a cost of around AUD$8.50-12.50 each, plus freight. Each net holds 6-8 oysters so even a relatively small pearl farm would have considerable financial outlay to change to a panel net based culture regime. A detailed cost-benefit analysis of the two husbandry options would be beneficial to pearl farmers.

8.6. Application of the results of this study

The cultured pearl industry in Fiji is relatively young and small. However, it has already developed an excellent international reputation for pearl quality and the unique colour range of its pearls (Anon, 2007; Southgate, 2015). These characteristics ensure continued international demand for Fiji pearls and provide a basis for continued expansion within the extremely competitive cultured 'black' pearl industry that is dominated by French Polynesia. However, if Fiji is to become more than a niche producer it will have to increase pearl production significantly while maintaining quality and reputation to increase market share. These challenges provided the basis for this study.

The major applications of the results of this study are outlined in Fig. 8.1. They are:

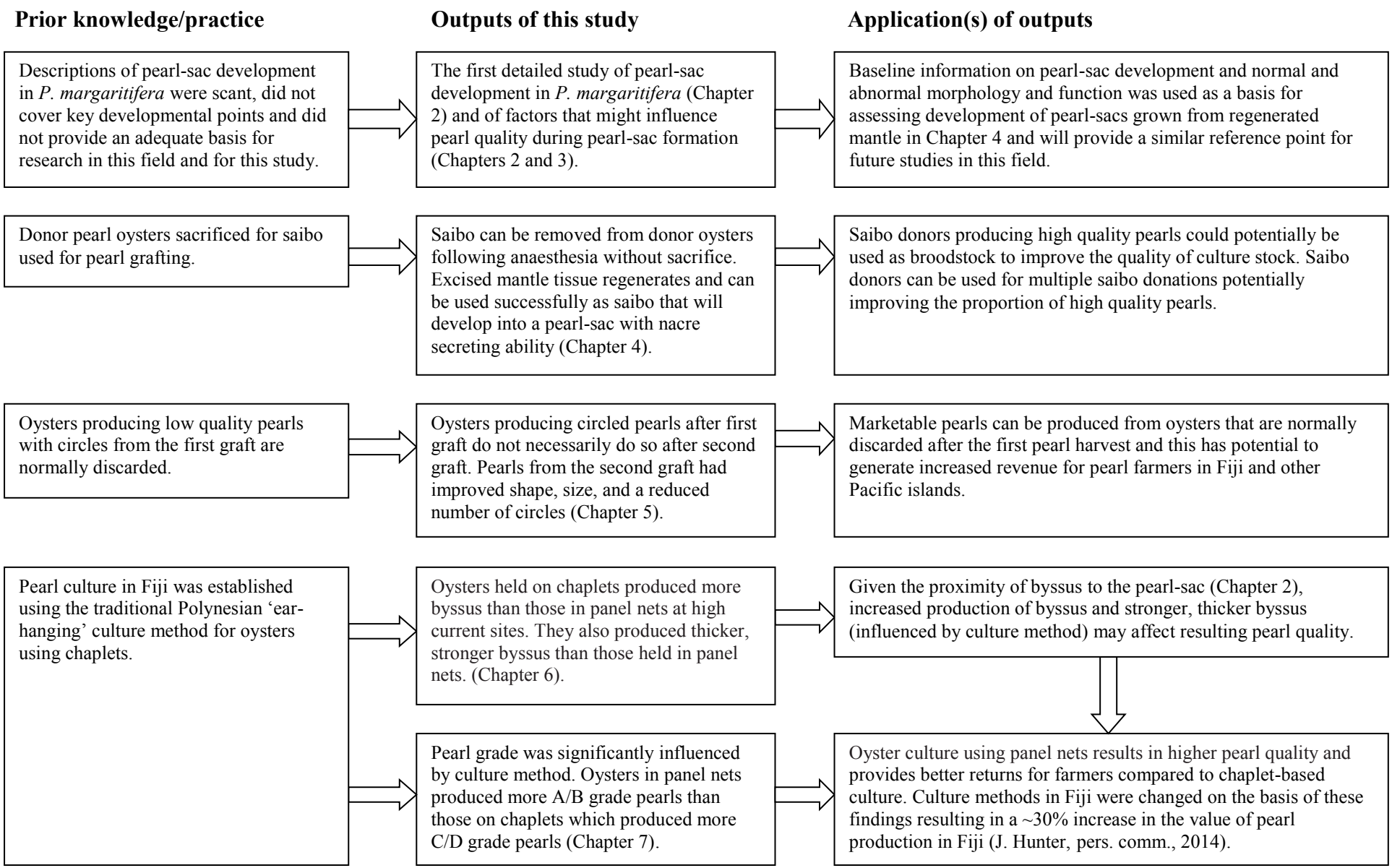
- potential use of saibo donors producing high quality pearls for multiple saibo donations potentially improving the proportion of high quality pearls;
- production of marketable pearls from oysters that are normally discarded after the first pearl harvest resulting in increased production and revenue; and
- change to a panel net-based culture system resulted in higher pearl quality and a ~30% increase in the value of pearls produced.

They provide a good basis for increased pearl production in Fiji and for future research in this field.

Further expansion of the cultured pearl industry in Fiji is supported by other research for development activities that include: (1) development of a national community-based spat collection program to ensure ongoing and sustainable supply of oysters to a growing industry (Anon, 2014); (2) a greater understanding of the genetic relatedness

of *P. margaritifera* populations in Fiji as a basis for developing appropriate guidelines for translocation of oysters within Fiji (Lal *et al.*, 2015); and (3) introduction of small-scale pearl culture to Fijian communities and development of pearl industry based livelihood opportunities (i.e. pearl and mother-of-pearl handicraft production) for coastal communities (Southgate, 2015).

Figure 8.1. Major outputs from this study and their applications.



Reference

Achari, G.P.K., 1982. Project profile of pearl culture. *Journal of Seafood Export* 14, 9-11.

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

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

Acosta-Salmón, H., Southgate, P.C., 2006. Wound healing after excision of mantle tissue from the Akoya pearl oyster, *Pinctada fucata*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 143, 264-268.

Alagarswami, K., 1970. *Pearl Culture in Japan and its Lessons for India*. In: Proceedings of the Symposium on Mollusca Part III. Marine Biological Association of India, India. pp. 975-998.

Alagarswami, K., 1987a. Technology of cultured pearl production. In: Alagarswami, K. (Ed.), *Pearl culture-CMFRI Bulletin No. 39*. Central Marine Fisheries Research, Conchi. pp. 98-106.

Alagarswami, K., 1987b. Cultured pearls-production and quality. In: Alagarswami, K. (Ed.), *Pearl Culture-CMFRI Bulletin No 39*. Central Marine Fisheries Research Institute, Cochin. pp. 107-111.

Anon, 2007. Justin Hunter and Pearls Fiji. *The International Pearling Journal* 16, 10-15.

Anon, 2014. *PARDI Capacity building in the South Pacific*, Canberra, pp. 32.

Anonymous, 2014a. *Pre-election economic and fiscal update 2014, The Cook Islands.* Available online at http://www.mfem.gov.ck/mfemdocs/treasury/budget-downloads/327-2014-pre-election-economic-and-fiscal-update/file. [Retrieved 24/03/2015]

Anonymous, 2014b. *The Pearl Forum, Inhorgenta, Munich*. Available online at http://media.nmm.de/29/inhorgenta-munich-2014-pearl-dossier_27618729.pdf. [Retrieved 24/03/2015]

Aoki, S., 1961. Some experiments on the nuclear insertion in pearl culture of the oyster (*Pinctada martensii*). V. Pearl sac formation in the material with a space between inserted nucleus and adjacent tissue. *Bulletin of National Pearl Resource Lab* 6, 647-656.

Arnaud-Haond, S., Goyard, E., Vonau, V., Herbaut, C., Prou, J., Saulnier, D., 2007. Pearl formation: Persistence of the graft during the entire process of biomineralization. *Marine Biotechnology* 9, 113-116.

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

Awaji, M., Machii, A., 2011. Fundamental studies on in vivo and in vitro pearl formation—Contribution of outer epithelial cells of pearl oyster mantle and pearl sacs. *Aqua-BioScience Monographs* 4, 1-39.

Babarro, J.M., Fernández Reiriz, M.J., Labarta, U., 2008. Secretion of byssal threads and attachment strength of *Mytilus galloprovincialis*: the influence of size and food availability. *Journal of the Marine Biological Association of the UK* 88, 783-791.

Babarro, J.M., Reiriz, M.J.F., 2010. Secretion of byssal threads in *Mytilus galloprovincialis*: quantitative and qualitative values after spawning stress. *Journal of Comparative Physiology B* 180, 95-104.

Babarro, J.M.F., Carrington, E., 2013. Attachment strength of the mussel *Mytilus galloprovincialis*: effect of habitat and body size. *Journal of Experimental Marine Biology and Ecology* 196, 188-196.

Barik, S., Jena, J., Ram, K.J., 2004. CaCO3 crystallization in primary culture of mantle epithelial cells of freshwater pearl mussel. *Current Science* 86, 730.

Bell, E., Gosline, J., 1996. Mechanical design of mussel byssus: material yield enhances attachment strength. *Journal of Experimental Biology* 199, 1005.

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* 212, 280-291.

Bondad-Reantaso, G.M., McGladdery, E.S., Berthe, C.J.F., 2007. *Pearl Oyster Health Management: a manual, FAO Fisheries Technical Paper*. Food and Agriculture Organization of the United Nations, Rome. pp. 120.

Cabral, P., Mizuno, K., Tauru, A., 1985. *Preliminary data on the spat collection of mother of pearl (Pinctada margaritifera, Bivalve, Mollusc) in French Polynesia*. In: Proceedings Vth International Coral Reef Congress, French Polynesian. pp. 177-182.

Carrington, E., 2002. Seasonal variation in the attachment strength of blue mussels: causes and consequences. *Limnology and Oceanography*, 1723-1733.

Cartier, L.E., Krzemnicki, M.S., Ito, M., 2012. Cultured pearl farming and production in the Federated States of Micronesia. *Gems & Gemology* 48, 108-122.

Cartwright, J.H., Checa, A.G., Rousseau, M., 2013. Pearls are self-organized natural ratchets. *Langmuir* 29, 8370-8376.

Chand, A., Naidu, S., Southgate, P.C., Theo, S., 2015. The relationship between tourism and the pearl and mother of pearl shell jewellery industries in Fiji. In: Pratt, S. and Harrison, D. (Eds.), *Tourism in Pacific Islands*. Routledge, Oxford. pp. 298.

Chang, S.-J., Tseng, S.-M., Chou, H.-Y., 2005. Morphological characterization via light and electron microscopy of the hemocytes of two cultured bivalves: a comparison study between the hard clam (*Meretrix lusoria*) and Pacific oyster (*Crassostrea gigas*). *Zoological Studies* 44, 144-153.

Clarke, M., McMahon, R.F., 1996. Effects of hypoxia and low-frequency agitation on byssogenesis in the freshwater mussel *Dreissena polymorpha* (Pallas). *The Biological Bulletin* 191, 413-420.

Cochennec-Laureau, N., Montagnani, C., Saulnier, D., Fougerouse, A., Levy, P., Lo, C., 2010. A histological examination of grafting success in pearl oyster *Pinctada margaritifera* in French Polynesia. *Aquatic Living Resources* 23, 131-140.

Coeroli, M., De Gaillande, D., Landret, J., 1984. Recent innovations in cultivation of molluscs in French Polynesia. *Aquaculture* 39, 45-67.

Cuif, J., Ball, A., Dauphin, Y., Farre, B., Nouet, J., Perez-Huerta, A., Salomé, M., Williams, C., 2008. Structural, mineralogical, and biochemical diversity in the lower part of the pearl layer of cultivated seawater pearls from Polynesia. *Microscopy and Microanalysis* 14, 405.

Dauphin, Y., Ball, A.D., Cotte, M., Cuif, J.P., Meibom, A., Salomé, M., Susini, J., Williams, C.T., 2008. Structure and composition of the nacre–prisms transition

in the shell of *Pinctada margaritifera* (Mollusca, Bivalvia). *Analytical and Bioanalytical Chemistry* 390, 1659-1669.

Dauphin, Y., Brunelle, A., Cotte, M., Cuif, J., Farre, B., Laprévote, O., Meibom, A., Salomé, M., Williams, C., 2010. A layered structure in the organic envelopes of the prismatic layer of the shell of the pearl oyster *Pinctada margaritifera* (Mollusca, Bivalvia). *Microscopy and Microanalysis* 16, 91.

de Nys, R., Ison, O., 2008. Biofouling. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elsevier, Amsterdam. pp. 527-553.

Dharmaraj, S., Chellam, A., Velayudhan, T., 1987a. Biofouling, boring and predation of pearl oyster. In: Alagarswami, K. (Ed.), *Pearl culture-CMFRI Bulletin No. 39*. Central Marine Fisheries Research Institute, Cochin. pp. 92-97.

Dharmaraj, S., Kandswami, D., Alagarswami, K., 1987b. Some aspects of physiology of Indian pearl oysters. In: Alagarswami, K. (Ed.), *Pearl culture-CMFRI Bulletin No. 39*. Central Marine Fisheries Research Institute, Cochin. pp. 21-28.

Dharmaraj, S., Velayudhan, T.S., Chellam, A., Victor, A.C.C., Gopinathan, C.P., 1991. Hatchery production of pearl oyster spat: Pinctada fucata. *Central Marine Fisheries and Research Institute* 49, 1-37.

Dix, T., 1972. Histochemistry of mantle and pearl sac secretory cells in *Pinctada maxima* (Lamellibranchia). *Australian Journal of Zoology* 20, 359-368.

Dix, T., 1973. Histology of mantle and pearl-sac of the pearl oyster *Pinctada maxima* (Lamellibranchia). *Journal of the Malacological Society of Australia* 2, 365-375.

Dolmer, P., Svane, I., 1994. Attachment and orientation of *Mytilus edulis* L. in flowing water. *Ophelia* 40, 63-74.

Doroudi, M.S., Southgate, P.C., Mayer, R.J., 1999. The combined effects of temperature and salinity on embryos and larvae of the black lip pearl oyster, *Pinctada margaritifera* (L.). *Aquaculture Research* 30, 271-277.

Doroudi, M.S., Southgate, P.C., 2002. The effect of chemical cues on settlement behaviour of blacklip pearl oyster (*Pinctada margaritifera*) larvae. *Aquaculture* 209, 117-124.

Elen, S., 2002. Identification of yellow cultured pearls from the black-lipped oyster *Pinctada margaritifera*. *Gems & Gemology* 38, 66-72.

Ellis, S., Haws, M., 1999. *Producing pearls using the black-lip pearl oyster (Pinctada margaritifera)*. Center for Tropical and Subtropical Aquaculture, Hilo. pp. 8.

Fassler, C.R., 1991. Farming jewels: The aquaculture of pearls. New opportunities arise as pearl culture spreads beyond Japan. *Aquaculture Magazine* 17, 32-58.

Fassler, C.R., 1997. *Review of investment opportunities in pearl farming*. SPC Pearl Oyster Bulletin 10, Noumea. pp. 46.

Fassler, C.R., 2002. *Recent developments in selected Pacific and Indian Ocean black pearl projects*. World Aquaculture Society. World Aquaculture 2002, Book of Abstracts, Beijing. pp. 218.

Fong, Q.S.W., Ellis, S., Haws, M., 2005. Economic feasibility of small-scale black-lipped pearl oyster (*Pinctada margaretifera*) pearl farming in the central Pacific. *Aquaculture Economics & Management* 9, 347-368.

Fougerouse, A., Rousseau, M., Lucas, S.J., 2008. Soft tissue anatomy, shell structure and biomineralisation. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elsevier, Amsterdam. pp. 77-102.

Fowler, J., Cohen, L., Jarvis, P., 1998. *Practical Statistics for Field Biology*. John Wiley & Sons Ltd, West Sussex. pp. 83-90.

Friedman, K.J., Southgate, P.C., 1999. Growout of blacklip pearl oysters, *Pinctada margaritifera*, on chaplets in suspended culture in Solomon Islands. *Journal of Shellfish Research* 18, 451-458.

Friedman, K.J., Bell, J.D., 2000. Shorter immersion times increase yields of the blacklip pearl oyster, *Pinctada margaritifera* (Linne.), from spat collectors in Solomon Islands. *Aquaculture* 187, 299-313.

Garcia-Gasca, A., Ochoa-Baez, J.R.I., Betancourt, M., 1994. Microscopic anatomy of the mantle of the pearl oyster *Pinctada magatianica* (Hanley, 1856). *Journal of Shellfish Research* 13, 85-92.

George, D.C., 1967. The cultured pearl, its history and development to the present day. *Australian Gemmologist* 8, 10-26.

Gervis, M.H., Sims, A.N., 1992. *The biology and culture of pearl oysters (Bivalvia: Pteriidae)*. Overseas Development Administration and International Center for Living Aquatic Resources Management, Mainilla. pp. 3-19.

Goebel, M., Dirlam, D.M., 1989. Polynesian black pearls. *Gems & Gemology* 25, 130-148.

Hänni, H., 2006. A short review of the use of'keshi'as a term to describe pearls. *The Journal of Gemmology* 30, 2.

Haoatai, H., Monypenny, R., 2011. Export demand for Tahitian black pearls. *Australasian Agribusiness Review* 19, 1-15.

Hawkins, A.J.S., Bayne, B.L., 1985. Seasonal variation in the relative utilization of carbon and nitrogen by the mussel *Mytilus edulis*: budgets, conversion efficiencies and maintenance requirements. *Marine Ecology Progress Series* 25.

Haws, M., Ellis, S., 2000. *Collecting black-lip pearl oyster spat*. Center for Tropical and Subtropical Aquaculture, Hilo. pp. 1-8.

Haws, M., 2002. *The basic methods of pearl farming: A layman's manual*. Hawaii Sea Grant, Center for Tropical and Subtropical Aquaculture, Hilo. pp. 1-15.

Heffernan, O., 2006. *Pearls of Wisdom.* SPC Pearl Oyster Bulletin 17, Noumea. pp. 13-14.

Hodgson, A.N., 1982. Studies on wound healing, and an estimation of the rate of regeneration, of the siphon of *Scrobicularia plana* (da Costa). *Journal of Experimental Marine Biology and Ecology* 62, 117-128.

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. pp. 111.

Hunt, H.L., Scheibling, R.E., 2001. Predicting wave dislodgment of mussels: variation in attachment strength with body size, habitat, and season. *Marine Ecology Progress Series* 213, 157-164.

Hynd, J.S., 1955. A Revision of the Australian Pearl-shells, Genus Pinctada (Lamelli-branchia). *Marine and Freshwater Research* 6, 98-138.

Ito, E., 1996. Study on the mechanism of formation of "circle pearls": An approach from the nacreous structure [in Japanese]. *Journal of the Gemmological Society of Japan* 21, 33-44.

Ito, M., 2009. Improving pearl quality by grafting and husbandry methods. *Aqua Tips* 20, 1-8.

Jabbour-Zahab, R., Chagot, D., Blanc, F., Grizel, H., 1992. Mantle histology, histochemistry and ultrastructure of the pearl oyster *Pinctada margaritifera* (L.). *Aquatic Living Resources* 5, 287-298.

Kawakami, K., 1953. Studies on pearl-sac formation II. The effect of water temperature and freshness of transplant on pearl-sac formation. *Annotationes Zoologicae Japonenses* 26, 217-223.

Knauer, J., Taylor, J.J.U., 2002. *The production of silver nacred 'saibo oysters' of the silver - or goldlip pearl oyster Pinctada maxima in Indonesia*. SPC Pearl Oyster Bulletin 15, Noumea. pp. 29-30.

Kono, M., Hayashi, N., Samata, T., 2000. Molecular mechanism of the nacreous layer formation in *Pinctada maxima*. *Biochemical and Biophysical Research Communications* 269, 213-218.

Ky, C.-L., Blay, C., Sham-Koua, M., Lo, C., Cabral, P., 2014a. Indirect improvement of pearl grade and shape in farmed *Pinctada margaritifera* by donor "oyster" selection for green pearls. *Aquaculture* 432, 154-162.

Ky, C.-L., Molinari, N., Moe, E., Pommier, S., 2014b. Impact of season and grafter skill on nucleus retention and pearl oyster mortality rate in *Pinctada margaritifera* aquaculture. *Aquaculture International* 22, 1689-1701.

Ky, C.-L., Nakasai, S., Molinari, N., Devaux, D., 2015. Influence of grafter skill and season on cultured pearl shape, circles and rejects in *Pinctada margaritifera* aquaculture in Mangareva lagoon. *Aquaculture* 435, 361-370.

Lal, M., Southgate, P.C., Jerry, D.R., Zenger, K.R., 2015. *Genome-wide SNPS reveal fine-scale population structure and connectivity in Fijian black-lip pearl oyster Pinctada margaritifera.* ISGA XII - The International Symposium on Genetics in Aquaculture XII, 21-27 June 2015, Santiago de Compostela, Spain. (Abstract).

Lane, I., Oengpepa, C., Bell, J., 2003. Production and grow-out of the black-lip pearl oyster *Pinctada margaritifera*. *Aquaculture Asia* VIII, 5-7.

Lee, C.Y., Lim, S.S.L., Owen, M.D., 1990. The rate and strength of byssal reattachment by blue mussels (*Mytilus edulis* L.). *Canadian journal of zoology* 68, 2005-2009.

Li, X., Huang, Z., 2009. Unveiling the formation mechanism of pseudo-single-crystal aragonite platelets in nacre. *Physical Review Letters* 102, 075502-075501-075502-075504.

Liu, Y., Shigley, J., Hurwit, K., 1999. Iridescent color of a shell of the mollusk *Pinctada margaritifera* caused by diffraction. *Optics Express* 4, 177-182.

López, C., Carballal, M.J., Azevedo, C., Villalba, A., 1997. Morphological characterization of the hemocytes of the clam, *Ruditapes decussatus* (Mollusca: Bivalvia). *Journal of Invertebrate Pathology* 69, 51-57.

Lucas, J.S., 2008. Environment Influences. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elservier, Armsterdam. pp. 187-229.

Ma, H., Zhang, B., Lee, I.S., Qin, Z., Tong, Z., Qiu, S., 2007. Aragonite observed in the prismatic layer of seawater-cultured pearls. *Frontiers of Materials Science in China* 1, 326-329.

Mamangkey, N.G.F., 2009. *Improving the quality of pearls from Pinctada maxima*. PhD Thesis, James Cook University, Townsville. pp. 1-166.

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

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

Mao Che, L., 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. *Marine Biology* 126, 509-519.

Matlins, A.L., 2002. *The Pearl Book: The Definitive Buying Guide: How to Select, Buy, Care for & Enjoy Pearls, 3rd edn*. Gemstone Press, Woodstock, VT. pp. 190-198.

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* 302, 175-181.

McGinty, E.L., Zenger, K.R., Taylor, J.U.U., Evans, B.S., Jerry, D.R., 2011. Diagnostic genetic markers unravel the interplay between host and donor oyster contribution in cultured pearl formation. *Aquaculture* 316, 20-24.

Miller, M., Stewart, J., 2013. Reproductive characteristics of the ocean leatherjacket, *Nelusetta ayraudi*. *Reviews in Fish Biology and Fisheries* 27, 87-101.

Mills, D., Tlili, A., Norton, J., 1997. Large-scale anesthesia of the silver-lip pearl oyster, *Pinctada maxima* Jameson. *Journal of Shellfish Research* 16, 573-574.

Miyamoto, H., Miyashita, T., Okushima, M., Nakano, S., Morita, T., Matsushiro, A., 1996. A carbonic anhydrase from the nacreous layer in oyster pearls. *Proceedings of the National Academy of Sciences* 93, 9657.

Miyashita, T., Takagi, R., 2011. Tyrosinase causes the blue shade of an abnormal pearl. *Journal of Molluscan Studies* 77, 312-314.

Miyoshi, T., Matsuda, Y., Komatsu, H., 1987. Fluorescence from pearls and shells of black lip oyster, *Pinctada margaritifera*, and its contribution to the distinction

of mother oysters used in pearl culture. *Japanese Journal of Applied Physics* 26, 1069-1072.

Moeser, G.M., Leba, H., Carrington, E., 2006. Seasonal influence of wave action on thread production in *Mytilus edulis*. *Journal of Experimental Biology* 209, 881-890.

Müller, A., 2005. *Cultured pearls-Update on global supply, demand, and distribution*. Presentation at the Swiss Gemmological Society, Sarnen.

Müller, A., 2009. *A brief analysis of the global, seawater cultured pearl industry, (past, present, future)*. Presentation at the 3rd European Gemmological Symposium, Bern.

Müller, A., 2013. *A brief Analysis of the global seawater cultured pearl industry (past, present, future)*. Presentation at Hong Kong Jewellery and Gem Fair (Asia World Expo), Hong Kong.

Murzyniec-Laurendeau, S., 2002. *La Perle de Tahiti: La fin de la Crise.* Master of Advance Studies thesis, University of French Polynesia, French Polynesia. pp. 125.

Nakahara, H., 1957. Some morphological features of pearl-sac tissues in relation to the normal and abnormal pearl production in the pearl-oyster (*Pinctada martensii*). *National Pearl Research Laboratory* 13, 268-270.

Norton, J.H., Dashorst, M., Lansky, T.M., Mayer, R.J., 1996a. An evaluation of some relaxants for use with pearl oysters. *Aquaculture* 144, 39-52.

Norton, J.H., Dashorst, M., Lansky, T.M., Mayer, R.J., 1996b. 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, D.J., 1975. Reviews. *The British Journal for the Philosophy of Science* 26, 353-355.

O'Connor, W.A., Lawler, N.F., 2004. Salinity and temperature tolerance of embryos and juveniles of the pearl oyster, *Pinctada imbricata* Röding. *Aquaculture* 229, 493-506.

Ponia, B., 2010. *A review of aquaculture in the Pacific Islands 1998-2007: Tracking a decade of progress through official and provisional statistics*. Secretariat of the Pacific Community, Noumea. pp. 12-14.

Poutiers, M.J., 1998. Bivalves (Acephala, Lamellibranchia, Pelecypoda). In: Carpenter, E.K. and Niem, H.V. (Eds.), *FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central Pacific*. Food and Agriculture Organisation, Rome. pp. 686.

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.

Price, H.A., 1982. An analysis of factors determining seasonal variation in the byssal attachment strength of *Mytilus edulis*. *Journal of the Marine Biological Association of the United Kingdom* 62, 147-155.

Rao, K.V., Rao, S.K., 1974. Pearl Oysters. In: Nair, V.R. and Rao, S.K. (Eds.), *The commercial molluscs of India*. ICAR Bulletin of Central Marine Fisheries Research Institute No. 25, Cochin. pp. 84-105.

Roberts, R.B., Rose, R.A., 1989. Evaluation of some shells for use a nuclei for round pearl culture. *Journal of Shellfish Research* 8, 387-389.

Rousseau, M., Lopez, E., Stempflé, P., Brendlé, M., Franke, L., Guette, A., Naslain, R., Bourrat, X., 2005. Multiscale structure of sheet nacre. *Biomaterials* 26, 6254-6262.

Rousseau, M., Meibom, A., Gèze, M., Bourrat, X., Angellier, M., Lopez, E., 2009. Dynamics of sheet nacre formation in bivalves. *Journal of Structural Biology* 165, 190-195.

Ruddell, C.L., 1971. The fine structure of oyster agranular amebocytes from regenerating mantle wounds in the Pacific oyster, *Crassostrea gigas*. *Journal of Invertebrate Pathology* 18, 260-268.

Ruiz-Rubio, H., Acosta-Salmón, H., Olivera, A., Southgate, P.C., Rangel-Davalos, C., 2006. The influence of culture method and culture period on quality of half-pearls (mabé') from the winged pearl oyster *Pteria sterna*, Gould, 1851. *Aquaculture* 254, 269-274.

Saucedo, P.E., Southgate, P.C., 2008. Reproduction, development and growth. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elservier, Amsterdam. pp. 37-75.

Scoones, S.J.R., 1996. *The development of the pearl sac in Pinctada maxima (Jameson, 1901) (Lamellibranchia:Pteriidae) and the implications for the quality of cultured pearls*. Master of Science, The University of Western Australia, Perth. pp. 1-80.

Seguin-Heine, M.-O., Lachance, A.-A., Genard, B., Myrand, B., Pellerin, C., Marcotte, I., Tremblay, R., 2014. Impact of open sea habitat on byssus attachment of

suspension-cultured blue mussels (*Mytilus edulis*). *Aquaculture* 426–427, 189-196.

Selin, N., Vekhova, E., 2004. Effects of environmental factors on byssal thread formation in some members of the family Mytilidae from the Sea of Japan. *Russian Journal of Marine Biology* 30, 306-313.

Shor, R., 2007. From single source to global free market: the transformation of the cultured pearl industry. *Gems & Gemology* 43, 200-226.

Simkiss, K., 1988. Molluscan skin (excluding cephalopods). *The Mollusca* 11, 11-34.

Sims, N.A., 1992. Abundance and distribution of the black-lip pearl oyster, *Pinctada margaritifera* (L.), in the Cook Islands, South Pacific. *Marine and Freshwater Research* 43, 1409-1421.

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.

Southgate, P.C., Beer, A.C., 1997. Hatchery and early nursery culture of the blacklip pearl oyster (Pinctada margaritifera L.). *Journal of Shellfish Research* 16, 561-568.

Southgate, P.C., Beer, A.C., 2000. Growth of blacklip pearl oyster (*Pinctada margaritifera*) juveniles using different nursery culture techniques. *Aquaculture* 187, 97-104.

Southgate, P.C., 2007. Overview of the cultured marine pearl industry. In: Bondad-Reantaso, M.G., McGladdery, S.E. and Berthe, F.C.J. (Eds.), *Pearl oyster health management-technical paper No. 503*. Food and Agriculture Organisation, Rome. pp. 7-17.

Southgate, P.C., 2008. Pearl Oyster Culture. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elsevier, Amsterdam. pp. 231-272.

Southgate, P.C., Strack, E., Hart, A., Wada, T.K., Monteforte, M., Carino, M., Langy, S., Lo, C., Acosta-Salmon, H., Wang, A., 2008. Exploitation and culture of major commercial species. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elservier, Armsterdam. pp. 303-354.

Southgate, P.C., 2011. *Pearl industry value chain review 2011 – Fiji Islands.* PARDI Report pp. 20.

Southgate, P.C., 2015. *Developing pearl industry based livelihoods in the western Pacific*. ACIAR Project Proposal, Australian Centre for International Agricultural Research, Canberra. pp. 51.

Steffani, C.N., Branch, G.M., 2003. Growth rate, condition, and shell shape of *Mytilus galloprovincialis*: responses to wave exposure. *Marine Ecology Progress Series* 246, 197-209.

Strack, E., 2006. *Pearls*. Ruhle-Diebener-Verlag GmbH & Co., Stuttgart. pp. 707.

Strack, E., 2008. Introduction. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elservier, Amsterdam. pp. 1-35.

Sud, D., Doumenc, D., Lopez, E., Milet, C., 2001. Role of water-soluble matrix fraction, extracted from the nacre of *Pinctada maxima*, in the regulation of cell activity in abalone mantle cell culture (*Haliotis tuberculata*). *Tissue and Cell* 33, 154-160.

Suzuki, M., Nagasawa, H., 2007. The structure–function relationship analysis of Prismalin-14 from the prismatic layer of the Japanese pearl oyster, *Pinctada fucata*. *FEBS Journal* 274, 5158-5166.

Suzuki, T., Yoshinaka, R., Mizuta, S., Funakoshi, S., Wada, K., 1991. Extracellular matrix formation by amebocytes during epithelial regeneration in the pearl oyster *Pinctada fucata*. *Cell and Tissue Research* 266, 75-82.

Taylor, J.J.U., Rose, R.A., Southgate, P.C., 1997. Byssus production in six age classes of the silver-lip pearl oyster, *Pinctada maxima* (Jameson). *Journal of Shellfish Research* 16, 97-101.

Taylor, J.J.U., 2002. *Producing golden and silver south sea pearls from Indonesian hatchery reared Pinctada maxima*. World Aquaculture 2002 Book of Abstracts. Beijing, P. R. of China, Apr 23–27, Baton Rouge LA. pp.

Taylor, J.J.U., Strack, E., 2008. Pearl production. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elsevier, Amsterdam. pp. 273-302.

Tisdell, C., Poirine, B., 2000. Socio-economics of pearl culture: Industry changes and comparisons focusing on Australia and French Polynesia. *World Aquaculture* 31, 30-61.

Tisdell, C.A., Poirine, B., 2008. Economics of pearl farmng. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elsevier, Amsterdam. pp. 473-494.

Torrey, D.R., Sheung, B., 2008. The Pearl Market. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elsevier, Amsterdam. pp. 357-366.

Tranter, D., 1958. Reproduction in Australian pearl oysters (Lamellibranchia). IV. *Pinctada margaritifera* (Linnaeus). *Marine and Freshwater Research* 9, 509-525.

Uwate, R.K., Kunatuba, P., Raobati, B., Tenakanai, C., 1984. *A review of aquaculture activities in the Pacific Islands Region*, Honululu. pp. 6-52.

Velayudhan, T.S., Chellam, A., Dharmaraj, S., Victor, A.C.C., Alagarswami, K., 2011. Histology of the mantle and pearl-sac formation in the Indian pearl oyster *Pinctada fucata* (Gould). *Indian Journal of Fisheries* 41, 70-75.

Wada, K., 1968. Machanism of growth of nacre in bivalvia. *Bulletin of National Pearl Resource Lab* 13, 1490-1539.

Wada, K., 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, T.K., Tëmkin, I., 2008. Taxonomy and phylogeny. In: Southgate, P.C. and Lucas, J.S. (Eds.), *The Pearl Oyster*. Elsevier, Amsterdam. pp. 37-66.

Wang, R.Z., Suo, Z., Evans, A.G., Yao, N., Aksay, I.A., 2001. Deformation mechanisms in nacre. *Journal of Materials Research* 16, 2485-2493.

Welladsen, H.M., Heimann, K., Southgate, P.C., 2011. The effects of exposure to near-future levels of ocean acidification on activity and byssus production of the Akoya Pearl Oyster, *Pinctada fucata*. *Journal of Shellfish Research* 30, 85-88.

Wells, F.E., Jernakoff, P., 2006. An assessment of the environmental impact of wild harvest pearl aquaculture (*Pinctada maxima*) in Western Australia. *Journal of Shellfish Research* 25, 141-150.

Wilt, F.H., Killian, C.E., Livingston, B.T., 2003. Development of calcareous skeletal elements in invertebrates. *Differentiation* 71, 237-250.

Witman, J.D., Suchanek, T.H., 1984. Mussels in flow: drag and dislodgement by epizoans. *Marine Ecology Progress Series* 16, 259-268.

Xie, L., Wang, X.-X., Li, J., 2010. The SEM and TEM study on the laminated structure of individual aragonitic nacre tablet in freshwater bivalve *H. cumingii* Lea shell. *Journal of Structural Biology* 169, 89-94.

Young, G.A., 1985. Byssus-thread formation by the mussel *Mytilus edulis*: effects of environmental factors. *Marine Ecology Progress Series* 24, 261-271.

Yukihira, H., Lucas, J.S., Klumpp, D.W., 2000. Comparative effects of temperature on suspension feeding and energy budgets of the pearl oysters *Pinctada margaritifera* and *P. maxima*. *Marine Ecology Progress Series* 195, 179-188.

Zardi, G., McQuaid, C., Nicastro, K., 2007. Balancing survival and reproduction: seasonality of wave action, attachment strength and reproductive output in indigenous Perna perna and invasive Mytilus galloprovincialis mussels. *Marine Ecology, Progress Series* 334, 155-163.

Zhao, B., Zhang, S., Qian, P.Y., 2003. Larval settlement of the silver-or goldlip pearl oyster *Pinctada maxima* (Jameson) in response to natural biofilms and chemical cues. *Aquaculture* 220, 883-901.