Genetic contribution to pearl formation

Thesis submitted by

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Manuscript from Chapter 2: 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. Contributions: ELM - Project design; sample collection; data generation & analysis;

manuscript preparation DRJ - Project design; review of final manuscript BSE - sample collection; review of final manuscript JJUT - review of final manuscript.

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Abstract

The silver, *Pinctada maxima*, and black-lip, *Pinctada margaritifera*, pearl oysters are two commercially important species which produce high-value "South Sea" pearls. Although pearl culture techniques were developed in the early 1950's and have been continually refined, a large proportion of the pearl harvest (~60%) from these two species still fails to be categorised as "gem" quality. The pearling industry stands to benefit substantially from improvements in pearl quality, as it is the proportion of "gem" quality pearls that largely contributes to the profitability of the industry. Despite research into innovative husbandry, nuclei implantation, and optimum grow-out environments, the industry has not seen dramatic increases in the proportion of high quality pearls from harvests. There is, however, the potential for genetic approaches to increase the proportion of "gem" quality pearls produced through selective breeding. Before targeted breeding programs can be developed though, there needs to be a strong understanding of the genetic basis of traits and this is currently lacking for pearls. To date, little research has focussed on the genetic processes behind pearl formation, a complex process potentially involving the genetic contribution from two individual oysters (host and donor oyster). Given the complexity of pearl production and the potential genetic contribution from two oyster genomes, without a clear understanding of the role of each oyster in the pearl biomineralisation process targeted selection cannot be effective. This thesis defines the respective roles of the host and donor oysters in pearl formation, by first examining their phenotypic contribution to pearl quality traits followed by a fine scale examination of their molecular contribution to the pearl biomineralisation process.

Prior to genetic improvement of pearl quality through selective breeding, the respective roles the donor and host oysters play in the determination of pearl quality traits must first be defined. Current pearl culture techniques do not permit differentiation between the host and donor oyster pearl phenotypes due to con-specifics being used as the host and donor oyster (allografts). One possible way to provide information on the contribution from the host and donor to pearl traits is by adopting a novel approach of using mantle grafts originating from one pearl oyster species implanted into a second recipient species that is closely related and characteristically has a different pearl phenotype (termed a xenograft). For the first time, this thesis definitively demonstrates the contribution from the donor and host oysters to pearl phenotypic traits through xenotransplantation of two closely related yet distinctly different pearl producing species, *P. maxima* and *P. margaritifera*. The results conclusively revealed that the donor oyster is the main contributor to pearl quality. In particular, pearl colour and size were strongly influenced by the donor oyster species used as xenografts. *P. maxima* donors produced larger, silver colour based pearls, whilst, *P. margaritifera* xenografts produced smaller, black colour based pearls. Through the novel approach of producing xenografted pearl oysters, this study demonstrates the potential of xenografts as a means to improve pearl quality traits such as pearl size, and highlights the role that donor oysters have in the realisation of pearl growth, colour and surface complexion.

In light of phenotypically detecting the donor oyster as the main contributor to pearl quality traits, the next logical question is what is happening at the molecular level in regard to the expression of biomineralisation related proteins that govern pearl formation. Whilst, studies have shown that genomic DNA from a mantle allograft remains present in the pearl sac at the time of pearl harvest, what remains unclear is whether biomineralisation genes from the

donor mantle allograft are transcriptionally active and contribute to pearl formation. One of the biggest impediments in determining whether the donor or host cells are transcriptionally active for biomineralisation genes in the pearl sac is discriminating between the gene products of the two potentially contributing oysters. Currently there is insufficient information on levels of intra-specific polymorphisms in putative biomineralisation genes to characterise gene products that may be derived from the host/donor oysters. This thesis took a powerful and novel approach in determining if the donor oyster cells remain transcriptionally active in the pearl sac, by xenografting two species of pearl oyster, *P. maxima* and *P. margaritifera*, which contain species-diagnostic gene differences. Diagnostic PCR tests revealed that donor oyster cells not only remained present in the pearl sac at the time of pearl harvest, but were found for the first time to be transcriptionally active in the expression of two biomineralisation genes, N44 and N66. These results support that the donor oyster is an important contributor to the biomineralisation process in pearl culture.

To further elaborate on what is happening at the molecular level in regard to the expression of biomineralisation related proteins that govern pearl formation, the pearl sac transcriptome of *P. maxima* and *P. margaritifera* was examined through high through-put RNA sequencing (Illumina GAII). Allografted and xenografted pearl sacs from two pearl oyster species with unique genomes, *P. maxima* and *P. margaritifera*, were produced. Putative molluscan biomineralisation-related genes identified within the sequenced allografted pearl sacs of both *P. maxima* and *P. margaritifera* revealed 19 biomineralisation genes similarly expressed in both species. This is the largest proportion of genes linked to the process of biomineralisation within the pearl sac to date. Based on the presence/absence of species diagnostic gene transcripts within xenografted pearl sacs, all genes examined were found to be expressed by the species used as the donor oyster. In one individual it also found that the host was

expressing *Linkine*. These results convincingly show for the first time that not only is the donor mantle tissue transcriptionally active, it is primarily responsible for the expression of biomineralisation genes in the pearl sac.

Outcomes of this thesis have provided a substantial advancement in the understanding of cultured pearl formation. By understanding the importance of the donor oyster to pearl formation and quality, this research provides grounds for a donor specific selective breeding program based on pearl growth, colour and surface complexion. Now that the major genes potentially involved in pearl biomineralisation have been identified in this thesis and the donor established as the main contributor to the expression of these genes, the next step is to identify the specific function of these genes that lead to different pearl quality traits. This will ensure pearl quality traits are not only selected upon based purely on phenotype, but an understanding of the molecular mechanisms underlying pearl traits to achieve maximum genetic gains. This thesis not only provides a solid foundation for elucidating the biological process of pearl formation in general, but it also provides valuable information that can be directly utilised for selective breeding programs in the cultured pearl industry to improve pearl quality.

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Chapter 1 General Introduction

1.1 Pearl culture overview

The term "pearl oyster" is generally applied to bivalves from the genera *Pinctada* and *Pteria* (Pteriidae) (Skelton and Benton, 1993). Pearl oysters from the genus *Pinctada* comprise 14 described species that are predominately distributed in tropical and subtropical shallow seawaters, particularly throughout the Indo-Pacific region (Gervis and Sims, 1992; Wada and Temkin, 2008). Although it is possible to produce pearls from species within *Pteria* the three main pearl oyster species the pearling industry targets for round pearl production all belong to *Pinctada*. These species are the silver-lip/gold-lip (*P. maxima*), black-lip (*P. margaritifera*), and Akoya (*P. fucata. P. martensii, P. imbricata, P. radiata*) marine pearl oysters (Southgate et al., 2008; Torrey and Sheung, 2008). Phylogenetic analyses of *Pinctada* separate these oysters into two distinct monophylogenetic clades, one comprising the large pearl oysters *P. maxima* and *P. margaritifera* and the other comprising species commonly referred to as the small or Akoya pearl oyster complex (*P. fucata. P. martensii, P. imbricata, P. radiata*) (Cunha et al., 2010). Within the large *Pinctada* clade, *P. maxima* and *P. margaritifera* have been shown to be phylogenetic sister species, based on the most complete attempt to resolve phylogenetic relationships to date using nuclear internal transcribed spacer markers (Yu and Chu, 2006; Yu et al., 2006).

Cultured round pearl production was developed in Akoya pearl oysters in the early 1900's (Taylor and Strack, 2008), providing the basis for a global commercial pearling industry currently worth US$640 million (Torrey and Sheung, 2008). Whilst many species produce

pearls, the larger and more valuable pearls are those called “South Sea” pearls, a name given to pearls produced by *P. maxima* and *P. margaritifera*. Pearls produced from these two species have base-tones that are “white/silver” or “black” in colour, respectively. The term “South Sea” pearls originated from the overlapping distribution these two species share in the Indo-Pacific region (Fig. 1.1) and as a marketing strategy implemented to differentiate between the smaller Akoya pearls (Gervis and Sims, 1992; Wada and Temkin, 2008). *P. margaritifera* is primarily farmed in the atoll lagoons of French Polynesia, with smaller scale production in the Cook Islands and other Pacific nations (Southgate et al., 2008). The leading pearl oyster species in terms of value, however, is *P. maxima*, with the majority of this species’ production being situated in Indonesia, Australia and the Philippines. Global production value of pearls derived from *P. maxima* is approximately US$248 million (Southgate et al., 2008).

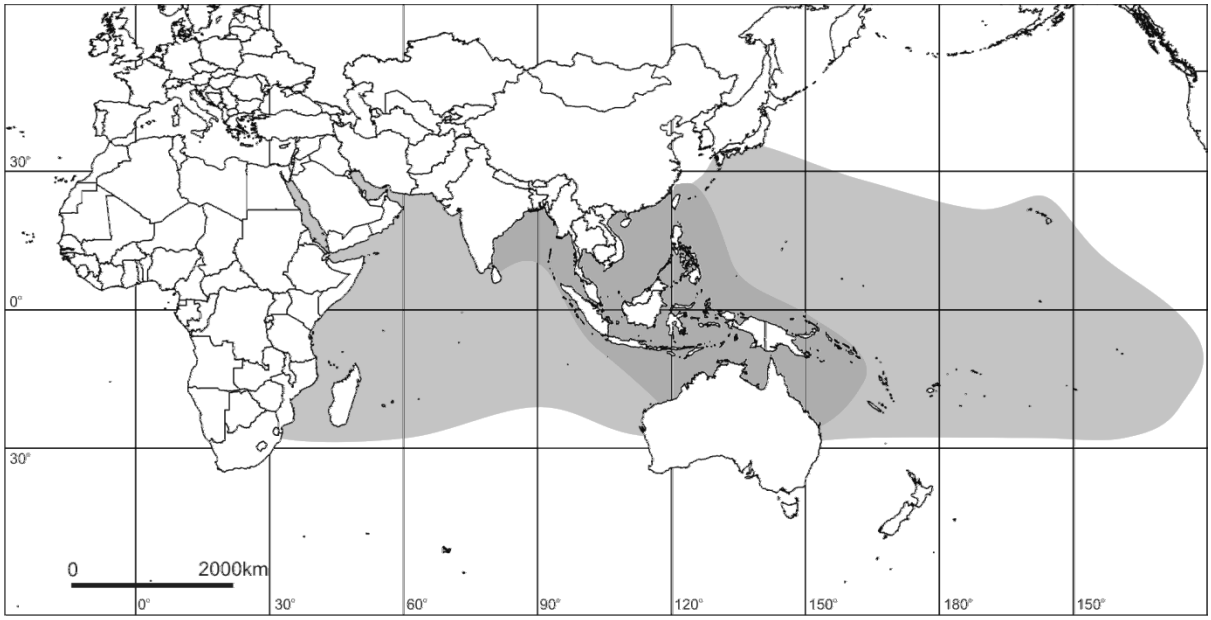

Figure 1.1: Approximate distribution of the Silver-lip (dark grey) and Black-lip (light grey) pearl oyster, *Pinctada maxima* and *Pinctada margaritifera* (Wada and Temkin, 2008).

Despite pearling being a mature aquaculture industry, the majority of pearls produced fall within “non-gem” quality grades. Alagarswami (1970) and Haws and Ellis (1999), estimated that for most pearl species only around 30% of pearls harvested can be categorised as high quality “gem” grade. Such low quantities of gem quality pearls is primarily a consequence of pearl grading being a complex process determined by variation in five pearl quality traits; size, colour, shape, lustre and surface complexion (Strack, 2006). Generally rounder, larger, smoother and more lustrous pearls achieve a high market value. Colour on the other hand is a more subjective quality characteristic and depends largely on the oyster species the pearl was derived from. Black pearls produced from *P. margaritifera* that carry a purple, green or pink overtone for example, are of greater value than pearls that appear silver or gray, whilst, pearls from *P. maxima* that are a white, silver, pink or deep golden colour hold the highest value of any marine pearl (Taylor and Strack, 2008). A variation in any of the pearl quality characteristics can dramatically alter the overall value of a pearl.

1.2 Biomineralisation and pearl formation

Pearl formation, whether natural or cultured, is an intriguing process that has been subject to much scientific speculation and research over the years. The biological mechanisms involved in pearl formation and the method in which a perfect pearl can be formed, however, is still somewhat a scientific enigma. Pearls are the result of an oyster’s capability to produce calcified shell material through secretions by the mantle tissue (Taylor and Strack, 2008). In cultured pearl formation a shell based nucleus is surgically implanted into the gonad of a host oyster along with a mantle graft excised from a donor oyster. The mantle graft then degenerates to a single epithelial cell layer that grows around the implanted shell nucleus to form a structure termed the “pearl sac”. The pearl sac then secretes shell matrix proteins leading to biomineralisation of calcium carbonate in the form of nacre from around two weeks post-pearl sac development (Kawakami, 1952a, b; Machii and Nakahara, 1967). Prior

to nuclei implantation, host and donor oysters are grown until they are mature enough to have reached a size to enable surgery, but still of an age where growth is vigorous (generally around two years of age, approximately 110 mm dorso-ventral shell height in *P. maxima* (J.J.U Taylor *pers. comm.,* 2009). Post operation, oysters are usually on-grown for a further 18-24 months before the pearl is harvested. How to reliably produce a cultured pearl has been established since the 1900s, however, there is much potential to improve pearl quality through a better understanding of the factors that directly influence the biomineralisation process in pearl development such as nacre secretion and nacre quality.

Biomineralisation is widespread among prokaryotic and eukaryotic organisms as a result of deposition of calcium carbonate (usually calcite or aragonite), calcium phosphate (usually apatite), or silica (opal). These biomineral compounds are found in most animals, functioning as supportive, protective or feeding structures (Bengtson, 1994). Pearl oysters and other molluscs have an extensive $CaCO_3$ biomineral structure which forms a shell protecting sensitive internal soft tissues. The fundamental structure of the shell is common to all pearl oysters and characteristic of other molluscs. The shell of *Pinctada* species consists of one protein layer and two $CaCO_3$ layers which are secreted by the mantle tissue; the outer periostracum (largely proteins), a prismatic layer (calcite) and inner nacreous (aragonite) layer (Fougerouse et al., 2008) (Fig. 1.2). Significant differences exist in the arrangement of $CaCO_3$ crystals among nacre-forming organisms, such as the axes along which these crystals are arranged and the way in which they are deposited. Studies have described gastropod nacre as “columnar”, with stacks of tablets forming along common axes, and bivalve nacre as “sheets” with the crystal tablets forming in a brick wall-like manner (Hedegaard, 1997; Hedegaard and Wenk, 1998; Chateigner et al., 2000).This structural diversity implies

substantial variation in the process of shell nacre formation which has sparked much interest into determining the growth dynamics of biological nacre formation.

The mollusc shell is not only made up of a mineral phase (calcium carbonate), but also an organic cell-free matrix comprising proteins, glycoproteins, lipids and polysaccharides, secreted by the external mantle epithelium. Although this matrix represents less than 2% of the total composition of the shell by dry weight (Weiner, 1986), it interacts with the crystal surface to orientate its nucleation and control crystal polymorphism in the form of aragonite or calcite (different structural layers of the shell) (Falini et al., 1996). Models of mollusc shell biomineralisation have therefore been proposed based on histochemical studies and ultrastructural observations of the shell, combined with biochemical analysis of the extracellular organic matrix. The current "chitin-silk fibroin gel proteins- acidic macromolecules" model proposed by Weiner and Traub (1984), updated by Addadi (2006) and recently reviewed by Furuhashi (2009), was established from mollusc nacre analysis. According to this model, the major components of biomineralisation are relatively hydrophobic silk proteins and a complex assemblage of hydrophilic proteins (many of which are unusually rich in aspartic acid), highly structured in a polysaccharide b-chitinous framework. These protein components of the organic matrix are thought to control various aspects of the biomineralisation process: the $CaCO_3$ crystal polymorphisms (calcite and aragonite) and the microstructures of shell layers (Marin, 2008). Therefore, research on the shell calcification process has mainly focused on shell proteins. Since the publication of the first complete amino-acid sequence of a nacre-shell protein in 1996 (Miyamoto et al., 1996), major advances in the field of molecular biology have led to the identification of an increasing number of shell matrix proteins, including: Nacrein, N14, N66, MSI17, MSI31, MSI60, Aspein and Lustrin_A (Miyamoto et al., 1996; Shen et al., 1997; Sudo et al., 1997;

Samata et al., 1999; Kono et al., 2000; Miyashita et al., 2000; Zhang et al., 2003; Tsukamoto et al., 2004; Miyamoto et al., 2005; Norizuki and Samata, 2008) (see reviews by Wilt et al., 2003; Samata, 2004). However, very little is known about the precise functional role of these biomineralisation-related proteins and therefore the molecular aspects of shell building are still far from being fully understood.

The edge of the mantle contains regions of specialised epithelial cells that secrete different types of $CaCO_3$ (the periostracum, prismatic and nacreous layers) for shell building (Wang et al., 2002). The edge of the mantle can be divided into three zones: the outer marginal zone, the pallial zone and the central zone (Chellam et al., 1991; Garcia-Gasca et al., 1994; Fig. 1.2). The periostracum and prismatic layers are secreted by one epithelial region of the outer epithelium of the mantle in the marginal zone and subsequently do not usually increase in thickness after secretion (Gervis and Sims, 1992; Garcia-Gasca et al., 1994). The nacreous layer on the other hand, is continuously secreted and thickened by the outer side of the pallial and central zones (Fougerouse et al., 2008). For example, gene expression in mantle tissue measured by real-time PCR, revealed that MSI60 and N16 are related to nacreous layer formation, whilst MSI31, Aspein and Prismalin-14 are related to prismatic layer formation (Tsukamoto et al., 2004; Takeuchi and Endo, 2006; Inoue et al., 2010). However, the precise role in the formation of the different $CaCO_3$ layers for most of these proteins remains unclear.

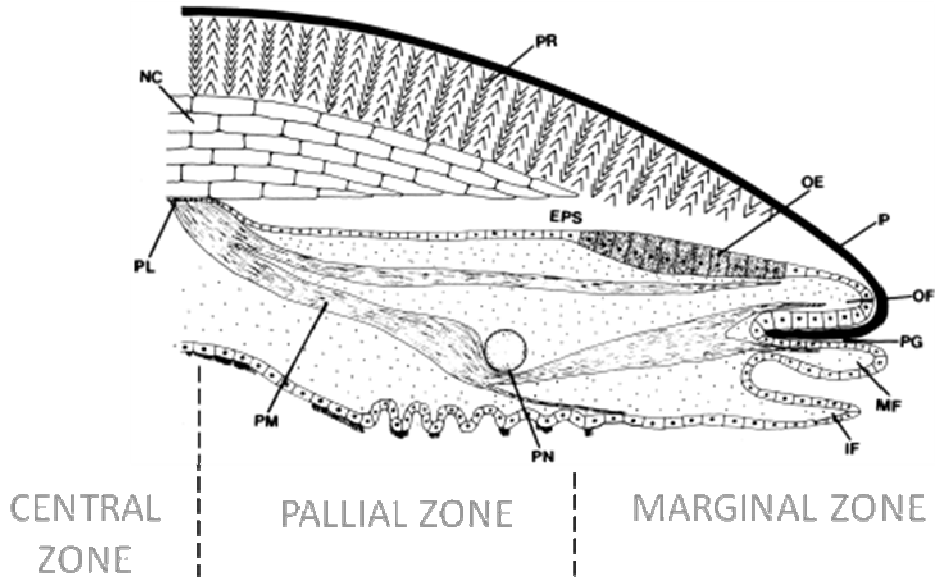

Figure 1.2: Diagrammatic cross-section of the growing outer edge of a bivalve shell and attached mantle tissue. *EPS*, extrapallial space; *MF*, middle fold of mantle; *NC*, nacreous shell layer; *OE*, outer epithelium of the mantle; *OF* outer fold of the mantle; *IF* inner fold of the mantle; *P*, periostracum; *PG*, periostracal groove; *PL*, pallial line; *PM*, pallial muscle; *PN*, pallial nerve; *PR*, prismatic shell layer (Wilt et al., 2003; Fougerouse et al., 2008).

1.3 Understanding pearl formation

Several proteins regulating nacreous shell matrix secretions have been identified in mantle tissue, although, very little is known about the role of these proteins in cultured pearl formation. Donor mantle tissue used for cultured pearl production is excised from the pallial zone which has been shown to secrete only the nacreous layer in shell formation (Sudo et al., 1997; Takeuchi and Endo, 2006; Taylor and Strack, 2008). Shell matrix proteins responsible for the nacreous layer formation in a shell are therefore thought to be important mediators for pearl formation (Bédouet et al., 2001, 2006). Interestingly, expression patterns of shell matrix proteins have been found to differ between mantle and pearl sac tissue. A recent study

demonstrated that N19 had significantly higher expression levels in the pearl sac compared to that of mantle tissue (Wang et al., 2009). Five other nacreous shell matrix proteins, including Nacrein, EFCBP, N16, ACCBP and MSI60, have shown high expression levels in mantle tissue with significantly lower expression levels in the pearl sac (Wang et al., 2009). The precise role of these nacreous shell matrix proteins in cultured pearl formation has yet to be defined. Also there is limited understanding on what regulates the expression of shell matrix proteins and why differential gene expression patterns appear to be evident between that in the natal mantle tissue compared to a pearl sac.

Only recently has the expression of shell matrix proteins been examined within the pearl sac and their potential influence on pearl formation is only starting to be elucidated (Inoue et al., 2009; Wang et al., 2009; Inoue et al., 2010). Our knowledge of shell matrix proteins within the pearl sac is restricted to research on one species, *P. fucata*, where nine previously known biomineralisation-related genes have been examined within the pearl sac of this species (MSI31, N16, Nacrein, MSI60, Prismalin-14, Aspein, EFCBP, ACCBP and N19). The levels of expression of six of these shell matrix proteins (MSI31, N16, Nacrein, MSI60, Prismalin-14 and Aspein) were found to differ within the pearl sac, with significantly higher expression levels of MSI31 in pearl sacs that produced low quality pearls compared to high quality pearls (Inoue et al., 2009). What is interesting, is that differences in the expression levels of all nine of these shell matrix proteins was observed between mantle and pearl sac tissue (Wang et al., 2009; Inoue et al., 2010). For example, within pearl sac cells low transcription levels of the Nacrein gene were evident compared to that of mantle tissue (Wang et al., 2009). Nacrein is a protein that regulates $CaCO_3$ supply and should therefore be present in high numbers for pearl development. This suggests that pearl sac cells, unlike mantle tissue,

rely on other resources for $CaCO_3$ supply. It has been reported that oyster hemocytes have the ability to bind calcium ions and mediate shell biomineralisation, therefore, the host oyster hemocytes could possibly be having an influence on pearl biomineralisation (Mount et al., 2004; Lee et al., 2008). There is currently a paucity of information on gene expression of shell matrix proteins within the pearl sac limiting our knowledge of pearl formation. More surprisingly, because pearl formation differs to that of shell formation in that two individuals are potentially involved in the pearl biomineralisation process, it has yet to be determined whether the host or donor oyster are responsible for the expression of biomineralisation-related genes contributing to pearl formation.

Understanding the genetic foundations behind the pearl biomineralisation process provides a solid foundation for elucidating the biological process in general, as well as providing valuable information that can be directly utilised by the cultured pearl industry through selective breeding and seeding approaches. For example, through selective breeding and seeding using oysters that hold particular genes linked to favourable pearl quality traits, pearl quality may be improved. Industry-wide interest towards genetic improvement of pearl oysters is rapidly growing, however, currently there has been no significant genetic improvement in pearl oysters from selective breeding despite pearl quality traits being shown to be heritable (Jerry, 2010). Since variation in pearl quality traits has a genetic basis, selective breeding and seeding for favourable traits may permit a substantial shift in the proportion of high-quality pearls, significantly improving profitability, although several fundamental issues must be addressed before selection can commence. The production of a cultured pearl is a unique, complex biological process potentially involving genetic contribution from two oysters (the host and donor oyster). Before any selective breeding programs can be established it is important to define the genetic contribution of these two

oysters to the expression of biomineralisation-related proteins and the various pearl quality traits.

1.4 Donor and host oyster contribution to pearl formation

It is generally considered that the mantle tissue from the donor oyster is primarily responsible for biomineralisation genes for shell formation, as well as pearl formation (Farn, 1986). Evidence from genotyping the pearl sac and comparing microsatellite alleles found with those in the donor and corresponding host oysters has shown that DNA originating from both the donor and host oyster is present in the pearl sac at pearl harvest (Arnaud-Haond et al., 2007). However, the precise genetic make-up of the pearl sac has yet to be determined with the role of donor and host cells and their active contribution to pearl development remaining unknown. A few phenotypic studies that have examined pearl colour agree that there is a tendency for shell nacre colouration in the donor oyster to influence overall pearl colour (Alagarswami, 1987a; Taylor, 2002; Wada and Komaru, 1996). An association between donor shell and pearl colour was observed in Akoya pearl oysters, *Pinctada fucata martensii* (Wada and Komaru, 1996). Through the implantation of mantle tissue derived from inbred white shelled oysters, Wada and Komaru (1996) showed the frequency of non-yellow pearls in a harvest to be lowered. In the above study and other similar experiments, implantations were based on allografts involving same nacre coloured hosts therefore there has been no separation of the donor and host contribution to pearl colour. The isolation of DNA from mantle donors in pearl sacs and realisation of phenotypic traits related to the donor oyster, like nacre colour, suggests that the donor oyster cells are actively involved in cultured pearl biomineralisation processes. Yet to date, no studies have shown donor mantle cells to be transcriptionally active in the pearl sac at pearl harvest, or conclusively defined the contribution of the donor oyster to various pearl quality traits.

To define the phenotypic contribution from the host and donor to pearl traits, mantle grafts originating from one pearl oyster species can be implanted into a second recipient species that characteristically has different pearl phenotypes (termed xenografts). Xenografts have been trialled in freshwater mussels where it was shown that pearl sac formation is viable (Kawakami, 1954; Panha and Kosavititkul, 1997; Wada, 1989). The pearl oyster species *Pinctada maxima* and its sister species *P. margaritifera* show unique pearl characteristics, particularly colouration, with *P. maxima* producing pearls with a base colour of gold or silver, whilst *P. margaritifera* predominately produce black colour based pearls. Using mantle xenografts originating from these two species for example, may help elucidate phenotypically the role the donor and host oysters play in pearl traits like colour.

Xeno-grafted mantle tissue may also resolve the problem of understanding the genetic contribution of the host and donor oyster to pearl formation. One of the biggest impediments in determining whether the donor or host cells are transcriptionally active for biomineralisation genes in the pearl sac is discriminating between the gene products of the two potentially contributing oysters. Currently there is insufficient information on levels of intra-species polymorphisms in putative biomineralisation genes to characterise gene products that may be derived from the host/donor oysters. Samata (2004) found Nacrein and N16 shell matrix gene sequences to have clear differences between *P. maxima* and *P. margaritifera*. Comparisons of Shermatrin genes showed differences in shell matrix gene sequence across three *Pinctada* species, *P. maxima, P. margaritifera* and *P. fucata* (Jackson et al., 2010). If xenografting two species of pearl oysters was viable in cultured marine pearl formation and if species-specific gene differences are present between these species for biomineralisation genes, then the use of inter-species xenografts would be a powerful, novel

approach in determining if the donor oyster cells remain transcriptionally active in the pearl sac.

1.5 Thesis aims and chapter summaries

Various techniques have been trialled to try to understand the biomineralisation process in molluscs, including microscopy, chemistry and molecular approaches. These approaches have provided much insight into the biomineralisation process of shell formation. However, little research has focused on understanding pearl formation, a process potentially involving two individuals that is still not clearly understood. This thesis evaluates whether the cells from the donor or host oyster are actively involved in the pearl formation process, by adopting a novel approach using xenografted mantle tissue. Here, mantle tissue originating from each of two pearl oyster species, *P. maxima* and *P. margaritifera*, was implanted into host oysters of the alternate species. This technique provided a definitive approach to determine the host and donor oyster contribution to pearl formation by 1) providing phenotypic differences between pearl quality traits from the host and donor oyster species, and 2) providing genetic polymorphisms in genes involved in biomineralisation between the host and donor oyster species.

Chapter 2 first examines the viability of xenografts between *P. maxima* and *P. margaritifera* for cultured pearl formation. This chapter also examines the impact xenografts have on pearl quality traits and investigates the respective role the host and donor oyster have in the expression of pearl quality traits.

Once the contribution of the host and donor oyster to pearl quality traits is defined, this thesis determines for the first time if the donor oyster cells are transcriptionally active for biomineralisation genes governing pearl formation. Chapter 3 examines if both DNA and cDNA gene products from biomineralisation genes involved in nacre formation, N66 (Kono et al., 2000) and N44 (NCBI Accession No. FJ913472.1), can be detected from the donor or host species in xenografted pearl sacs.

To further elaborate on the contribution of the donor and host oyster to the expression of biomineralisation-related genes, Chapter 4 uses species-specific single nucleotide polymorphisms (SNP) to detect and differentiate biomineralisation associated gene transcripts expressed in xenografted pearl sacs. This was carried out using high-throughput next-generation sequencing (Illumina GAII), which aided in the initial identification of all biomineralisation-related genes expressed within the allografted pearl sacs and in the examination of which species diagnostic SNP was present within the xenografted pearl sacs.

The results of this study are synthesized and the advantages of understanding the contribution from the host and donor oyster to pearl formation are discussed in Chapter 5. This Chapter also addresses directions for future research to further our understanding of pearl formation.

Each of the data chapters in this thesis contains a stand-alone Introduction, Materials & Methods, Results and Discussion section. At the time of thesis submission, two data chapters have been accepted for publication in peer-reviewed journals and are presented as published

herein, with minimal modification and minor re-formatting. The final data chapter has been submitted for publication.

Chapter 2 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

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2.1 Introduction

Pearl quality is measured as five variables; size, shape, colour, lustre and surface complexion. Larger, unblemished, lustrous and spherical pearls fetch the highest market price. Depending on the pearl oyster species, colour is a more subjective indicator of value, with white 'South Sea' pearls, especially those with a pink overtone, holding the greatest value of any marine pearl with similar quality characteristics (Taylor and Strack, 2008). Despite pearling being a mature industry, the majority of pearls produced cannot be sold as high quality, due to blemishes or other defects. For example, it has been estimated that only 30% of pearls harvested can be categorised as high quality (Gervis and Sims, 1992). Despite research into innovative husbandry, nuclei implantation and identification of optimum environments (Rose and Baker, 2003; Yuxiang and Fu-Liang, 2003; Lucas, 2008), the industry has not seen dramatic increases in the proportion of high quality pearls from harvests with only around 30% of pearls harvested categorised as high quality (Alagarswami, 1970; Haws and Ellis, 1999). Further research is required to increase our understanding of the complex processes involved in producing a cultured pearl.

One major gap in our knowledge is on what contribution genetic factors have in the realisation of pearl quality traits (Wada and Jerry, 2008). Despite commercial hatchery

production being commonplace in most pearl oyster industries, the application of modern genetic breeding methods as an approach to increase the proportion of high quality pearls has been rare. Our knowledge of the genetic basis of important traits in pearl oysters is largely restricted to the heritability of shell traits and their phenotypic correlations with pearl weight and colour (Velayudan et al., 1996; Wada, 1984, 1986). The production of a cultured pearl, however, is a unique, complex biological process potentially involving genetic contribution from two oysters (the host and donor). No study to date has conclusively defined the contribution of these two oysters to the various pearl quality traits.

Production of a cultured pearl involves surgical implantation of a mantle graft (termed saibo) originating from a donor oyster, along with a spherical shell bead (nucleus) into the gonad of a second recipient oyster (host). The mantle tissue from the donor oyster then degenerates to a single outer epithelial cell layer which grows around the implanted nuclei to form a pearl sac, which is responsible for secreting nacre (Kawakami, 1952a, b). Until recently, it was not known if the donor oyster cells actually persisted in the pearl sac until time of pearl harvest. Genotyping the pearl sac and comparing microsatellite alleles found with those in the corresponding host oysters has shown, however, that DNA originating from the donor oyster can still be detected in the pearl sac at pearl harvest (Arnaud-Haond et al., 2007). The role these donor cells have in the realisation of the various pearl quality traits, or indeed even if the DNA detected is actively transcribed, is yet to be determined.

One possible way to improve pearl quality whilst providing information on the contribution from the host and donor to pearl traits is by using mantle grafts originating from one pearl oyster species implanted into a second recipient species (termed xenografts). This technique

has yet to be explored in pearl oysters. The pearl oyster species *Pinctada maxima* and its sister species *P. margaritifera* show unique pearl characteristics, particularly colouration, with *P. maxima* producing pearls with a base colour of gold or silver, whilst *P. margaritifera* predominately produce black colour based pearls. Using mantle xenografts originating from these two species may help elucidate the role the donor and host oysters play in pearl traits like colour, as well as providing a unique opportunity to see if desirable traits of both species can be combined to enhance overall pearl quality. Xenografts have been trialled in freshwater mussels where it was shown that pearl sac formation is viable (Kawakami, 1954; Panha and Kosavititkul, 1997; Wada, 1989). However, how xenografts influence pearl production and overall quality has not been explored in any mollusc species.

This study used xenografted mantle tissue originating from each of two pearl oyster species, *P. maxima* and *P. margaritifera*, when implanted into host oysters of the alternate species to a) examine the impact xenografts have on pearl quality and b) to further our understanding of the respective role the host and donor oyster have in the expression of pearl traits.

2.2 Materials and methods

2.2.1 Experimental animals

Adult black-lip (*P. margaritifera*) (N = 80, shell height: 14.6 – 16.1 cm) and silver-lip (*P. maxima*) (N = 80, shell height: 10.5 – 11.7 cm) pearl oysters were sourced from West Papuan Province (1º13’N, 130º54’E), and Bali (8º23’S, 115º14E), Indonesia, respectively. *P. margaritifera* oysters were sourced from the wild, whilst *P. maxima* were hatchery produced. *P. margaritifera* oysters were transported by boat to the Bali site three months prior to

nucleus implantation to allow the oysters to adjust to environmental conditions. Both oyster species are native to the Indo-Pacific region (Gervis and Sims, 1992) and the site where the experiments were undertaken. All experimental oysters were held together in 16 pocket panel nets on a single commercial long-line. Three weeks prior to implantation, panels were covered with mesh to reduce the oyster's metabolic rate and gametogenic activity, making them less likely of rejecting implanted nuclei (Gervis and Sims, 1992).

Donor mantle tissue was excised from 10 *P. maxima* and 10 *P. margaritifera* oysters. Excised mantle tissue from each oyster was then cut into eight segments and four segments used as allografts (species controls) and xenografts (experimental treatments) respectively. Eighty host oysters from each species were implanted (Fig. 2.1). By implanting the same donor in both oyster species we were able to control for any individual genetic effect of the donor oyster on pearl quality. According to the gonad size of the host oyster appropriately sized nuclei (ranging from 5.76 - 7.88 mm and 0.28 - 0.73 g in size and weight, respectively) were implanted along with the mantle tissue. In total four groups were created; black-lip pearl oyster hosts with black-lip donors (BB); black-lip hosts with silver-lip donors (BS); silver-lip hosts with black-lip donors (SB); silver-lip hosts with silver-lip donors (SS). Mean nuclei size was similar across all groups (SS: 6.52 mm; BB: 6.24 mm; BS: 6.43 mm; SB: 6.22 mm). The same technician was used to implant nuclei for each group. Following implantation, the 160 host oysters were randomly placed in ten 16 pocket panel nets. Host oysters were x-rayed at 3, 6 and 9 months post operation to estimate pearl nuclei retention rates. Fourteen months post operation, pearls were harvested and individual pearl quality traits graded according to the collaborating commercial organisations criteria.

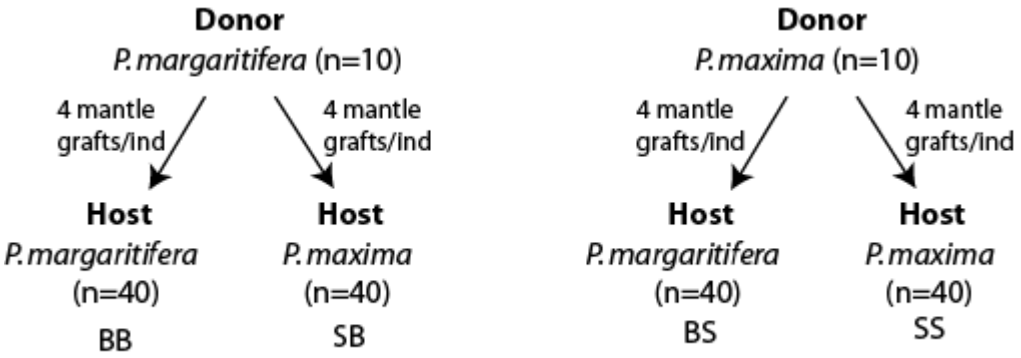


Figure 2.1: Experimental design involving implantation of allografts (controls) and xenografts (treatment) into the two host oyster species, *P. maxima* and *P. margaritifera.* In the figure, BB refers to a black-lip host with a black-lip donor; BS, a black-lip host with silver-lip donor; SB, a silver-lip host with black-lip donor; SS, a silver-lip host with silver-lip donor.

2.2.2 Data analyses

The nuclei retention rates of experimental treatments and controls were compared using a chi-squared test. Keshi pearls (small irregular pearls resulting from rejection of the pearl nuclei) were counted as rejected nuclei in this analysis. A Fisher's exact test was used to determine if there was an effect of individual donor oyster on nuclei retention rates within each group. Donor oysters were removed from this analysis if two or less replicates remained.

All keshi and pearls that were classed as rejects (i.e., had organic or prismatic blemishes on the surface) were removed from the pearl quality data set before analyses. Shape and colour traits were grouped into broad categories for ease of data analyses. Here shape was condensed to four categories; round (R), button (B), baroque (BQ) or drop (D), and colour categorised into white (w), black (b), cream (c), pink (p), yellow (y) or silver (s). Complexion

(grades A1, A2, B1, B2, C1, C2) and lustre (grades 1, 2, 3) were kept as initially classified. Lustre was graded based on the ability of the pearl surface to reflect and refract light, whilst complexion was graded on how many blemishes were present.

Nacre deposition (mm) and weight (g) were estimated by subtracting the initial implanted nucleus size and weight from those of the harvested pearls respectively. Nacre growth and weight differences and the effect of the host and donor on these traits were evaluated using a two-way ANOVA. Assumptions of ANOVA were assessed by the Shapiro–Wilk test for normality of residuals and Levene's test for homogeneity of variance. Where ANOVA demonstrated significant differences among groups, a Tukey's HSD post hoc test was applied to identify heterogenous subsets within the data. Log linear analyses were used to examine the effect host and donor oyster had on the categorical traits, pearl complexion, lustre, shape and colour. Correlations between pearl quality traits were assessed using a Spearman's rank test. All statistical analyses were performed using the S-Plus 8.0 statistical software program (Insightful Corporation).

2.3 Results

2.3.1 Nuclei retention

Nuclei retention rates did not differ significantly among the four groups ($\chi^2 = 5.64$, df = 3, $P > 0.05$) suggesting similar retention between allografted and xenografted oysters. The SS group had the highest retention rate at 83%, whereas all other groups had a similar lower retention rate from 61-63% (BB: 63%; BS: 61%; SB: 62%). There was also no bias caused by individual donor oysters on nuclei retention within each group (Fisher's exact test, $P > 0.05$).

Mortalities between the two host oyster species were different with 31% of *P. margaritifera* and 10% of *P. maxima* dying throughout the experiment. These mortalities are within the ranges often observed in the culture of these two species (Table 2.1).

Table 2.1: Mortality and nuclei retention rates post operation for allograft and xenograft *P. margaritifera* and *P. maxima* hosts and comparisons to previous published studies.

Species	**Mortality**	**Nuclei retention**	**Source**
P. margaritifera	Up to 30%	60%	Strack (2006), Cochennec-Laureau et al. (2010).
	31%	63%	This study
P. maxima	2-10%	70-90%	Taylor and Strack (2008)
	10%	83%	This study
P. margaritifera host with *P. maxima* donor (BS)	21%	61%	This study
P. maxima host with *P. margaritifera* donor (SB)	7%	62%	This study

2.3.2 Nacre growth

Nacre growth was significantly different among the groups (Table 2.2). The mean size of pearls produced from the SS control was significantly larger (46%) than those from the BB control (Fig. 2.2). When *P. margaritifera* was the host oyster the xenograft treatment (BS) produced 61% more nacre growth than that seen in the comparable allograft (BB), and 28% more growth than in the *P. maxima* allograft (SS). Conversely the xenograft where the host oyster was *P. maxima* (SB) grew 48% less nacre than that of the SS allograft (Fig. 2.2). Overall, it was found that the donor oyster species had a significant influence on nacre

growth, with no significant effect of the host species, nucleus size, or host/donor interaction evident (Table 2).

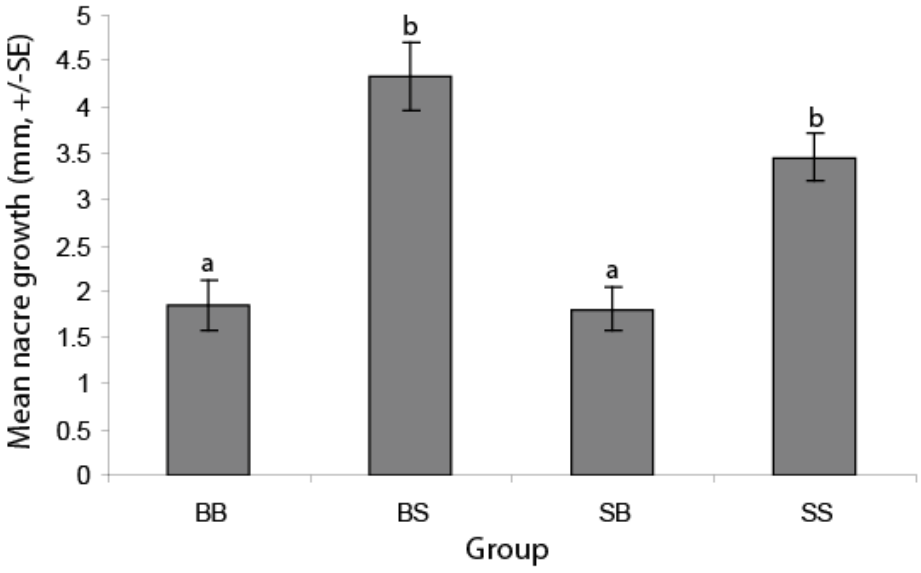


Figure 2.2: Mean nacre growth (mm, ±SE) (after adjustment for nucleus size implanted) of pearls resulting from allografts and xenografts into host *P. margaritifera* (black-lip oyster) and *P. maxima* (silver-lip oyster). See Fig. 2.1 or the text for group category explanation. Different superscripts in Fig. represent heterogeneous groups ($p < 0.05$).

2.3.3 Nacre weight

Nacre weight was significantly different among the groups (Table 2.2). The mean weight of pearls produced from the SS control was significantly heavier (57%) than that of the BB control (Fig. 2.3). When *P. margaritifera* was the host oyster the xenograft treatment (BS) produced 62% heavier nacre than that seen in the comparable allograft (BB), and 10% heavier nacre than in the *P. maxima* allograft (SS). Conversely, the nacre weight of the

xenograft, where the host oyster was *P. maxima* (SB), was 54% less than that of the SS allograft (Fig. 2.3). Similarly to nacre growth the donor oyster species had a significant influence on nacre weight, with no significant effect of the host species, nucleus size, or host/donor interaction evident (Table 2.2).

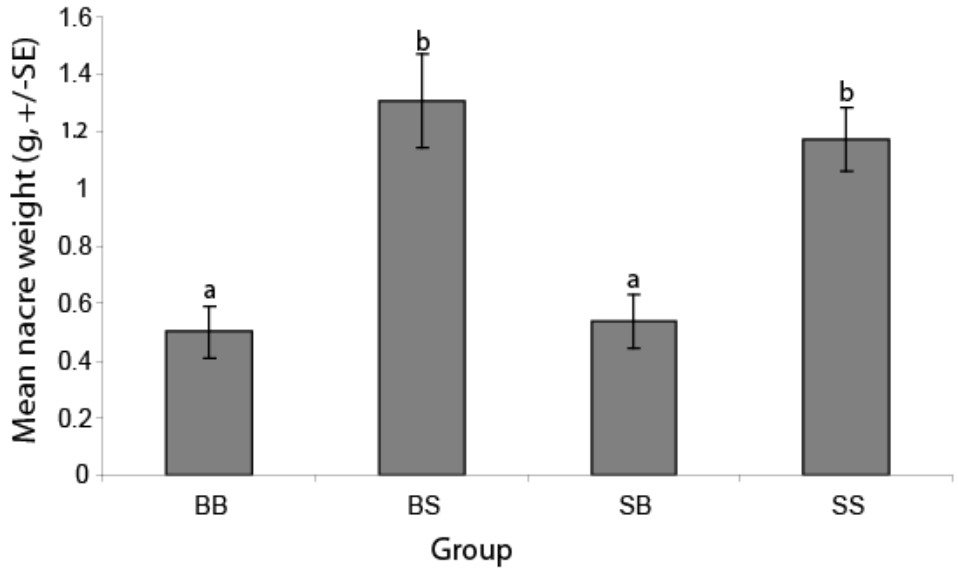


Figure 2.3: Mean nacre weight (g, ±SE) (after adjustment for nucleus weight implanted) of pearls resulting from allografts and xenografts into host *P. margaritifera* (black-lip oyster) and *P. maxima* (silver-lip oyster). See Fig. 2.1 or the text for group category explanation. Different superscripts in the figure represent heterogeneous groups ($p < 0.05$).

2.3.4 Pearl colour

Significant differences were observed in the proportion of the various coloured pearls produced amongst the four groups (Table 2.2). Analysis of the contribution of the host and donor oysters demonstrated that donor oyster species significantly influences this quality

trait, while the host oyster species appears to have little impact (Table 2.2). Here it was observed that in the majority of cases when a *P. maxima* donor was used the resulting pearl exhibited silver base colours consistent with that of *P. maxima*, whilst *P. margaritifera* donors produced coloured pearls consistent with that of *P. margaritifera*, regardless of the host oyster species (Fig. 2.4).

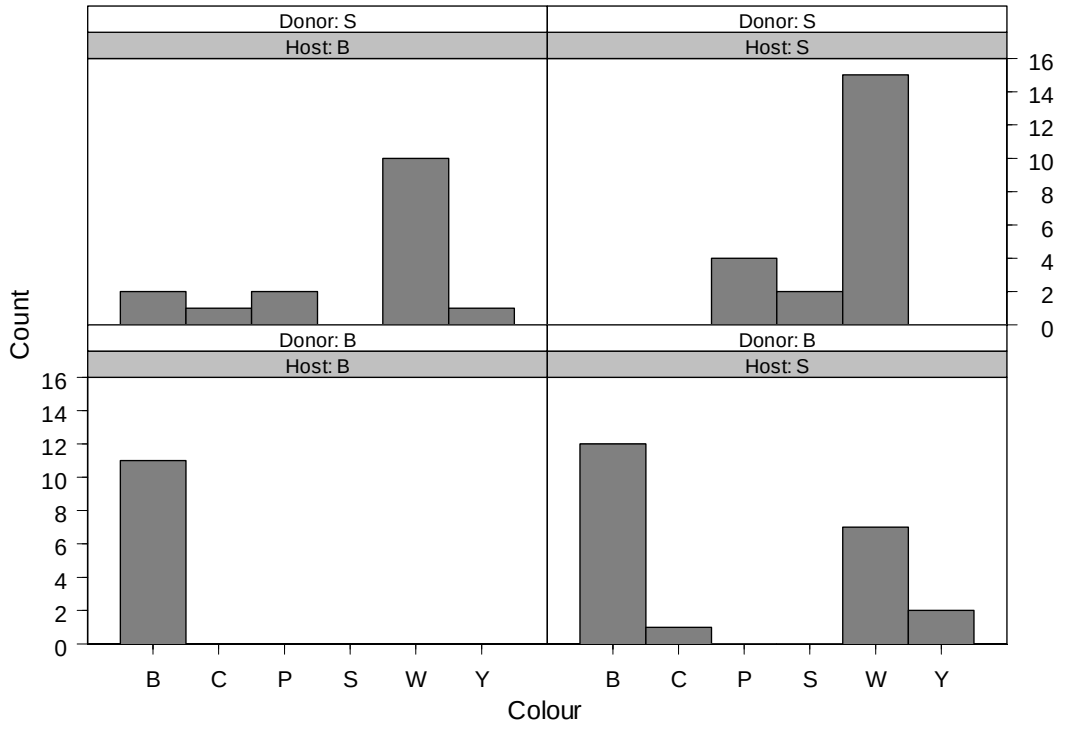


Figure 2.4: The various coloured pearls (B: Black, C: Cream, P: Pink, S: Silver, W: White, Y: Yellow) produced as a result of mantle xenografts and allografts in *P. margaritifera* (black-lip oyster (B)) and *P. maxima* (silver-lip oyster (S)) host oysters.

2.3.5 Pearl shape

Significant differences in pearl shape were present among the different groups (Table 2.2). The frequency of different shaped pearls was influenced by the host oyster species, whereas

the donor species had little influence (Table 2.2). *P. maxima* hosts produced a higher proportion of round pearls than those of *P. margaritifera*, regardless of the donor species used (Fig. 2.5). Caution needs to be exercised in interpreting this result however, as pearl shape may have been significantly influenced by the ability of the technician to implant the nucleus into the gonad of *P. margaritifera*, a species they do not normally operate on at the commercial farm used. Recent heritability estimates for *P. maxima* indicate that shape exhibits low levels of additive genetic variance suggesting that other factors like implantation technique may dramatically influence the shape of the pearl (Jerry, unpublished data).

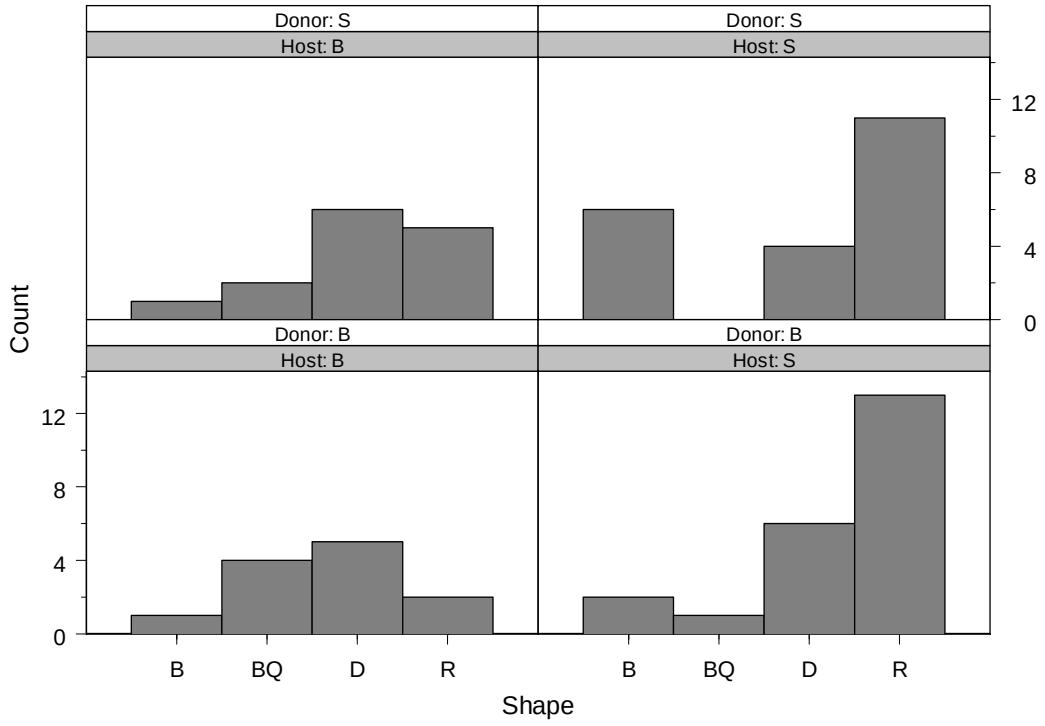


Figure 2.5: The various shaped pearls (B: Button, BQ: Baroque, D: Drop, R: Round) produced as a result of mantle xenografts and allografts in *P. margaritifera* (black-lip oyster (B)) and *P. maxima* (silver-lip oyster (S)) host oysters.

2.3.6 Pearl complexion

The grade of pearl complexion was significantly different among the various groups (Table 2.2). The effect of donor species on pearl complexion was highly significant, whereas the host species had no apparent influence on this pearl quality trait (Table 2.2). However, this trend appears to be largely driven by the SB group where a larger proportion of lower grades were observed for this trait (Fig. 2.6). *P. maxima* where used as the donor generally produced a higher complexion grade pearl than that of *P. margaritifera* donors (Fig. 2.6).

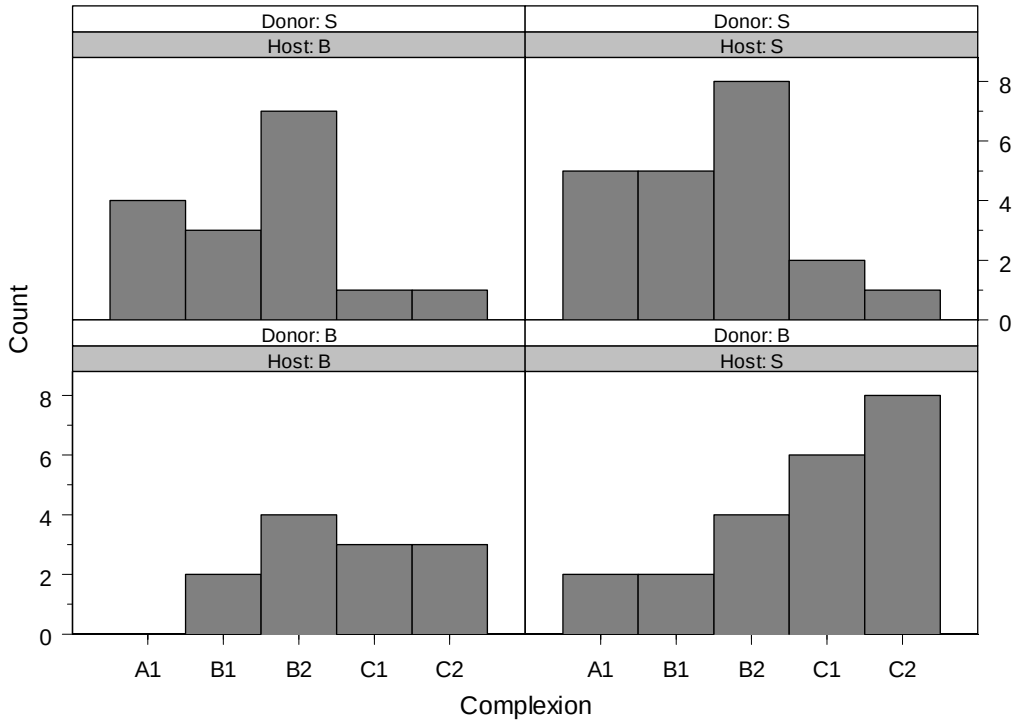


Figure 2.6: The various pearl complexion grades (ordered from highest (A1) to lowest (C2) grade) produced as a result of mantle xenografts and allografts in *P. margaritifera* (black-lip oyster (B)) and *P. maxima* (silver-lip oyster (S)) host oysters.

2.3.7 Pearl lustre

No significant differences in pearl lustre grades were evident amongst the various groups, or due to the host or donor species (Fig. 2.7, Table 2.2).

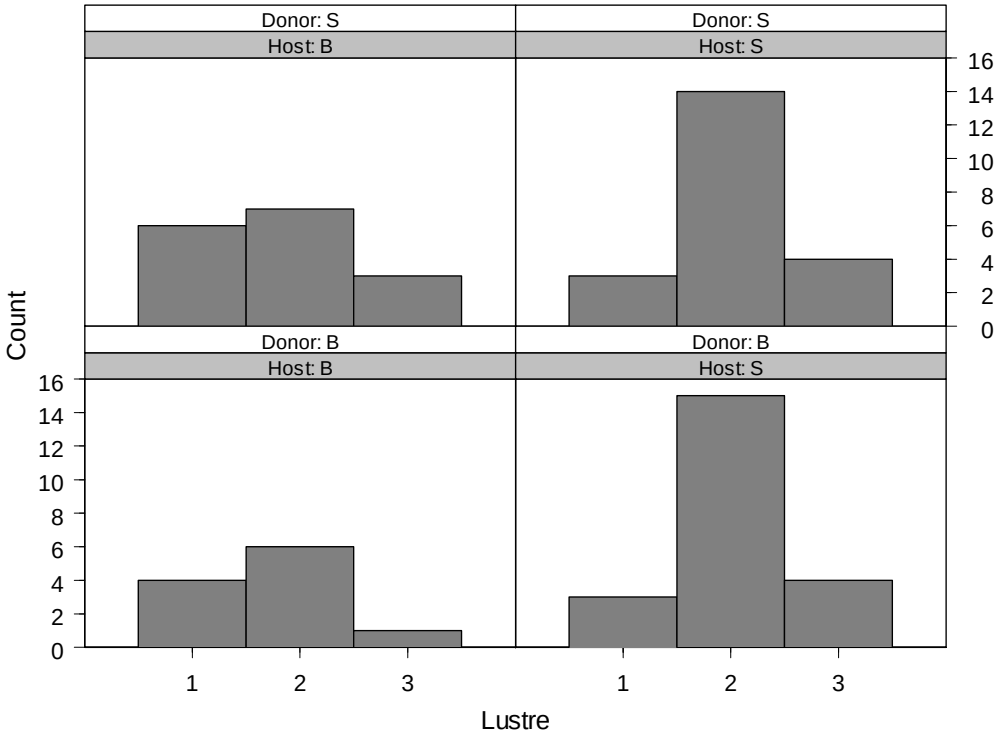


Figure 2.7: The various grades of pearl lustre (ordered from highest (1) to lowest (3)) produced as a result of mantle xenografts and allografts in *P. margaritifera* (black-lip oyster (B)) and *P. maxima* (silver-lip oyster (S)) host oysters.

Table 2.2: Analysis of variance and log linear statistics outlining the effect of mantle grafts, host and donor oyster, and implanted nucleus on the pearl quality traits size, weight, shape, colour, complexion and lustre.

Trait	**Independent Variables**	**Deviance**	***F***	***df***	**Sig.**
Size	Treatment	-	19.1	3, 67	***
	Host	-	3.19	1, 67	ns
	Donor	-	50.33	1, 67	***
	Nucleus size	-	0.02	1, 67	ns
	Host: Donor	-	1.86	1, 67	ns
Weight	Treatment	-	14.71	3, 64	***
	Host	-	0.27	1, 64	ns
	Donor	-	41.73	1, 64	***
	Nucleus weight	-	0.3	1, 64	ns
	Host: Donor	-	0.18	1, 64	ns
Shape	Treatment	19.46	-	9, 67	*
	Host	5.97	-	3, 67	**
	Donor	1.76	-	3, 67	ns
	Host: Donor	0	-	3, 67	ns
Colour	Treatment	56.82	-	15, 67	***
	Host	52.45	-	5, 67	ns
	Donor	3.31	-	5, 67	***
	Host: Donor	0	-	5, 67	ns
Complexion	Treatment	21.72	-	12, 67	*
	Host	1.35	-	4, 67	ns
	Donor	17.86	-	4, 67	***
	Host: Donor	2.52	-	4, 67	ns
Lustre	Treatment	5.41	-	6, 67	ns
	Host	4.52	-	2, 67	ns
	Donor	0.38	-	2, 67	ns
	Host: Donor	0.51	-	2, 67	ns

ns = not significant at $p>0.05$, * = significant at $p<0.05$, ** = significant at $p<0.01$, *** = significant at $p<0.001$

2.3.8 Correlations between traits

Significant correlations were found between some of the pearl quality traits. Pearl complexion had a weak positive relationship with pearl lustre ($r_s = 0.27$, $P < 0.05$), whilst pearl complexion was negatively correlated with both nacre growth ($r_s = -0.55$, $P < 0.001$)

and nacre weight (r_s = -0.61, $P < 0.001$). As expected, nacre growth had a strong positive relationship with nacre weight (r_s = 0.97, $P < 0.001$).

2.4 Discussion

This study used xenografts from two *Pinctada* pearl oyster species, *P. maxima* (silver-lip) and *P. margaritifera* (black-lip), to a) examine if xenografts can produce a viable pearl and, if so, whether there are differences in quality between pearls originating from allografts or xenografts, and b) further our understanding of the contribution donor and host oysters respectively have in pearl formation. In our study we have shown that the use of xenografts did not significantly influence nuclei retention, but did affect pearl quality and that the donor oyster plays a significant role in realisation of several pearl quality traits.

2.4.1 Nacre deposition

The rate of nacre deposition is an important factor potentially influencing several pearl quality traits. Generally the greater the nacre deposition rate for oysters implanted with the same size nuclei the larger the resultant pearl should be and the higher the pearl value (given consistency in the other quality traits). Consequently there may be advantages to the pearling industry in using oysters which exhibit rapid nacre deposition. In our study, we observed differences in the level of nacre deposition (as measured by nacre thickness and weight) among the various xenograft and allograft groups. Although not significant, *P. margaritifera* hosts implanted with a xenograft (BS) produced pearls with thicker nacre when compared to both species allografts. The ultimate causes of these differences in nacre deposition rate are unknown. *P. margaritifera* is an energetic bivalve which maximises energy gain under low

suspended particulate matter (SPM) concentrations (<3 mg l¯1) (Yukihira et al., 1998). Yukihira et al. (1998) reported significantly higher algal clearance rates compared to *P. maxima* under low SPM. If there was a low SPM count over the period these oysters were grown, the higher energy gain from *P. margaritifera*, combined with the high biomineralisation capabilities observed in *P. maxima* (Kono et al., 2000; Müller, 1997; Strack, 2006), may have contributed to the greater nacre deposition we observed in the BS xenograft. Further environmental tests are required to test the levels of SPM in the water at the time of this experiment to investigate this theory. Alternatively, increased nacre deposition may have resulted from a positive interaction effect when these two oyster genotypes are simultaneously expressed in the pearl sac (similar to that of heterosis when two species are hybridised). Given the differences in nacre deposition observed here and the importance of pearl size to the value of a pearl, further use of xenografts should be explored as an approach to increase overall pearl size in these two species. However, caution needs to be exercised on the possible effect fast nacre deposition may have on other pearl quality traits (discussed below).

2.4.2 Pearl colour

Based on our results, the mantle tissue derived from the donor oyster appears to be one of the most critical determinants of pearl colour. In our study we found that the distinctive base colours of the donor oyster from each of the *Pinctada* species were reflected in the colour of the pearl produced. This finding was most salient when mantle tissue from the black-lip pearl oyster was implanted into silver-lip hosts. Generally the pearl produced from this xenograft had a black base colour, a colour not seen in the host species. Similarly, in the reciprocal xenograft silver-based pearls were primarily produced. Although research on colour

determination in pearls is limited, those few studies which have addressed aspects of pearl colour have similar findings to this current thesis and suggest a tendency for shell nacre colouration in the donor oyster to influence overall pearl colour (Alagarswami, 1987a; Taylor, 2002; Wada and Komaru, 1996). For example, Wada and Komaru (1996) observed an association between donor shell and pearl colour in Akoya pearl oysters, *Pinctada fucata martensii,* whereby the frequency of non-yellow pearls in a harvest was lowered through the implantation of mantle tissue derived from inbred white shelled oysters, rather than using brown shelled donors. However, separation of the donor contribution to pearl colour from that of host oysters in the above study and other similar experiments have been inconclusive to date due to implantations being based on allografts involving same nacre coloured hosts. The use of xenografts involving two species which produce distinctively different base-coloured pearls is an effective approach to elucidate the relative contribution of host and donor oysters to pearl colour. Our results show conclusively for the first time that the donor oyster is the primary determinant of pearl colour.

2.4.3 Surface complexion and its correlation to growth

The use of different mantle donor species had a significant impact on pearl surface complexion. Generally, implantation with *P. maxima* mantle tissue produced pearls with smoother complexions (i.e., higher grades) than that of *P. margaritifera* donor tissue, regardless of the host oyster species. However, across all grafts a negative correlation between nacre deposition and surface complexion was observed. This suggests that techniques to improve nacre deposition have potential to adversely impact on other pearl quality traits such as complexion. As with any genetic improvement method, a strong understanding of genetic correlations is necessary to avoid inadvertent selection against non-

target traits, and accordingly, the use of xenografts to improve nacre deposition should be further evaluated for their effect on pearl complexion and other traits.

The negative correlation between nacre deposition and complexion observed, however, may not be purely restricted to xenografts, as negative correlations between these two traits has also been noted within the scientific literature (Alagarswami, 1987b; Snow et al., 2004). At a practical level, pearl farmers also appear to be aware of the impact of rapid nacre deposition on pearl quality and restrict pearl harvest to winter when nacre deposition is at its slowest. This practice is believed to produce pearls with an overall smooth complexion and high lustre. While the underlying factors leading to pearls with poor surface complexions are yet to be identified, Snow et al. (2004) hypothesise that pearls with a smooth surface and brilliant lustre are produced when consistent and regular crystal formation occurs. Nacre-based crystal formation is a complex biomineralisation process involving numerous genes (Kono et al., 2000; Miyamoto et al., 1996, 2005; Miyashita et al., 2000; Norizuki and Samata, 2008; Samata et al., 1999; Shen et al., 1997; Sudo et al., 1997; Suzuki et al., 2009; Tsukamoto et al., 2004; Wang et al., 2009; Zhang et al., 2003). Therefore, while xenografts have potential as a rapid way to improve individual pearl quality traits such as growth, the use of multi-trait selection approaches which take into account quantitative genetic control and associated negative correlations may be on the whole a more effective strategy to improve pearl quality and value. What is still required before this type of selection can proceed, however, are accurate estimates on the additive genetic contribution of donor and host oysters to the various pearl quality traits.

2.5 Conclusion

The use of xenografts is a novel approach to help us understand the complex process involved in the realisation of a gem quality pearl. Here xenografts have proven particularly useful in highlighting the importance of the donor oyster to the realisation of pearl quality traits, predominantly colour. They also have potential to provide rapid improvements in pearl growth. However, further research is needed prior to the use of xenografts in commercial pearl aquaculture, in particular, more information is required on their effect on overall pearl value.

Chapter 3 Diagnostic genetic markers unravel the interplay between host and donor oyster contribution in cultured pearl formation

3.1 Introduction

Cultured pearl production involves a complex biological process that is not yet completely understood. Cultured pearl production involves surgical implantation of a mantle allograft originating from a donor oyster along with a shell bead nucleus into the gonad of a recipient oyster from the same species (termed the host oyster). The donor mantle tissue then degenerates to a single epithelial cell layer, which proliferates around the seed nuclei to form a pearl sac (Kawakami, 1952a, b). Pearl sac epithelia begin to secrete shell matrix proteins, which subsequently deposit $CaCO_3$ for pearl formation up to two weeks post pearl sac development (Machii and Nakahara, 1967). Due to cell differentiation during the initial stages of pearl sac development, the fate of the mantle graft cells and their exact role in pearl formation cannot easily be determined by classical histological methods (Kawakami, 1952a, b; Herbaut et al., 2000; Cochennec-Laureau et al., 2010), leaving a lack of resolution as to the genetic makeup of the pearl sac at pearl harvest.

Until recently, it was unknown if donor oyster mantle cells actually persisted in the pearl sac until the time of pearl harvest, or whether they were rejected in the initial immunological response from the host during pearl sac formation. Evidence from genotyping the pearl sac using diagnostic microsatellite genetic markers in *Pinctada margaritifera* has shown that

DNA originating from the donor oyster as well as host oyster cells can be detected in the pearl sac at pearl harvest (Arnaud-Haond et al., 2007). The presence of the host cells detected in pearl sac samples could be the result of contamination from surrounding tissue. However, because the precise genetic make-up of the pearl sac has yet to be determined, the role of donor and host cells (i.e. whether the cells from the donor or host oyster are transcriptionally active in the biomineralisation process) and their respective contribution to pearl development remains unknown. The importance of the donor oyster to the realisation of pearl quality traits like colour has also been shown through examination of the phenotype of pearls produced from xenografting two distinct coloured pearl oyster species, *P. maxima* and *P. margaritifera* (Chpt 2, Section 2.3.4). Here, *P. maxima* host oysters seeded with mantle tissue from the black-lip pearl oyster *P. margaritifera* produced black-coloured pearls – a colour not seen in pearls produced in pure culture of *P. maxima*. The isolation of mantle donor specific DNA and realisation of phenotypic traits related to the donor oyster such as nacre colour, strongly suggest that the donor oyster cells are actively involved in cultured pearl biomineralisation processes. However, to date no study has shown that donor mantle cells remain transcriptionally active in the pearl sac until pearl harvest.

Numerous proteins regulating nacreous shell matrix secretion have been identified in the mantle tissue of the pearl oyster [e.g. N66 (Kono et al., 2000), Nacrein (Miyamoto et al., 1996, 2005), P10 (Zhang et al., 2006), N19 (Yano et al., 2007), N16 (Samata et al., 1999)], with some being found to be localised specifically to the mantle tissue and not present in gonadal tissue (Wang et al., 2009). Therefore, if donor oyster mantle cells are expected to remain transcriptionally active in the pearl sac, these tissue specific genes may be good biomarkers to identify whether the donor or host oyster cells are transcriptionally active and

contribute to the biomineralisation process. Because we know these genes are not present in gonad tissue (Wang et al., 2009), expression of these genes from the host oyster in the pearl sac would not be expected, unless a form of cell potency is occurring whereby the host oyster cells are taking on a functional role similar to that of mantle cells.

One of the biggest impediments to determining whether the donor or host cells are transcriptionally active for biomineralisation genes in the pearl sac is discriminating between the gene products of the two potentially contributing oysters. Currently there is insufficient information on levels of intra-specific polymorphisms in putative biomineralisation genes to characterise gene products that may be derived from the host and/or donor oysters. One approach that may resolve these problems of lack of intra-specific polymorphisms in putative biomineralisation genes is to use xeno-grafted mantle tissue from two closely related species where inter-specific gene sequence differences are present. Mantle grafts between two species, *P. maxima* and *P. margaritifera* (so called xenografts), have been shown to result in pearl sac formation and pearl development (Chpt 2, Section 2.3.1). If species-specific gene differences are present for biomineralisation genes, then the use of inter-specific xenografts and the examination of what species-specific biomineralisation gene homologue is being expressed in the pearl sac will be a powerful, novel, approach in determining if the donor oyster cells remain transcriptionally active through pearl development.

We tested the hypothesis that cells originating from the mantle tissue of the donor oyster not only remain in the pearl sac during development as previously found (Arnaud-Haond et al., 2007), but that they also are actively engaged in pearl formation through the expression of biomineralisation genes. We did this through designing *P. maxima* and *P. margaritifera*

species-diagnostic primers for two biomineralisation genes believed to be involved in nacre formation, N66 (Kono et al., 2000) and N44 (GenBank Accession No. FJ913472.1), and examining if both DNA and mRNA gene products from the donor species could be detected in the pearl sac when it was used as a xenograft.

3.2 Materials and Methods

3.2.1 Experimental animals

A description of the experimental animals used in the current chapter and the technique used to produce allografted and xenografted oysters is reported in Chpt 2, Section 2. The xenografted oysters were able to form pearl sacs and produce pearls, therefore, the pearl sac, mantle and gonad tissue were extracted (on the same day over a 6 h period) from each of the allografts and xenografts at the time of pearl harvest (14 months post operation) and preserved in RNAlater (Ambion^{TM}) held at 4ºC for 24 h then stored at -20°C.

3.2.2 Extraction of nucleic acids and cDNA generation

Genomic DNA was extracted from the pearl sac and mantle tissue of eight oysters from each of the allografts (Ss, Bb) and nine oysters from the xenograft when *P. maxima* was the donor oyster (Bs), using a high throughput extraction procedure described by Lind et al. (2009).

3.2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from the pearl sac of both allografts [Ss (*n*=10), Bb (*n*=10)] and both xenografts [Bs (*n*=10), Sb (*n*=10)] and the gonads of both allografts from the same

individuals in which the pearl sac was excised [Ss (*n*=10), Bb (*n*=10)]. RNA was extracted by dissecting the inner layers of the pearl sac and homogenizing 10-100 mg of tissue in 1 ml of Ultraspec RNA (Biotecx, Houston, TX). RNA was then separated from the organic material (DNA and protein) using 0.1 volume 1-bromo-3-chloropropane and precipitated by adding 0.5 volumes isopropyl alcohol and 0.5 volumes RNA precipitation solution (1.2 M sodium chloride, 0.8M disodium citrate) (Biotecx, Houston, TX; Sambrook and Russell, 2001). The quality of RNA and presence of contamination (inorganic material, genomic DNA) was examined visually by inspection of the total RNA (incorporating mRNA and rRNA) on an agarose gel (at a total RNA concentration of 200 ng/µl). RNA contamination was also measured by absorbance ratios $OD_{260/280}$ (RNA range: 1.95-2.08) and $OD_{260/230}$ (Commonly pure range: 2-2.2) on a Nanodrop spectrophotometer (Nanodrop Technology, Wilmington, DE). RNA was quantified by absorbance at 260 nm on the Nanodrop spectrophotometer.

To reduce any residual traces of DNA contamination a total of 10 µg of total RNA for each sample was treated with a Turbo DNA-free kit (Ambion, Austin, TX), including a sodium acetate precipitation. First strand complementary DNA (cDNA) was synthesized from approximately 2-3 µg of DNAse treated RNA using Superscript III first-strand synthesis supermix with 50 µM oligo(dT)20 (Invitrogen, Carlsbad, CA, USA). The 20 µl cDNA reaction involved incubation at 50°C for 50 min followed by deactivation at 85°C for 5 min. For verification of complete DNA removal, –RT controls were created by splitting one sample per treatment (group or tissue type) in half and performing a cDNA synthesis reaction with the remaining half without the reverse transcriptase. The RNA strand was digested from all cDNA synthesis reactions using 1 µl of RNAse cocktail (Ambion, Austin, TX). An aliquot

of each cDNA sample was diluted to a final standardized concentration of 2 ng/µl prior to PCR analyses.

3.2.4 PCR of species diagnostic targeted biomineralisation genes

Initially, no species diagnostic gene sequence information was available for *P. maxima* and *P. margaritifera* for any candidate nacre genes. A species-specific forward primer, N66 3F (ATT AAA GGG TCA TTG CGA GAA C), was designed for *P. maxima* based on sequence variations in alleles found at the N66 domain between *P. maxima* and *P. margaritifera* (Kono et al., 2000) (unpublished sequence data). This forward primer was situated 700 bp from the 5' start of the N66 mRNA sequence. The reverse primer, N66-R2 (GAC GAT ACA TCC TCC GCT AAA G), was used as it has previously been shown to be conserved across *P. maxima* and *P. margaritifera* species (Smith-Keune and Jerry, 2009). A conserved primer set was also developed for the two species (N66 Fcons: GGC AAC AAT GGA AAC AAC GGA and N66 R2 primers) to verify that genomic DNA was amplifiable if present in all individuals used (unpublished sequence data). This forward primer was situated 770 bp from the 5' start of the N66 mRNA sequence. PCR primers for the analysis of the target gene N44 (N44 Fmarg: TAG TTG CTT TAA GGC ATG TGG AAG; N44 R: CCC GTT TCC ACC ATT GTT ACC AT) were developed using Primer3 v0.4.0 based on the assembly and characterization of the N44 gene. Raw sequences were attained from a next-generation mRNA sequencing database (Illumina GAII) for both *P. maxima* and *P. margaritifera* (unpublished sequence data). The forward primer was situated 223 bp and the reverse primer was located 1195 bp from the 5' start of the N44 mRNA sequence.

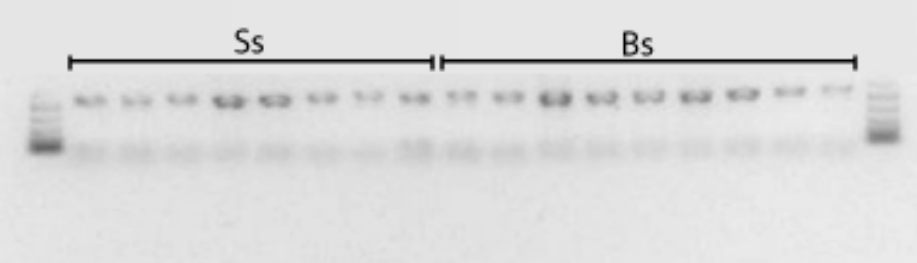
Polymerase chain reaction (PCR) was performed in 15 µl volumes with final concentrations of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1X PCR buffer**,** 0.5 U/µl of *Taq* DNA polymerase (Bioline) and 10 ng of gDNA or 4 ng of cDNA. The thermocycler program for both N66 and N44 began with an initial denaturation step at 95ºC for 5 min, 35 cycles of 95ºC for 45 sec, 57.5ºC for 30 sec, and 72ºC for 45 sec, followed by a final extension step of 10 min at 95ºC. PCR fragments were visualized on a 1.5% TBE agarose gel.

3.3 Results

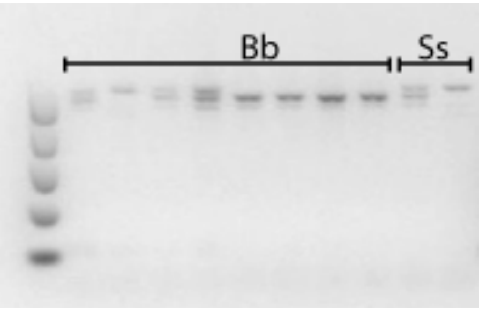
3.3.1 Pinctada maxima specific N66 genomic primers

P. maxima N66 species-specific primers (N66 Fmax and N66 R2 primers) produced a PCR product of expected size for all eight *P. maxima* allografts (Ss, Fig. 3.2a), whilst no specific amplification was detected in any of the eight *P. margaritifera* allografts (Bb, Fig. 3.2c). This demonstrates that a PCR product was only produced where *P. maxima* genomic DNA was present in the pearl sac tissue. A PCR product was produced for all nine xenografted pearl sacs where *P. maxima* was the donor oyster, with this set of N66 *P. maxima* specific primers (Bs, Fig. 3.2a). The viability of the DNA from *P. margaritifera* and *P. maxima* (D) allografts (Bb, Ss) was verified using a conserved primer set (N66 Fcons and N66 R2 primers) that amplifies in both species (Fig. 3.2b). Overall it was found that the donor oyster cells remain present in the pearl sac at the time of pearl harvest. No PCR product was present in the negative controls for both N66 Fcons, N66 R2 and N66 Fmax, N66 R2 primer sets.

3.2a) N66 Fmax and N66 R2 primers



3.2b) N66 Fcons and N66 R2 primers



3.2c) N66 Fmax and N66 R2 primers

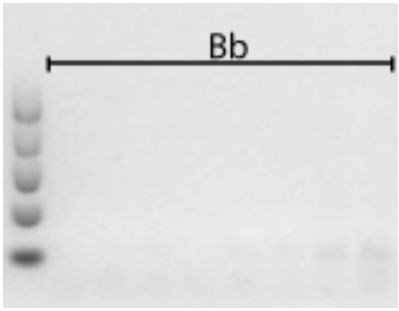


Figure 3.2: Electrophoretic 1.5% agarose gel showing presence or absence of PCR product for the nacreous gene, N66, in the pearl sac genomic DNA of *Pinctada maxima* and *Pinctada margaritifera* allografts and xenografts (standard bp sizes: 766, 500, 300, 150, 50). A species-specific primer set for *P. maxima* (N66 3F and N66 R2 primers) amplified eight *P. maxima* allografts (Ss) and nine xenografts where *P. maxima* was the donor oyster (Bs) (3.2a). A conserved primer set for the two species (N66 1F and N66 R2 primers) verified that DNA from eight *P. margaritifera* (Bb) and two *P. maxima* (Ss) individuals was viable (3.2b). To verify that the *P. maxima* primer set (N66 3F and N66 R2) was species-specific, these primers were applied to the DNA of the same eight *P. margaritifera* individuals used in 3.2b (Bb, 3.2c).

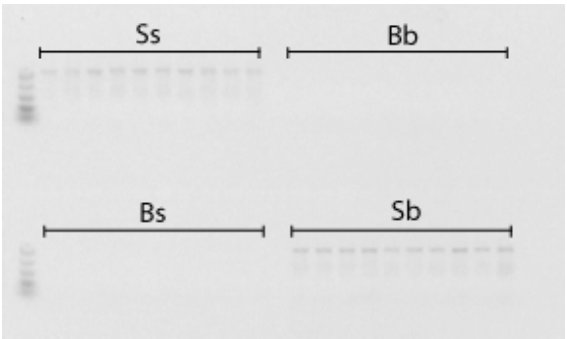
3.3.2 Pinctada maxima specific N66 transcriptomic primers

The *P. maxima* species-specific N66 primer set was only found to produce a PCR product where the N66 gene was expressed from *P. maxima* tissue (Fig. 3.3). The *P. maxima* primer set produced a cDNA PCR product for all 10 *P. maxima* allografts (Ss) and all 10 xenografts where *P. maxima* was the donor oyster (Bs). Whilst, no amplification occurred in any of the 10 *P. margaritifera* allografts (Bb) or any of the 10 xenografts where *P. margaritifera* was the donor oyster (Sb) (Fig. 3.3a).

3.3.3 Pinctada margaritifera specific N44 transcriptomic primers

P. margaritifera species-specific N44 primers were only found to produce a PCR product where the N44 gene was expressed from *P. margaritifera* tissue (Fig. 3.3). The *P. margaritifera* species-specific primers (N44 Fmarg and N44 R primers) produced a cDNA PCR product for all 10 *P. margaritifera* allografts (Bb) and all 10 xenografts where *P. margaritifera* was the donor oyster (Sb). Whilst, no amplification occurred in any of the 10 *P. maxima* allografts (Ss) or any of the 10 *P. maxima* xenografts where *P. maxima* was the donor oyster (Bs) (Fig. 3.3b).

3.3a) N66 Fmax and N66 R2 primers



3.3b) N44 Fmarg and N44 R primers

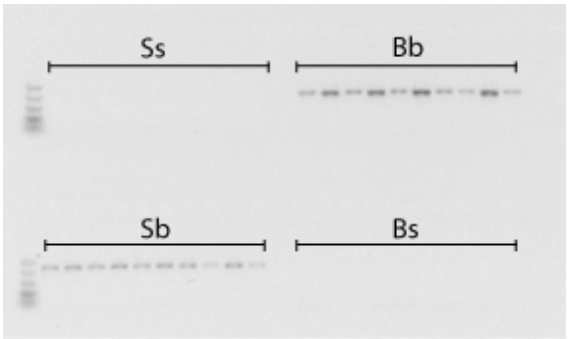


Figure 3.3: Electrophoretic 1.5% agarose gel showing presence or absence of PCR product for two nacreous genes, N66 and N44, in the pearl sac transcriptome of *Pinctada maxima* and *Pinctada margaritifera* allografts and xenografts (standard bp sizes: 766, 500, 300, 150, 50). The N66 *P. maxima* species-specific primer set (N66 Fmax and N66 R2 primers) and a N44 *P. margaritifera* species-specific primer set (N44 Fmarg and N44 R primers) were each applied to 10 *P. maxima* allografts (Ss; 3.3a and 3.3b), 10 *P. margaritifera* allografts (Bb; 3.3a and 3.3b), 10 xenografts where *P. maxima* was the donor oyster (Bs; 3.3a and 3.3b) and 10 xenografts where *P. margaritifera* was the donor oyster (Sb; 3.3a and 3.3b).

3.3.4 Data integrity

There was no specific amplification of nacre genes N66 and N44 in the gonad transcriptome or in the –RT samples. No PCR product was present in the negative controls for both N66 and N44 primer sets.

3.4 Discussion

This study produced xenografts from two *Pinctada* pearl oyster species to examine if the donor cells remain present in the pearl sac at pearl harvest and, if so, whether the donor or host cells remained transcriptionally active and contributed to pearl formation. Our study has not only confirmed that the cells from the donor oyster survived the initial host immunological response and persisted in the pearl sac, as shown by DNA analysis, but the donor cells were also transcriptionally active in secreting nacreous shell matrix proteins contributing to pearl formation.

The survival of grafted mantle tissue from another individual let alone another species through the entire process of pearl formation implies an exceptional rate of graft success and a complex biological cooperation between two unique genotypes in the biomineralisation process. The grafting procedure induces an immunological reaction in the host oyster, whereby cells differentiate making it difficult to track the different cells during pearl sac formation (Cochennec-Laureau et al., 2010). Accordingly, earlier histological studies were unable to clearly verify graft success and the persistence of the grafted donor cells (Herbaut et al., 2000). At the time of early histological studies (Herbaut et al., 2000), it was unknown whether the donor cells survived the immunological response of the host oyster and persisted to form the pearl sac or whether the host cells were responsible for pearl sac formation. Evidence from genotyping the pearl sac has shown that the donor oyster cells persisted in the pearl sac until pearl harvest. In the study by Arnaud-Haond et al. (2007), three co-dominant polymorphic microsatellite markers were used to screen for a foreign genotype in allografted pearl sacs compared with the host oyster muscle tissue. Through a novel approach of using a xenografted pearl sac containing the genome of two different species, our study supports the findings of this previous work. Our study found that species diagnostic gene sequences of the donor oyster were found in the pearl sac at harvest, supporting the hypothesis that donor cells are actively involved in pearl biomineralisation.

The persistence of the donor oyster DNA in the pearl sac supports the observations of professional grafters that some pearl phenotypes are influenced by the donor oyster. A few studies have found a tendency for shell nacre colour of the donor oyster to influence the overall colour of the pearl produced (Alagarswami, 1987a; Wada and Komaru, 1996; Taylor, 2002). The influence of the donor oyster on pearl quality traits was definitively demonstrated when the donor oyster was found to have a significant influence on pearl growth, colour and

surface complexion using xenografted oysters (Chpt 2, Section 2). This supporting information from previous studies showing the influence of the donor on pearl quality traits and the opinion of professional seeding technicians provides evidence that the DNA from the donor oyster is not only present, but is actively contributing to the biomineralisation process.

This present study detected two nacreous shell matrix protein gene transcripts from the donor oyster in the pearl sac, with no expression of these genes from the host oyster. No nacreous shell matrix protein transcripts were detected in the gonad tissues in our study which has also been confirmed for six other shell matrix proteins (Nacrein, EFCBP, N16, ACCBP, MSI60, N19; Wang et al., 2009), therefore, any detection of nacreous shell matrix protein transcripts in the pearl sac could then only be due to either secretions from the donor, or possibly host oyster gonad cells which have transformed into cells capable of biomineralisation (i.e. a form of cell potency). Due to cell differentiation following the grafting process, it has been difficult to track the cells from the host and donor oysters into pearl sac formation using histological methods (Herbaut et al., 2000). The host has the potential to produce shell matrix protein transcripts through the biological process of cell potency, whereby, the cells of the gonad may have the capacity to differentiate into different cell types (i.e. take on the functional role of mantle cells) as an immunological reaction to the introduction of mantle cells during the grafting process. A high proliferation rate and high content of saccharides in the central zone of mantle tissue, characteristic of stem cells, was found from an investigation into cell proliferation and differentiation in the mantle of *Pinctada fucata* (Fang et al., 2008). To date, however, there has been no stem cell research in invertebrates (including molluscs) and investigations into shell matrix protein expression in the pearl sac (Inoue et al., 2009, 2010; Wang et al., 2009) have yet to examine the potential for host cells to be contributing to

shell matrix protein secretion and therefore pearl formation. However, due to the species diagnostic sequences amplified in the xenografted pearl sacs in this study, the concept of cell potency has been ruled out in the pearl sacs analysed here for two nacreous shell matrix proteins. In this study, detection of species-specific transcripts confirmed the donor oyster was the only possible contributor to the secretion of nacreous shell matrix proteins N66 and N44, showing that the cells from the donor oyster are the likely cells actively contributing to the biomineralisation process and pearl formation. Although this idea of cell potency in the gonads of the host oyster is adventurous, it deserves further research using more shell matrix proteins to rule out some host contribution to pearl formation.

3.5 Conclusion

This study, through the use of xenografts, has clearly shown that the cells from the donor oyster remain in the pearl sac and are transcriptionally active for two genes (N66, N44) believed to be involved in pearl biomineralisation. Through the examination of what donor oyster species DNA was present in the pearl sac of xenografted oysters, we have shown that the cells from the donor oyster persist in the pearl sac until pearl harvest. What's more interesting, however, is that examining gene products from xenografts have shown for the first time that the cells originating from the donor oyster actively secrete nacreous shell matrix proteins and likely contribute to the biomineralisation process of pearl development. No evidence for host expression of these genes was found. Further research into host and donor expression of more than two nacreous shell matrix proteins would be beneficial in evaluating overall host and donor oyster potential for contributing to pearl formation.

Chapter 4 Transcriptome analysis of biomineralisation-related genes within the pearl sac: Host and donor oyster contribution.

4.1 Introduction

In molluscs the mantle epithelium is the tissue responsible for shell formation. The mantle creates the shell indirectly, with the mantle epithelium not touching the surface of calcification. Instead, the organic material (organic matrix) secreted by the mantle tissue is thought to be the regulator of shell calcification (Fougerouse et al., 2008). A number of proteins have been isolated from the organic matrix using biochemical and molecular approaches and their functions have been discussed based on their primary and predicted secondary structures, expression patterns and results from *in vitro* experiments (Miyamoto et al., 1996; Shen et al., 1997; Sudo et al., 1997; Samata et al., 1999; Kono et al., 2000; Mann et al., 2000; Miyashita et al., 2000; Weiss et al., 2001; Zhang et al., 2003; Tsukamoto et al., 2004; Gotliv et al., 2005). It is generally conceived that due to a pearl having the same nacre constitution as the inside of a pearl oyster shell and because a cultured pearl is produced by surgical implantation of a mantle allograft from a donor oyster, that the shell matrix proteins responsible for nacreous shell formation produced by the mantle are also responsible for pearl formation (Farn, 1986).

The relative genetic contribution from the donor and host oyster to nacre secretion, however, has not been defined. Until recently, the fate of the mantle allograft following the grafting process, where it degenerates to a single epithelial cell layer prior to pearl sac formation

around the nucleus, was uncertain. This was due to the immunological reaction of the host oyster causing early histological efforts to be unable to track the cells of the mantle tissue past the grafting process due to cell differentiation (Kawakami, 1952a, b; Herbaut et al., 2000; Cochennec-Laureau et al., 2010). However, genotyping the pearl sac using microsatellite genetic markers in *Pinctada margaritifera* recently confirmed that DNA originating from the donor oyster can still be detected in the pearl sac at pearl harvest (Arnaud-Haond et al., 2007). Also the influence of the donor oyster on pearl phenotypes such as colour has been shown through examination of pearl quality traits produced from xenografting two distinct coloured pearl producing species. Here, black-coloured pearls were produced from *P. maxima* (silver-lip) host oysters seeded with mantle tissue from *P. margaritifera* (black-lip) donor oysters, a colour not present in *P. maxima* pearls (Chpt 2, Section 3). Molecular work (Chpt 3, Section 3), has also shown through the use of xenografts in these two species, that two shell matrix proteins are expressed only by the donor oyster within the pearl sacs of *P. maxima* and *P. margaritifera*. The phenotypic evidence that pearl traits such as nacre colour are related to the donor oyster, and the molecular verification that the donor oyster expresses two shell matrix proteins (N44, N66) within the pearl sac at pearl harvest, demonstrates that the donor oyster cells are not only present throughout the pearl development process, but are also likely to be actively involved in cultured pearl formation. To fully elucidate the extent to which the donor oyster contributes to pearl formation, the origin of more biomineralisation-related genes expressed within the pearl sac needs to be examined.

One of the biggest impediments in determining whether biomineralisation genes in the pearl sac are transcriptionally derived from donor or host cells is being able to first identify all biomineralisation-related genes expressed in the pearl sac at pearl harvest. Previous

technology applied to examine the expression of biomineralisation-related genes has predominantly relied upon the examination of genes on an individual basis (e.g. through real time PCR) (Wang et al., 2009; Inoue et al., 2010). Recent developments in high-throughput mRNA sequencing using next-generation sequencing platforms now provides a potential way to simultaneously examine all biomineralisation genes that are being expressed at one time.

The second impediment in determining donor or host cell pearl biomineralisation gene activity is being able to discern the origin of gene transcripts. To date there is a lack of data on intra-specific polymorphisms in biomineralisation genes to allow the characterisation of gene products derived from individual oysters that were used as donors or hosts. One novel approach to resolve the problem of discriminating between gene products from the host and donor oysters that we use herein and in previous work (Chpt 3, Section 3) is to produce xeno-grafted pearl sacs from two closely related species where inter-specific sequence differences in homologous biomineralisation genes are present. Mantle grafts between two species, *P. maxima* and *P. margaritifera* (so called xenografts), have previously been shown to result in pearl sac formation and pearl development (Chpt 2, Section 2.3.1). Where species-specific gene differences are present between these species for homologous biomineralisation genes, then the use of xenografts can be used to unequivocally ascertain whether the host or donor cells are transcriptionally active for the relevant gene through detecting the species-specific transcript present.

In the present study we used high-throughput mRNA sequencing (Illumina GAII) derived from allografted *P. maxima* and *P. margaritifera* pearl sacs to detect putative biomineralisation genes expressed in pearl sac tissue. Based on sequence differences between

these two pearl oysters for homologous biomineralisation genes, we then identified single nucleotide polymorphisms (SNP) that were species-diagnostic for each gene. Finally, biomineralisation gene transcripts and associated species diagnostic SNPs from *P. maxima* /*P. margaritifera* xenografted pearl sacs were used to determine for the first time whether host or donor derived cells are primarily responsible for the expression of biomineralisation genes in pearl sac tissue.

4.2 Materials and Methods

4.2.1 Experimental animals

A description of the experimental animals used in the current chapter and the technique used to produce allografted and xenografted oysters is reported in Chpt 2, Section 2. At the time of pearl harvest the inner layers of the pearl sac were excised from host oysters (on the same day over a 6 hr period), along with gonadal tissue from separate oysters which had not been previously seeded with a pearl (*P. maxima* N=10 and *P. margaritifera* N=10). Tissue samples were preserved in RNAlater (Ambion^{TM}) stored at -20 °C.

4.2.2 RNA sequencing and transcriptome assembly

Total RNA was extracted from five oyster pearl sacs within each group (Ss, Bb, Sb and Bs) following the methods of Chpt 3, Section 2. Individual RNA from each group was then quantified and pooled together, and sent to a service provider for sequencing (Macrogen Inc, Korea) using Illumina RNA-seq 100 bp paired-end read length sequencing technology (http://www.illumina.com/systems/genome_analyzer_iix.ilmn). Each group was bar-coded and pooled prior to being sequenced on two channels. The sequencing generated more than

14GB of raw sequence data with 30-40 M sequence reads per group. *P. maxima* (Ss) and *P. margaritifera* (Bb) sequence data was assembled into contigs using ABYSS 1.20 (Simpson et al., 2009). Following initial parameter optimisation to maximise transcript coverage, the final assembly parameters incorporated a trim quality threshold q=15, k-mer size k=54, seed length s=200 and all other options at default settings. The resulting assemblies produced approximately 65,000 contigs (> 200 bp), N50 of ~500 bp and maximum contig length of ~7000 bp for each species.

4.2.3 Identification of biomineralisation-related proteins in Pinctada maxima and Pinctada margaritifera pearl sac EST library

Candidate genes that were most likely to be related to biomineralisation in *Pinctada* species were identified in closely related taxa from the literature or public online databases. In total 188 bivalve putative biomineralisation genes were indentified in the public domain. These 188 biomineralisation genes were then blasted against the Ss and Bb assembled sequence contigs to obtain a list of detectable gene transcripts expressed within the pearl sacs of both *P. maxima* and *P. margaritifera* (Blast-2.2.23+, E-value $\leq 10^{-3}$). Partial transcripts from 19 putative biomineralisation genes were detected within pearl sacs from these two species.

4.2.4 SNP design and in-silico SNP analysis

The ability to detect species specific biomineralisation transcripts is imperative when determining if the host and/or donor is contributing to pearl formation. To achieve this, the 19 biomineralisation genes identified within the pearl sacs of *P. maxima* and *P. margaritifera* were examined for the presence of species-diagnostic sequence variation. This was carried

out by first identifying all available raw sequence reads from both species that blast to the 19 biomineralisation gene sequences (Blast-2.2.23+, E-value $\leq 10^{-3}$). These raw sequence reads were then assembled together using MIRA v3.2.1 (http://sourceforge.net/projects/mira-assembler/) with optional parameters (-AL:egp=no, -CO:asir=yes) allowing for multiple strains/species sequences to be assembled and clustered together. A sequence contig assembly file (ace) incorporating both species assembled reads was generated and used to investigate species diagnostic variation (using the software SNPStation, http://code.google.com/p/snpstation/) by screening for fixed variation differences between the species reads, while also maintaining conserved flanking sequence within a species for primer/probe design. The diagnostic SNPs were then validated by screening against the full Ss and Bb raw sequence reads (i.e. some reads may have been excluded in contig assembly) as well as from other available independent data sets that used different sequencing technology (454 sequencing platform) for both *P. maxima* and *P. margaritifera*. The independent *P. maxima* sequence dataset comprised mantle tissue from 120 individual oysters containing 1.3 million sequence reads with an average sequence length of 340 bp (unpublished sequence data), whilst, the independent *P. margaritifera* data set was based on mantle tissue from 12 individual oysters and 276 738 sequence reads with an average sequence length of 234 bp (Joubert et al., 2010). To screen for SNPs within databases, a sliding window over 41 bp encompassing the SNPs was produced and a Linux grep script was used to extract exact sequence matches from databases.

Once validated, species diagnostic SNPs were examined in xenograft derived pearl sac transcripts (Bs, Sb) to identify the species responsible for expressing each biomineralisation gene. Through this approach we were able to unravel whether the host or donor oyster were

putatively genetically contributing to pearl nacre formation in pearl sac tissue through the expression of biomineralisation genes.

4.2.5 Validation of host and donor biomineralisation transcripts

Four biomineralisation genes showed transcripts to have originated from the host oyster based on the SNP analysis (MSI60, Calreticulin, Linkine and PfCHS1; Fig. 4.2). This may have resulted either because the pearl sac samples were contaminated with surrounding gonad cells that always expressed these genes, or because the host gonad cells within the pearl sac were specifically expressing these genes. To test which of these two possibilities was responsible for host transcripts detected, conserved PCR primers were designed that amplified regions encompassing the diagnostic interspecific SNPs in these four biomineralisation genes (Table 4.1). These conserved primers were first amplified from cDNA prepared as below (section 4.2.6) from pure gonadal tissue which had not been previously seeded with a pearl (*P. maxima* N=10 and *P. margaritifera* N=10). Two genes (MSI60, Calreticulin) were shown to be expressed in gonad tissue regardless of whether it had been seeded with a pearl nucleus. The remaining two genes (Linkine and PfCHS1) were not detectable in normal gonad tissue. To confirm the initial SNP data which indicated that the host oyster expressed these two genes in pearl sac, PCR was performed on individual pearl sacs (Ss N=2, Bb N=2, Bs N=5, Sb N=5) using conserved primers (Table 4.1). Following several attempts at PCR amplification the concentration of PfCHS1 was found to be too weak for sequencing, therefore, the PCR product for Linkine only was purified with an ammonium acetate (7.5 M) precipitation and sequenced in both directions at a commercial facility (Macrogen, Korea).

Table 4.1: *P. maxima* and *P. margaritifera* conserved primer sequences for four putative biomineralisation genes that amplify across species-diagnostic SNPs.

Primer name	**Primer sequence 5' – 3'**
MSI60_F	GAAGTCCTGATCCTGAGGAGGA
MSI60_R	CCCCCAACCCTAATGTTGTTAA
Linkine_F	TAGGACTTTGATTGCAGTCATG
Linkine_R	CTCTTCTTGAGTTTGCAGCAAT
Calreticulin_F	TAAGCAGATTGATAATCCAGCA
Calreticulin_R	CTCCCCATGTTTCTTTTCCTAC
PfCHS1_F	TTCGTCCATTTTGCTCTAATGA
PfCHS1_R	GAGTCGGAAGAGTGAACCACAG

4.2.6 cDNA synthesis and PCR conditions

First strand complimentary DNA (cDNA) was synthesised from extracted total RNA (Section 4.2.2) in pearl sac and gonad tissue samples using the methods reported in Chapter 3, Section 2. Polymerase chain reaction (PCR) was performed in 20 µl volumes with final concentrations of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 µM of each primer, 1X PCR buffer, 0.5 units of *Taq* DNA polymerase (Bioline) and 4 ng of cDNA. The thermocycler programme for MSI60, Calreticulin, Linkine and PfCHS1 began with an initial denaturation step at 94 ºC for 3 min, 35 cycles of 94 ºC for 30 sec, 53 ºC for 45 sec, and 72 ºC for 45 sec, followed by a final extension step of 2 min at 72 ºC. PCR fragments were visualized on a 1.5% TBE agarose gel.

4.3 Results

4.3.1 Biomineralisation genes expressed within the pearl sac

Putative molluscan biomineralisation genes were identified from public databases (N=188) to determine which genes were expressed within the pearl sac of *P. maxima* and *P. margaritifera* and potentially contributing to pearl formation. Of the 188 putative molluscan biomineralisation genes in public databases, 19 were expressed in the pearl sacs of allografted *P. maxima* and *P. margaritifera* (Table 4.2). More biomineralisation genes are potentially present, although, they are not seen in the transcriptome coverage of our sequence dataset. The majority of genes identified have been shown to be specifically linked to nacre formation (i.e. N14, N19, N33, N44, N66, Nacrein, Pearlin, PfCHS1, Pif177 and PMMG1).

Table 4.2: List of biomineralisation genes expressed in the pearl sacs of *P. maxima* and *P. margaritifera* and their putative function.

Candidate Gene	**Accession #**	**Function**	**Reference**
Calmodulin	AY341376.1	Calcium metabolism	(Li et al., 2004)
Calreticulin	EF551334.1	Calcium binding, Periostracum and Prismatic	(Fan et al., 2008)
Linkine	EF183520.1	SMP	(Joubert et al., 2010)
M45	AF513719.1	Expressed in mantle	
ML7A7	DW986406.1	Shell secretome	(Jackson et al., 2006)
MSI60	D86074.1	Nacre formation	(Takeuchi and Endo, 2006)
N14	AB032612.1	Nacre formation	(Kono et al., 2000)
N19	AB332326.1	Nacre formation	(Yano et al., 2007)
N33	FJ913471.1	Nacre formation	
N44	FJ913472.1	Nacre formation	
N66	AB032613.1	Nacre formation	(Kono et al., 2000)
Pearlin	AB020779.1	Nacre formation	(Miyashita et al., 2000)
Perline	DQ665305.3	SMP	
PfCHS1	AB290881.1	Nacre formation	(Suzuki et al., 2007)
Pif177	AB236929.1	Nacre formation	(Suzuki, 2009)

PMMG1	FJ386386.1	Nacre formation	(Wang et al., 2010)
Pmshem-1	AB429365.1	SMP	
Shematrin-8	EF160119.1	SMP	
Shematrin-9	EF160120.1	SMP	

SMP: Shell Matrix Protein

4.3.2 Validation of species diagnostic SNPs

When evaluating species-specific variation, there was no detection of non-target species sequence variation in either *P. margaritifera* or *P. maxima* sequence datasets. The average number of sequence reads that contained *P. maxima* diagnostic SNPs within this *P. maxima* database was 813 (±SE 27.8) and 270 (±SE 18.4) for the *P. margaritifera* SNPs within the *P. margaritifera* database. Furthermore, the evaluation of the SNPs used in this experiment on alternative sequencing datasets containing 120 and 12 different individuals for the *P. maxima* (unpublished sequence data) and *P. margaritifera* (Joubert et al., 2010) databases respectively (454 sequencing platform), provided further evidence that the inter-species SNPs from this study were indeed species diagnostic. The average number of sequence reads that contained *P. maxima* diagnostic SNPs within this other *P. maxima* database was 103 (±SE 9.15) and 62 (±SE 17.81) for the *P. margaritifera* SNPs within the *P. margaritifera* database.

4.3.3 Donor and host oyster contribution to biomineralisation gene expression

All putative biomineralisation genes (N=7) were found to be expressed by the donor oyster (Fig. 4.2). Three of these genes N66, Perline and N44, were solely expressed by the donor with no expression from the host oyster. Here, the *P. maxima* diagnostic SNPs only detected expression of N66, Perline and N44 in the xenografts where *P. maxima* was the donor oyster

(Bs) and the *P. margaritifera* diagnostic SNPs only detected expression from the xenografts where *P. margaritifera* was the donor oyster (Sb) (Fig. 4.2).

For four of the seven biomineralisation genes (Linkine, PfCHS1, MSI60 and Calreticulin), both donor and host oyster transcripts were detected within the xenografted pearl sacs (Bs, Sb; Fig. 4.2). Here, *P. margaritifera* SNPs detected expression of Linkine, PfCHS1, MSI60 and Calreticulin in the xenografts where *P. margaritifera* was the donor and host oyster (Sb, Bs) and *P. maxima* SNPs detected expression in the xenografts where *P. maxima* was the donor and host oyster (Bs, Sb), with the exception of Linkine (Fig. 4.2). Gene transcripts from Calreticulin and MSI60, however, were detected in gonad tissue samples from *P. maxima* and *P. margaritifera*. No specific amplification of Linkine and PfCHS1 transcripts were detected in the gonad samples.

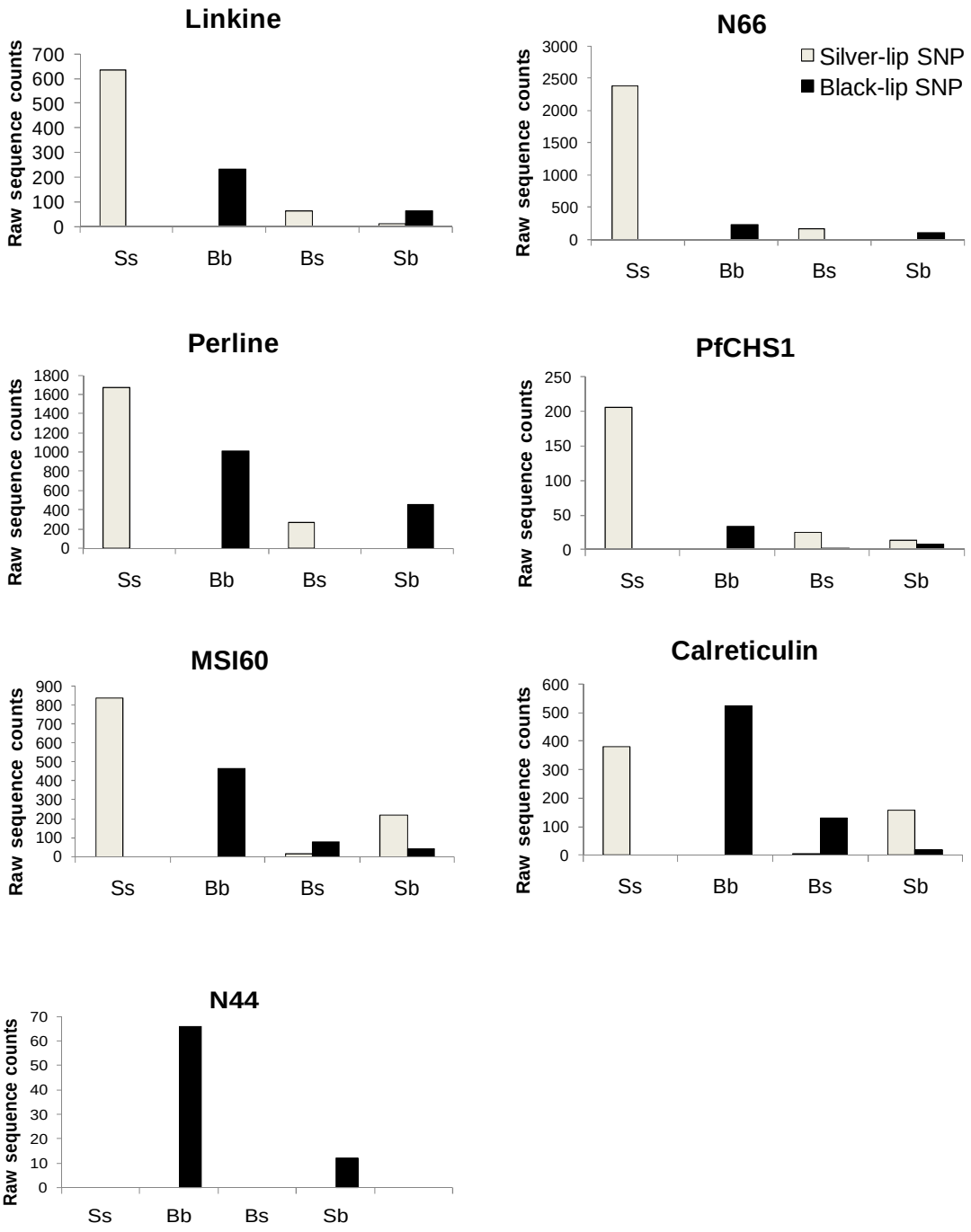


Figure 4.2: Raw sequence counts of species diagnostic SNPs for putative biomineralisation genes Linkine, N66, Perline, PfCHS1, MSI60, Calreticulin and N44 in pearl sac tissue from allografted (Ss, Bb) and xenografted (Bs, Sb) *P. maxima* and *P. margaritifera* (Illumina GAII mRNA sequencing).

4.3.4 Illumina GAIIx data integrity

To further confirm the expression of biomineralisation genes from the host oyster and to validate the sequencing data (Illumina GAII), a highly informative region (40 bp in length) of Linkine was sequenced that contained five known species diagnostic single nucleotide polymorphisms (SNPs). Individuals from the allografted pearl sacs (Ss, N=2; Bb, N=2) were also sequenced to validate that the SNPs were species diagnostic, followed by sequencing of individuals from the xenografted pearl sacs (Sb, N=5; Bs, N=5) to determine whether the host or donor oyster species diagnostic SNPs were present (Table 4.3). All *P. margaritifera* allografted pearl sacs (Bb) showed an A nucleotide at a particular SNP site, whilst *P. maxima* allografted pearl sacs (Ss) had a T nucleotide at the SNP site. All five xenografted pearl sacs, where *P. maxima* was the donor oyster (Bs), had a *P. maxima* diagnostic SNP (T). Whilst four of the xenografts where *P. margaritifera* was the donor oyster (Sb) possessed the *P. margaritifera* SNP (A). However, one of these xenografted pearl sacs where *P. margaritifera* is the donor possessed the *P. maxima* diagnostic SNP (T), suggesting that the host was expressing Linkine in this individual (Table 4.3). The other four diagnostic SNPs within the region sequenced for Linkine showed the same pattern as the above mentioned SNP site.

Table 4.3: Species diagnostic single nucleotide polymorphism (SNP) present in the gene Linkine for allografted *P. maxima* and *P. margaritifera* groups (Ss, N=2; Bb, N=2) and two xenograft groups, where *P. margaritifera* is the donor oyster (Sb, N=5) and *P. maxima* is the donor oyster (Bs, N=5).

Group	SNP
Bb $_{N=2}$	A
Ss $_{N=2}$	T
Bs $_{N=5}$	T
Sb $_{N=4}$	A
Sb $_{N=1}$	T

4.4 Discussion

This study used transcriptome profiling to a) identify putative biomineralisation genes expressed in the pearl sacs of *P. maxima* and *P. margaritifera* and, b) determine which of these genes originate from the host and/or donor oyster. Our study found 19 of the 188 putative molluscan biomineralisation genes to be expressed within the pearl sacs of *P. maxima* and *P. margaritifera*. For the first time, we also showed that the majority of biomineralisation gene transcripts are derived from the mantle tissue of donor oysters used in the pearl seeding. This suggests that the donor oyster is the main genetic contributor to the secretion of the necessary regulatory proteins governing pearl formation.

4.4.1 Pearl sac gene expression

This study presents the first comprehensive sequencing effort of a pearl sac for a pearl producing species. Through the use of high throughput Illumina GAII sequencing we were able to examine for the first time 188 putative biomineralisation genes expressed in the pearl sacs of *P. maxima* and *P. margaritifera* at pearl harvest and therefore potentially contributing to the biomineralisation process of pearl formation. Previous to this study, the expression of only nine putative biomineralisation genes had been identified within the pearl sac of a pearl

oyster species, *P. fucata* (MSI31, N16, Nacrein, MSI60, Prismalin-14, Aspein, EFCBP, ACCBP and N19). These studies compared expression patterns of these shell matrix proteins showing differences in expression levels within the pearl sac and between the pearl sac and mantle tissue (Inoue et al., 2009, 2010; Wang et al., 2009). In the present study, we found 19 putative biomineralisation genes similarly expressed in both species examined indicating little divergence in the biomineralisation processes of pearl formation between these two species. The closeness of these two species has been previously highlighted using nuclear internal transcribed spacer markers (Yu and Chu, 2006; Yu et al., 2006). However, the present study is the first to highlight that the process of pearl formation may be very similar between these two species.

4.4.2 Donor oyster expression

All detectable biomineralisation genes were expressed by the donor oyster tissue. This clearly demonstrates that the original donor mantle tissue survives the immunological response from the host oyster and actively secretes some of the necessary biomineralisation proteins that govern pearl formation. This confirms at a molecular level previous studies that have shown phenotypically that the donor is the main contributor to pearl quality traits, in particular colour and nacre deposition rate (Wada and Komaru, 1996; Chpt 2, Section 3). For example, through the use of xenografts involving two species which produce distinctively different base-coloured pearls, *P. maxima* and *P. margaritifera*, it was conclusively shown that the donor oyster is responsible for the colour of a pearl (Chpt 2, Section 2.3.4). It has also been shown on the molecular level through the use of species diagnostic markers that gene products from the donor oyster species could be detected for two nacreous shell matrix

proteins, N66 and N44, within the pearl sac of xenografted *P. maxima* and *P. margaritifera* (Chpt 3, Section 3).

Three of the seven genes found to be expressed by the donor oyster in this study were previously described as being specifically involved in the formation of the nacreous layer (N66 (Kono et al., 2000), N44 (Accession No. FJ913472.1) and MSI60 (Takeuchi and Endo, 2006)). This result is expected because the donor mantle tissue, which is excised for cultured pearl production, is taken from the pallial zone of the mantle which has been shown to secrete only the nacreous layer of the inner shell (Sudo et al., 1997; Takeuchi and Endo, 2006). Therefore, as a result of the donor tissue being excised from the pallial zone of the mantle tissue in this study, it can be concluded that the genes found to be expressed in the pearl sac by the donor oyster are related specifically to the formation of the nacreous biomineralisation layer. Additionally, only one of the two shell mineralised layers (i.e. calcite or nacreous aragonite layers) are being secreted in pearl formation, that of nacre. Very little is known about the specific functional role of most biomineralisation-related genes, with many shell matrix proteins yet to be localised to specific parts of the mantle which are known to be responsible for the secretion of the different layers of shell/pearl formation or extracted directly from these layers (periostracum, prismatic and nacre layers) (Fougerouse et al., 2008). According to Takeuchi and Endo (2006), MSI60 was found to be strongly expressed in the mantle pallial, concluding that this gene is related to nacreous layer formation. Our study supports this suggestion where MSI60 was found to be expressed by the donor oyster within the pearl sac, suggesting that because the donor tissue originated from the mantle pallial, MSI60 is related to nacreous layer formation. However, four of the seven biomineralisation-related genes found to be expressed by the donor oyster within the pearl sac of *P. maxima* and *P. margaritifera* (Calreticulin, Linkine, PfCHS1 and Perline), have yet

to be defined as contributing to nacreous layer formation. Calreticulin for example, showed strong hybridization signals in the inner fold, middle fold and outer fold of the mantle edge, a zone that is known to secrete the periostracum and prismatic layers, through in situ hybridization of PCRT mRNA in mantle tissue (Fan et al., 2008). In our study, Calreticulin was found to be expressed by the donor oyster within the pearl sac at pearl harvest. Therefore it can be surmised that Calreticulin also may play a role in the secretion of the nacreous layer. Through identifying biomineralisation-related genes expressed by the donor oyster from xenografted pearl sacs of *P. maxima* and *P. margaritifera*, our study has identified four genes (Calreticulin, Linkine, PfCHS1 and Perline) putatively involved in the formation of the nacreous biomineralisation layer, which were previously undescribed.

4.4.3 Host oyster expressing Linkine – turning on gene pathways

The host oyster was found to express four putative biomineralisation genes, MSI60, Calreticulin, Linkine and PfCHS1. Transcripts of two putative biomineralisation genes, MSI60 and Calreticulin, were detected in gonad tissue, conflicting a previous study that found MSI60 was not expressed within the gonads of *P. fucata* (Wang et al., 2009). Due to these two genes being expressed by the gonad, evaluation of host expression of these genes within the pearl sac was difficult due to the possibility of gonad tissue contamination within pearl sac samples. Therefore, Linkine, a gene found to be expressed by the donor and host oyster and not expressed in the gonad, was sequenced to validate host expression of this gene within individual pearl sacs. Here, it was discovered that Linkine was expressed by the host oyster in one individual. Recently, direct evidence was provided of Linkine's implication in the shell biomineralisation process. By extracting shell matrix proteins from decalcified shell powder, Joubert et al. (2010) definitively showed that Linkine is part of the calcifying matrix,

which is embedded within the biomineral structures in the shell of *P. margaritifera*. Therefore, because a cultured pearl forms within the gonads of a host oyster, the host cells that were found to be expressing Linkine within the pearl sac must have originated from the gonad tissue. However, Linkine was not found to be expressed in gonad tissue. One hypothesis as to why the host was found to express Linkine is that the cells from the gonad are migrating into the pearl sac during its development and the mantle cells are turning on gene pathways within the host cells, causing them to express this putative biomineralisation gene.

4.5 Conclusion

This study is the first to examine the transcriptome profile of a pearl sac using high-throughput sequencing (Illumina GAII). Here, 19 putative molluscan biomineralisation genes were identified as being expressed within the pearl sac of *P. maxima* and *P. margaritifera* at pearl harvest. Furthermore, through the novel approach of producing xenografts from *P. maxima* and *P. margaritifera*, this study has clearly shown that the donor oyster is the main contributor to the expression of putative biomineralisation genes governing pearl formation. However, the process of pearl formation could be more complex than we think, with the biomineralisation gene Linkine found to be expressed by the host oyster in one individual. More research is required into the potential for the host to express biomineralisation genes and contribute to pearl formation. The expression levels of the 19 putative biomineralisation genes found to be expressed within the pearl sac also need to be examined to determine what level of association these genes have with pearl formation.

Chapter 5 General Discussion

Pearl production is a complex process that involves the biological co-operation of two individuals, a host and a donor oyster. This thesis defined the respective roles of these two oysters in pearl formation, by first examining their phenotypic contribution to pearl quality traits followed by a fine scale examination of their molecular contribution to the pearl biomineralisation process. It was unequivocally shown that the donor oyster tissue not only survives the grafting process, but is the main contributor to the expression of putative biomineralisation genes and pearl quality traits. The donor oyster therefore has a major influence in pearl formation.

5.1 Defining host and donor oyster contribution to pearl phenotype

Given that the most commercially significant trait affecting the profitability of a pearling company is pearl quality, it is important to understand how a pearl is formed. Significant research has gone into optimising pearl culture techniques to try to improve pearl quality such as, nuclei implantation techniques and identification of optimum environments (Yuxiang and Fu-Liang, 2003; Lucas, 2008; Mamangkey and Southgate, 2009). However, little research effort has gone into understanding the complex genetic processes behind producing a cultured pearl. Before pearl quality can be improved, the contribution genetic factors have in the realisation of pearl quality traits needs to be understood (Wada and Jerry, 2008). Our knowledge of the genetic basis of important traits in pearl oysters is largely restricted to the heritability of shell traits and their phenotypic correlations with pearl weight and colour

(Velayudan et al., 1996; Wada, 1984, 1986). The production of a cultured pearl, however, is a unique, complex biological process potentially involving genetic contribution from two oysters (the host and donor). No study to date has conclusively defined the contribution of these two oysters to the various pearl quality traits. Chapter 2 of this thesis defines the role the host and donor oysters play in the realisation of pearl phenotype, in particularly pearl colour and size.

The results of this thesis show conclusively for the first time that the donor oyster is the primary determinant of pearl colour. Previous research on pearl colour determination has agreed with our findings and suggested a tendency for shell nacre colouration in the donor oyster to influence overall pearl colour (Alagarswami, 1987a; Taylor, 2002; Wada and Komaru, 1996). Separation of the donor contribution to pearl colour from that of host oysters in these previous studies, however, has been inconclusive to date due to implantations being based on allografts involving same nacre coloured hosts. Through the use of xenografts involving two species which produce distinctively different base-coloured pearls, the results of this thesis were able to definitively show that the colour of a pearl is due to the donor oyster.

Nacre growth was also conclusively shown to be dependent on the donor oyster. No previous research has examined the contribution from the donor and host oysters to nacre growth. It is well known that pearls produced by *P. maxima* are the largest of all pearl oyster species (9-20 mm; Strack, 2006). Therefore, by using xenografts involving *P. maxima* and *P. margaritifera* which produce different sized pearls, nacre growth is a strong diagnostic trait which was used to show the donor oyster is primarily responsible for this pearl quality trait. Xenografts using *P. margaritifera* hosts and *P. maxima* donors produced pearls with a faster nacre growth rate

when compared to both species allografts. Therefore, xenografts using these two species may be an effective approach to increase the overall size of silver pearls. However, a negative correlation between nacre deposition and surface complexion was found in our study, suggesting that techniques to improve nacre deposition have potential to adversely impact on other pearl quality traits such as complexion. Consequently, caution needs to be exercised when selecting donors for fast nacre deposition.

The outcomes from this chapter significantly improved the understanding of the realisation of pearl quality traits by defining the relative contribution of the donor and host oysters. By understanding the importance of the donor oyster to the majority of pearl quality traits, this research provides grounds for a donor specific selective breeding program based on pearl growth, colour and surface complexion. This work also demonstrates the potential of xenografts as a means to improve pearl quality traits such as pearl size.

5.2 Host and donor molecular contribution to pearl formation

In light of detecting the donor oyster as the main contributor to pearl quality traits, the next logical question is what is happening at the molecular level in regard to the expression of biomineralisation related proteins that govern pearl formation. In molluscs, the mantle epithelium secretes an organic matrix consisting of inorganic calcium carbonate polymorphs and organic biopolymers (structural proteins) which regulate shell calcification (Fougerouse et al., 2008). Therefore, a number of proteins have been isolated from the shell, shell organic matrix and mantle tissue using biochemical and molecular approaches to try to elucidate the process of shell formation. The functions of these proteins have been discussed based on their primary and predicted secondary structures, expression patterns and results from *in vitro* experiments (Miyamoto et al., 1996; Shen et al., 1997; Sudo et al., 1997; Samata et al., 1999;

Kono et al., 2000; Mann et al., 2000; Miyashita et al., 2000; Weiss et al., 2001; Zhang et al., 2003; Tsukamoto et al., 2004; Gotliv et al., 2005). Only recently has the expression of shell matrix proteins been examined within the pearl sac (Inoue et al., 2009, 2010; Wang et al., 2009). Cultured pearl production is a complex biomineralisation process, potentially involving the genetic contribution from two individual oysters, the host and donor oysters. To help resolve the interplay between host and donor genetic contribution in pearl formation, xenografts were produced in Chapters 3 and 4, using two *Pinctada* species, *P. maxima* and *P. margaritifera*. Through the novel approach of producing xenografts, Chapter 3 of this thesis first examined whether the donor oyster DNA persisted in the pearl sac until the time of pearl harvest. More interestingly, for the first time this chapter examined whether the donor and/or host cells were transcriptionally active in the expression of two biomineralisation genes, N66 and N44, and potentially contributing to pearl formation. Diagnostic DNA tests revealed that donor oyster cells not only remained present in the pearl sac at the time of pearl harvest, but were found for the first time to be transcriptionally active in the expression of two biomineralisation genes, confirming the results of Chapter 2 that the donor oyster is an important contributor to the pearl biomineralisation process. Chapter 4 of this thesis then endeavoured to gain a comprehensive understanding of pearl formation by examining which of the molluscan shell biomineralisation genes are expressed within the pearl sacs of two *Pinctada* species, *P. maxima* and *P. margaritifera*, and potentially contributing to the pearl biomineralisation process. To further elaborate on the host and donor oyster contribution to the expression of biomineralisation genes, the presence/absence of species diagnostic gene transcripts within the xenografted pearl sacs was further examined.

This study presents the first comprehensive sequencing effort of a pearl sac for a pearl

producing species. Throughout the extensive literature on molluscan biomineralisation proteins, their potential influence on the biomineralisation process of pearl formation is only starting to be elucidated. Because the donor graft used for cultured pearl formation is derived from the mantle tissue, which is the sole tissue responsible for the expression of biomineralisation genes, it has been assumed that genes within the mantle that are responsible for shell formation are also responsible for pearl formation. However, differences have been shown in the expression levels of biomineralisation genes within the mantle tissue compared to the pearl sac. Therefore, it is important to examine the genes expressed within the pearl sac when investigating pearl formation. Previous to this study, the expression of only nine putative biomineralisation genes had been identified within the pearl sac of a pearl oyster species, *P. fucata* (MSI31, N16, Nacrein, MSI60, Prismalin-14, Aspein, EFCBP, ACCBP and N19). This thesis identified 19 biomineralisation genes similarly expressed within the pearl sacs of *P. maxima* and *P. margaritifera* oysters, indicating little divergence in the biomineralisation processes of pearl formation between these two species.

Pearl production potentially involves the genetic contribution from two individuals, the host and donor oyster. General perception is that the donor oyster is the main contributor to pearl formation due to cultured pearl production involving the surgical implantation of a mantle graft (the tissue responsible for proteins that produce a shell) from the donor oyster (Farn, 1986). The importance of the donor oyster to the realisation of pearl quality traits like colour was definitively shown in Chapter 2 through examination of the phenotype of pearls produced from xenografting two distinct coloured pearl oyster species, *P. maxima* and *P. margaritifera*. Here, *P. maxima* host oysters seeded with mantle tissue from the black-lip pearl oyster *P. margaritifera* produced black-coloured pearls – a colour not seen in pearls produced in pure culture of *P. maxima*. In light of detecting the donor specific transcripts for

all genes examined within the pearl sac in Chapters 3 and 4, this thesis has clearly shown that the donor oyster is the main contributor to the expression of putative biomineralisation genes. This is the first time donor oyster cells have been shown to be actively expressing biomineralisation-related genes and contributing to pearl formation. This clearly demonstrates that the original donor mantle tissue survives the immunological response from the host oyster and actively secretes some of the necessary biomineralisation proteins that govern pearl formation and confirms at a molecular level Chapter 2 that demonstrated phenotypically that the donor is the main contributor to pearl quality traits.

Very little is known about the specific functional role of most biomineralisation-related genes. Specific parts of the mantle are known to be responsible for the secretion of the different layers of shell/pearl formation (periostracum, prismatic and nacre layers) (Fougerouse et al., 2008), yet many shell matrix proteins have yet to be localised to or extracted directly from these layers. The donor mantle tissue, which is excised for cultured pearl production, is taken from the pallial zone of the mantle which has been shown to secrete only the nacreous layer of the inner shell (Sudo et al., 1997; Takeuchi and Endo, 2006). Therefore, it can be concluded that the genes found to be expressed in the pearl sac by the donor oyster in this thesis, are related specifically to the formation of the nacreous biomineralisation layer. This thesis identified four genes (Calreticulin, Linkine, PfCHS1 and Perline) putatively involved in the formation of the nacreous biomineralisation layer which were previously undescribed, enhancing our knowledge of the formation of the nacreous layer and the biomineralisation process in general.

However, the process of pearl formation could be more complex than we think. In this thesis, the biomineralisation gene Linkine was found to be expressed by the host oyster in one

individual. Recently, direct evidence was provided of Linkine's implication in the shell biomineralisation process by extracting shell matrix proteins from decalcified shell powder (Joubert et al., 2010). Because a cultured pearl forms within the gonads of a host oyster, the host cells that were found to be expressing Linkine within the pearl sac must have originated from the gonad tissue. However, Linkine was not found to be expressed in gonad tissue. One hypothesis as to why the host was found to express Linkine is that the cells from the gonad are migrating into the pearl sac during its development and the mantle cells are turning on gene pathways within the host cells, causing them to express this putative biomineralisation gene. However, due to the host only being found to express one biomineralisation gene in one individual in this thesis, more research is required into the potential for the host to express biomineralisation genes and contribute to pearl formation.

Understanding the role the donor and host oyster have at the molecular level of cultured pearl formation provides a solid foundation for elucidating the biological process in general, as well as providing valuable information that can be directly utilised for selective breeding programs by the cultured pearl industry. By examining the transcriptome profile of the pearl sacs of *P. maxima* and *P. margaritifera* oysters, this thesis identified the largest proportion of genes linked to the process of biomineralisation within the pearl sac to date. It is also the first to highlight that the process of pearl formation may be very similar between these two species. Furthermore, through the novel approach of producing xenografts from *P. maxima* and *P. margaritifera*, this study has clearly shown that the donor oyster is the main contributor to the expression of putative biomineralisation genes governing pearl formation. However, the process of pearl formation could be more complex than thought, with the biomineralisation gene Linkine found to be expressed by the host oyster in one individual. This research has provided valuable information into elucidating the pearl biomineralisation

process by identifying the potential genes expressed in pearl sac tissue and the contribution to the expression of these genes by the donor and host oyster.

5.3 Future directions and concluding remarks

Considering one individual host oyster was detected expressing a putative biomineralisation-related gene (Linkine) within the pearl sac, more research is required into the potential for the host to express biomineralisation genes and contribute to pearl formation. Xenografting two species of pearl oysters that have a greater genetic distance may provide the opportunity to examine a larger number of biomineralisation genes if the graft is still genetically similar enough to survive the grafting process. One of the biggest impediments in determining whether the donor or host cells are transcriptionally active for biomineralisation genes in the pearl sac is discriminating between the gene products of the two potentially contributing oysters. By examining a xenografted pearl sac with greater genetic distance between species used, more species diagnostic transcripts may be found in a greater range of biomineralisation genes. Further unravelling of the host and donor oyster genetic interplay to cultured pearl formation will not only provide a greater understanding of the pearl biomineralisation process, but will provide valuable information for selective breeding programs to improve pearl quality.

Once the contribution to the expression of biomineralisation-related genes is accountable to the host and/or donor oyster, the next logical step is to elucidate the specific biological function of these genes leading to different pearl quality traits. This will ensure pearl quality traits are not only selected upon based purely on phenotype, but an understanding of the molecular mechanisms underlying pearl traits to achieve maximum genetic gains. The

transcript levels of the 19 putative biomineralisation genes found to be expressed within the pearl sac in this research need to be examined to determine what level of association these genes have with pearl formation and their precise functional roles. Additionally, it is possible that the other biomineralisation genes were not found within the pearl sac in this research due to limitations in sequencing coverage. More genes with low coverage may have been uncovered with more in-depth sequencing and by normalising sequenced genes.

In conclusion, the body of work entailed in this thesis has provided significant advances in our understanding of genetic factors affecting pearl formation and quality. The outcomes of this thesis have not only furthered our understanding of the pearl biomineralisation process in general, but have created a sound foundation from which pearl quality can begin to be improved with the knowledge of what genes are likely to be influencing pearl formation and the contribution the host and donor oysters provide in biomineralisation and to various pearl quality traits. This research has demonstrated for the first time that the donor oyster is transcriptionally active in the expression of biomineralisation genes that govern the process of pearl formation and has a major influence on pearl quality traits. This demonstrates the importance of the donor oyster in cultured pearl production of *P. maxima* and *P. margaritifera* and the need for a donor oyster specific selective breeding program to improve pearl quality in these industries.

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Appendix 1

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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

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ABSTRACT

Mantle xenografts between two *Pinctada* pearl oyster species, *P. maxima* (silver-lip pearl oyster) and *P. margaritifera* (black-lip pearl oyster), were used to examine their influence on pearl quality and to further our understanding of the respective contribution host and donor oysters have in pearl formation. Here, host oysters were implanted with mantle tissue originating from either the same species (allograft) or the other species (xenograft) and pearl formation allowed to proceed for 14 months, where after pearls were harvested and nuclei retention and pearl quality traits (nacre deposition/weight, shape, colour, complexion and lustre) recorded. Results showed that xenografts did not significantly affect pearl sac formation and subsequent nuclei retention ($\chi^2 = 5.64$, $df = 3$, $P > 0.05$), but did influence pearl colour, complexion, shape, nacre deposition and nacre weight ($P < 0.05$). Nacre deposition and weight were found to be higher in xenografts comprising of a *P. maxima* donor and *P. margaritifera* host, than in the reciprocal xenograft and allografts. Pearl colour and complexion were also strongly influenced by the donor oyster species used as xenografts, with *P. maxima* host oysters implanted with mantle from *P. margaritifera* producing generally black colour based pearls. Conversely, *P. margaritifera* hosts implanted with *P. maxima* mantle produced silver colour based pearls. This study demonstrates the potential of xenografts as a means to improve pearl quality traits such as pearl size, and highlights the role that donor oysters have in the realisation of pearl growth, colour and surface complexion.

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Diagnostic genetic markers unravel the interplay between host and donor oyster contribution in cultured pearl formation

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Diagnostic genetic markers unravel the interplay between host and donor oyster contribution in cultured pearl formation

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ABSTRACT

To produce a cultured pearl, a mantle allograft originating from a donor oyster is surgically implanted along with a shell bead nucleus into the gonad of a recipient oyster from the same species (termed the host oyster). Whilst, studies have shown that genomic DNA from a mantle allograft remains present in the pearl sac at the time of pearl harvest, what remains unclear is whether biomineralisation genes from the donor mantle allograft are transcriptionally active and contribute to pearl formation. To help resolve the interplay between host and donor genetic contribution in pearl formation, xenografts were produced, using two *Pinctada* species, *P. maxima* and *P. margaritifera*, to examine which species-specific nacreous genes (N66 and N44) were expressed in the pearl sac. Diagnostic DNA tests revealed that donor oyster cells not only remained present in the pearl sac at the time of pearl harvest, but were found for the first time to be transcriptionally active in the expression of biomineralisation genes, N44 and N66. These results confirm that the donor oyster is an important contributor to the biomineralisation process in pearl culture.

Understanding the role the donor and host oyster have in cultured pearl formation provides a solid foundation for elucidating the biological process in general, but it also provides valuable information that can be directly utilised for selective breeding programs in the cultured pearl industry.

1. Introduction

Cultured pearl production involves a complex biological process that is not yet completely understood. Cultured pearl production involves surgical implantation of a mantle allograft originating from a donor oyster along with a shell bead nucleus into the gonad of a recipient oyster from the same species (termed the host oyster). The donor mantle tissue then degenerates to a single epithelial cell layer, which proliferates around the seed nuclei to form a pearl sac (Kawakami, 1952a, b). Pearl sac epithelia begin to secrete shell matrix proteins, which subsequently deposit $CaCO_3$ for pearl formation up to two weeks post pearl sac development (Machii and Nakahara, 1967). Due to cell differentiation during the initial stages of pearl sac development, the fate of the mantle graft cells and their exact role in pearl formation cannot easily be determined by classical histological methods (Kawakami, 1952a, b; Herbaut et al., 2000; Cochennec-Laureau et al., 2010), leaving a lack of resolution as to the genetic makeup of the pearl sac at pearl harvest.

Until recently, it was unknown if donor oyster mantle cells actually persisted in the pearl sac until the time of pearl harvest, or whether they were rejected in the initial immunological response from the host during pearl sac formation. Evidence from genotyping the pearl sac using diagnostic microsatellite genetic markers in *Pinctada margaritifera* has shown that DNA originating from the donor oyster as well as host oyster cells can be detected in the pearl sac at pearl harvest (Arnaud-Haond et al., 2007). The presence of the host cells detected in pearl sac samples could be the result of contamination from surrounding tissue. However, because the precise genetic make-up of the pearl sac has yet to be determined, the role of donor and host cells (i.e. whether the cells from the donor or host oyster are transcriptionally

active in the biomineralisation process) and their respective contribution to pearl development remains unknown. The importance of the donor oyster to the realisation of pearl quality traits like colour has also been shown through examination of the phenotype of pearls produced from xenografting two distinct coloured pearl oyster species, *P. maxima* and *P. margaritifera* (McGinty et al., 2010). Here, *P. maxima* host oysters seeded with mantle tissue from the black-lip pearl oyster *P. margaritifera* produced black-coloured pearls – a colour not seen in pearls produced in pure culture of *P. maxima*. The isolation of mantle donor specific DNA and realisation of phenotypic traits related to the donor oyster such as nacre colour, strongly suggest that the donor oyster cells are actively involved in cultured pearl biomineralisation processes. However, to date no study has shown that donor mantle cells remain transcriptionally active in the pearl sac until pearl harvest.

Numerous proteins regulating nacreous shell matrix secretion have been identified in the mantle tissue of the pearl oyster [e.g. N66 (Kono et al., 2000), Nacrein (Miyamoto et al., 1996, 2005), P10 (Zhang et al., 2006), N19 (Yano et al., 2007), N16 (Samata et al., 1999)], with some being found to be localised specifically to the mantle tissue and not present in gonadal tissue (Wang et al., 2009). Therefore, if donor oyster mantle cells are expected to remain transcriptionally active in the pearl sac, these tissue specific genes may be good biomarkers to identify whether the donor or host oyster cells are transcriptionally active and contribute to the biomineralisation process. Because we know these genes are not present in gonad tissue (Wang et al., 2009), expression of these genes from the host oyster in the pearl sac would not be expected, unless a form of cell potency is occurring whereby the host oyster cells are taking on a functional role similar to that of mantle cells.

One of the biggest impediments to determining whether the donor or host cells are transcriptionally active for biomineralisation genes in the pearl sac is discriminating between the gene products of the two potentially contributing oysters. Currently there is insufficient information on levels of intra-specific polymorphisms in putative biomineralisation genes to characterise gene products that may be derived from the host and/or donor oysters. One approach that may resolve these problems of lack of intra-specific polymorphisms in putative biomineralisation genes is to use xeno-grafted mantle tissue from two closely related species where inter-specific gene sequence differences are present. Mantle grafts between two species, *P. maxima* and *P. margaritifera* (so called xenografts), have been shown to result in pearl sac formation and pearl development (McGinty et al., 2010). If species-specific gene differences are present for biomineralisation genes, then the use of inter-specific xenografts and the examination of what species-specific biomineralisation gene homologue is being expressed in the pearl sac will be a powerful, novel, approach in determining if the donor oyster cells remain transcriptionally active through pearl development.

We tested the hypothesis that cells originating from the mantle tissue of the donor oyster not only remain in the pearl sac during development as previously found (Arnaud-Haond et al., 2007), but that they also are actively engaged in pearl formation through the expression of biomineralisation genes. We did this through designing *P. maxima* and *P. margaritifera* species-diagnostic primers for two biomineralisation genes believed to be involved in nacre formation, N66 (Kono et al., 2000) and N44 (GenBank Accession No. FJ913472.1), and examining if both DNA and mRNA gene products from the donor species could be detected in the pearl sac when it was used as a xenograft.

2. Materials and Methods

2.1 Experimental animals

P. margaritifera and *P. maxima* adult oysters were sourced from wild West Papuan Province (1°13'N, 130°54'E) stocks, and from a Balinese hatchery (8°23'S, 115°14E), respectively. *P. margaritifera* oysters were transported in the hold of a commercial pearl oyster transport vessel and upon arrival in Bali were transferred into 16-pocket panel nets suspended on a long-line alongside the *P. maxima* stock used for this experiment. Oysters were then left undisturbed for three months prior to nuclei implantation to allow both species to adjust to environmental conditions. Three weeks prior to implantation, panels were covered with mesh to lower oyster metabolic rate and gametogenic activity to reduce the chance of implanted nuclei rejection (Gervis and Sims, 1992).

Host oysters from both *P. maxima* (n=80) and *P. margaritifera* (n=80) were implanted with either an allograft (i.e. intra-species - Ss, Bb) or a xenograft (i.e. cross-species - Sb, Bs) (Fig. 1). Mantle tissue was excised from donor *P. maxima* (n=10) and *P. margaritifera* (n=10) oysters and cut into eight segments per individual. Four of these segments were each used as allografts (species controls) and four used as xenografts (experimental treatment) (Fig. 1). Individual genetic effect of the donor oyster on pearl quality was controlled for by implanting tissue from the same donor in both oyster species. According to the gonad size of the host oyster appropriately sized nuclei (ranging from 5.76–7.88 mm to 0.28–0.73 g in size and weight, respectively) were implanted together with the mantle tissue. In total, four groups were created; black-lip pearl oyster hosts with blacklip donors (Bb); black-lip hosts with silver-lip donors (Bs); silver-lip hosts with black-lip donors (Sb); silver-lip hosts with silver-

lip donors (Ss). Following implantation, the 160 host oysters were randomly placed in ten 16 pocket panel nets.

The xenografted oysters were able to form pearl sacs and produce pearls, therefore, the pearl sac, mantle and gonad tissue were extracted (on the same day over a 6 h period) from each of the allografts and xenografts at the time of pearl harvest (14 months post operation) and preserved in RNAlater (Ambion™) held at 4°C for 24 h then stored at -20°C.

2.2 Extraction of nucleic acids and cDNA generation

Genomic DNA was extracted from the pearl sac and mantle tissue of eight oysters from each of the allografts (Ss, Bb) and nine oysters from the xenograft when *P. maxima* was the donor oyster (Bs), using a high throughput extraction procedure described by Lind et al. (2009).

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from the pearl sac of both allografts [Ss (n=10), Bb (n=10)] and both xenografts [Bs (n=10), Sb (n=10)] and the gonads of both allografts from the same individuals in which the pearl sac was excised [Ss (n=10), Bb (n=10)]. RNA was extracted by dissecting the inner layers of the pearl sac and homogenizing 10-100 mg of tissue in 1 ml of Ultraspec RNA (Biotecx, Houston, TX). RNA was then separated from the organic material (DNA and protein) using 0.1 volume 1-bromo-3-chloropropane and precipitated by adding 0.5 volumes isopropyl alcohol and 0.5 volumes RNA precipitation solution (1.2 M sodium chloride, 0.8M disodium citrate) (Biotecx, Houston, TX; Sambrook and Russell, 2001). The quality of RNA and presence of contamination (inorganic material, genomic DNA) was

examined visually by inspection of the total RNA (incorporating mRNA and rRNA) on an agarose gel (at a total RNA concentration of 200 ng/µl). RNA contamination was also measured by absorbance ratios $OD_{260/280}$ (RNA range: 1.95-2.08) and $OD_{260/230}$ (Commonly pure range: 2-2.2) on a Nanodrop spectrophotometer (Nanodrop Technology, Wilmington, DE). RNA was quantified by absorbance at 260 nm on the Nanodrop spectrophotometer.

To reduce any residual traces of DNA contamination a total of 10 µg of total RNA for each sample was treated with a Turbo DNA-free kit (Ambion, Austin, TX), including a sodium acetate precipitation. First strand complementary DNA (cDNA) was synthesized from approximately 2-3 µg of DNAse treated RNA using Superscript III first-strand synthesis supermix with 50 µM oligo(dT)20 (Invitrogen, Carlsbad, CA, USA). The 20 µl cDNA reaction involved incubation at 50°C for 50 min followed by deactivation at 85°C for 5 min. For verification of complete DNA removal, –RT controls were created by splitting one sample per treatment (group or tissue type) in half and performing a cDNA synthesis reaction with the remaining half without the reverse transcriptase. The RNA strand was digested from all cDNA synthesis reactions using 1 µl of RNAse cocktail (Ambion, Austin, TX). An aliquot of each cDNA sample was diluted to a final standardized concentration of 2 ng/µl prior to PCR analyses.

2.4 PCR of species diagnostic targeted biomineralisation genes

Initially, no species diagnostic gene sequence information was available for *P. maxima* and *P. margartifera* for any candidate nacre genes. A species-specific forward primer, N66 3F (ATT AAA GGG TCA TTG CGA GAA C), was designed for *P. maxima* based on sequence variations in alleles found at the N66 domain between *P. maxima* and *P. margaritifera* (Kono

et al., 2000) (unpublished sequence data). This forward primer was situated 700 bp from the 5' start of the N66 mRNA sequence. The reverse primer, N66-R2 (GAC GAT ACA TCC TCC GCT AAA G), was used as it has previously been shown to be conserved across *P. maxima* and *P. margaritifera* species (Smith-Keune and Jerry, 2009). A conserved primer set was also developed for the two species (N66 Fcons: GGC AAC AAT GGA AAC AAC GGA and N66 R2 primers) to verify that genomic DNA was amplifiable if present in all individuals used (unpublished sequence data). This forward primer was situated 770 bp from the 5' start of the N66 mRNA sequence. PCR primers for the analysis of the target gene N44 (N44 Fmarg: TAG TTG CTT TAA GGC ATG TGG AAG; N44 R: CCC GTT TCC ACC ATT GTT ACC AT) were developed using Primer3 v0.4.0 based on the assembly and characterization of the N44 gene. Raw sequences were attained from a next-generation mRNA sequencing database (Illumina Solexa GAII) for both *P. maxima* and *P. margaritifera* (unpublished sequence data). The forward primer was situated 223 bp and the reverse primer was located 1195 bp from the 5' start of the N44 mRNA sequence.

Polymerase chain reaction (PCR) was performed in 15 µl volumes with final concentrations of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1X PCR buffer, 0.5 U/µl of *Taq* DNA polymerase (Bioline) and 10 ng of gDNA or 4 ng of cDNA. The thermocycler program for both N66 and N44 began with an initial denaturation step at 95°C for 5 min, 35 cycles of 95°C for 45 sec, 57.5°C for 30 sec, and 72°C for 45 sec, followed by a final extension step of 10 min at 95°C. PCR fragments were visualized on a 1.5% TBE agarose gel.

3. Results

3.1 P. maxima specific N66 genomic primers

P. maxima N66 species-specific primers (N66 Fmax and N66 R2 primers) produced a PCR product of expected size for all eight *P. maxima* allografts (Ss, Fig. 2a), whilst no specific amplification was detected in any of the eight *P. margaritifera* allografts (Bb, Fig. 2c). This demonstrates that a PCR product was only produced where *P. maxima* genomic DNA was present in the pearl sac tissue. A PCR product was produced for all nine xenografted pearl sacs where *P. maxima* was the donor oyster, with this set of N66 *P. maxima* specific primers (Bs, Fig. 2a). The viability of the DNA from *P. margaritifera* and *P. maxima* (D) allografts (Bb, Ss) was verified using a conserved primer set (N66 Fcons and N66 R2 primers) that amplifies in both species (Fig. 2b). Overall it was found that the donor oyster cells remain present in the pearl sac at the time of pearl harvest. No PCR product was present in the negative controls for both N66 Fcons, N66 R2 and N66 Fmax, N66 R2 primer sets.

3.2 P. maxima specific N66 transcriptomic primers

The *P. maxima* species-specific N66 primer set was only found to produce a PCR product where the N66 gene was expressed from *P. maxima* tissue (Fig. 3). The *P. maxima* primer set produced a cDNA PCR product for all 10 *P. maxima* allografts (Ss) and all 10 xenografts where *P. maxima* was the donor oyster (Bs). Whilst, no amplification occurred in any of the 10 *P. margaritifera* allografts (Bb) or any of the 10 xenografts where *P. margaritifera* was the donor oyster (Sb) (Fig. 3a).

3.3 P. margaritifera specific N44 transcriptomic primers

P. margaritifera species-specific N44 primers were only found to produce a PCR product where the N44 gene was expressed from *P. margaritifera* tissue (Fig. 3). The *P. margaritifera* species-specific primers (N44 Fmarg and N44 R primers) produced a cDNA PCR product for all 10 *P. margaritifera* allografts (Bb) and all 10 xenografts where *P. margaritifera* was the donor oyster (Sb). Whilst, no amplification occurred in any of the 10 *P. maxima* allografts (Ss) or any of the 10 *P. maxima* xenografts where *P. maxima* was the donor oyster (Bs) (Fig. 3b).

3.4 Data integrity

There was no specific amplification of nacre genes N66 and N44 in the gonad transcriptome or in the –RT samples. No PCR product was present in the negative controls for both N66 and N44 primer sets.

4. Discussion

This study produced xenografts from two *Pinctada* pearl oyster species to examine if the donor cells remain present in the pearl sac at pearl harvest and, if so, whether the donor or host cells remained transcriptionally active and contributed to pearl formation. Our study has not only confirmed that the cells from the donor oyster survived the initial host immunological response and persisted in the pearl sac, as shown by DNA analysis, but the donor cells were also transcriptionally active in secreting nacreous shell matrix proteins contributing to pearl formation.

The survival of grafted mantle tissue from another individual let alone another species through the entire process of pearl formation implies an exceptional rate of graft success and a complex biological cooperation between two unique genotypes in the biomineralisation process. The grafting procedure induces an immunological reaction in the host oyster, whereby cells differentiate making it difficult to track the different cells during pearl sac formation (Cochennec-Laureau et al., 2010). Accordingly, earlier histological studies were unable to clearly verify graft success and the persistence of the grafted donor cells (Herbaut et al., 2000). Evidence from genotyping the pearl sac has shown that the donor oyster cells persisted in the pearl sac until pearl harvest. Here, three co-dominant polymorphic microsatellite markers were used to screen for a foreign genotype in allografted pearl sacs compared with the host oyster muscle tissue (Arnaud-Haond et al., 2007). Through a novel approach of using a xenografted pearl sac containing the genome of two different species, our study supports the findings of this previous work. Here, we found that species diagnostic gene sequences of the donor oyster were found in the pearl sac at harvest, supporting the hypothesis that donor cells are actively involved in pearl biomineralisation.

The persistence of the donor oyster DNA in the pearl sac supports the observations of professional grafters that some pearl phenotypes are influenced by the donor oyster. A few studies have found a tendency for shell nacre colour of the donor oyster to influence the overall colour of the pearl produced (Alagarswami, 1987a; Wada and Komaru, 1996; Taylor, 2002). The influence of the donor oyster on pearl quality traits was definitively demonstrated when the donor oyster was found to have a significant influence on pearl growth, colour and surface complexion using xenografted oysters (McGinty et al., 2010). This supporting information from previous studies showing the influence of the donor on pearl quality traits

and the opinion of professional seeding technicians provides evidence that the DNA from the donor oyster is not only present, but is actively contributing to the biomineralisation process.

This present study detected two nacreous shell matrix protein gene transcripts from the donor oyster in the pearl sac, with no expression of these genes from the host oyster. No nacreous shell matrix protein transcripts were detected in the gonad tissues in our study which has also been confirmed for six other shell matrix proteins (Nacrein, EFCBP, N16, ACCBP, MSI60, N19; Wang et al., 2009), therefore, any detection of nacreous shell matrix protein transcripts in the pearl sac could then only be due to either secretions from the donor, or possibly host oyster gonad cells which have transformed into cells capable of biomineralisation (i.e. a form of cell potency). Due to cell differentiation following the grafting process, it has been difficult to track the cells from the host and donor oysters into pearl sac formation using histological methods (Herbaut et al., 2000). The host has the potential to produce shell matrix protein transcripts through the biological process of cell potency, whereby, the cells of the gonad may have the capacity to differentiate into different cell types (ie. take on the functional role of mantle cells) as an immunological reaction to the introduction of mantle cells during the grafting process. A high proliferation rate and high content of saccharides in the central zone of mantle tissue, characteristic of stem cells, was found from an investigation into cell proliferation and differentiation in the mantle of *Pinctada fucata* (Fang et al., 2008). To date, however, there has been no stem cell research in invertebrates (including molluscs) and investigations into shell matrix protein expression in the pearl sac (Inoue et al., 2009, 2010; Wang et al., 2009) have yet to examine the potential for host cells to be contributing to shell matrix protein secretion and therefore pearl formation. However, due to the species diagnostic sequences amplified in the xenografted pearl sacs in this study, the concept of cell

potency has been ruled out in the pearl sacs analysed here for two nacreous shell matrix proteins. In this study, detection of species-specific transcripts confirmed the donor oyster was the only possible contributor to the secretion of nacreous shell matrix proteins N66 and N44, showing that the cells from the donor oyster are the likely cells actively contributing to the biomineralisation process and pearl formation. Although this idea of cell potency in the gonads of the host oyster is adventurous, it deserves further research using more shell matrix proteins to rule out some host contribution to pearl formation.

5. Conclusion

This study, through the use of xenografts, has clearly shown that the cells from the donor oyster remain in the pearl sac and are transcriptionally active for two genes (N66, N44) believed to be involved in pearl biomineralisation. Through the examination of what donor oyster species DNA was present in the pearl sac of xenografted oysters, we have shown that the cells from the donor oyster persist in the pearl sac until pearl harvest. What's more interesting, however, is that examining gene products from xenografts have shown for the first time that the cells originating from the donor oyster actively secrete nacreous shell matrix proteins and likely contribute to the biomineralisation process of pearl development. No evidence for host expression of these genes was found. Further research into host and donor expression of more than two nacreous shell matrix proteins would be beneficial in evaluating overall host and donor oyster potential for contributing to pearl formation.

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Figures

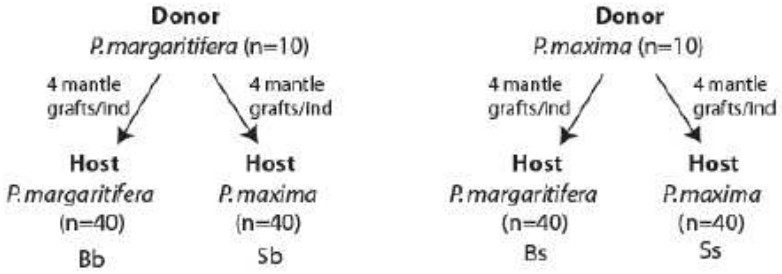
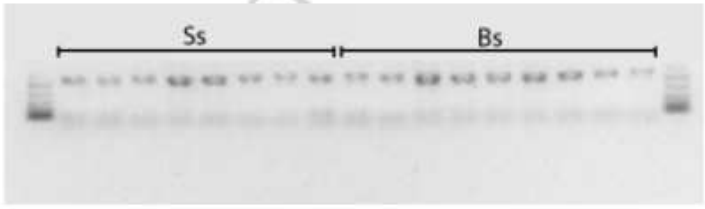
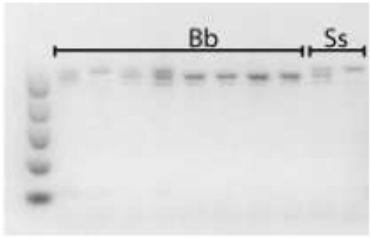


Fig. 1: Experimental design involving implantation of allografts (controls) and xenografts (treatment) into the two host oyster species, *Pinctada maxima* and *Pinctada margaritifera*. In the Fig. Bb refers to a black-lip host oyster with a black-lip donor oyster; Bs, a black-lip host with silver-lip donor; Sb, a silver-lip host with black-lip donor; Ss, a silver-lip host with silver-lip donor.

2a) N66 Fmax and N66 R2 primers



2b) N66 Fcons and N66 R2 primers



2c) N66 Fmax and N66 R2 primers

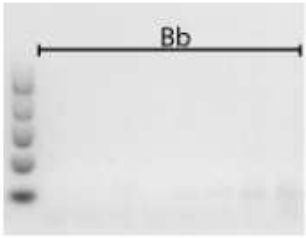
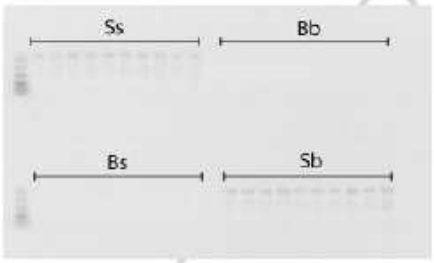


Figure 2: Electrophoretic 1.5% agarose gel showing presence or absence of PCR product for the nacreous gene, N66, in the pearl sac genomic DNA of *Pinctada maxima* and *Pinctada margaritifera* allografts and xenografts (standard bp sizes: 766, 500, 300, 150, 50). A species-specific primer set for *P. maxima* (N66 3F and N66 R2 primers) amplified eight *P. maxima* allografts (Ss) and nine xenografts where *P. maxima* was the donor oyster (Bs) (2a). A conserved primer set for the two species (N66 1F and N66 R2 primers) verified that DNA from eight *P. margaritifera* (Bb) and two *P. maxima* (Ss) individuals was viable (2b). To verify that the *P. maxima* primer set (N66 3F and N66 R2) was species-specific, these primers were applied to the DNA of the same eight *P. margaritifera* individuals used in 2b (Bb, 2c).

3a) N66 Fmax and N66 R2 primers



3b) N44 Fmarg and N44 R primers

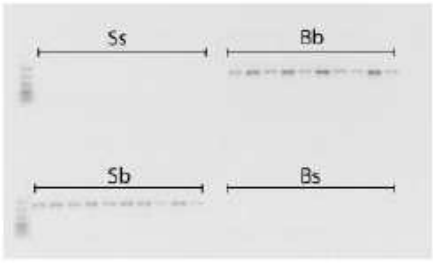


Figure 3: Electrophoretic 1.5% agarose gel showing presence or absence of PCR product for two nacreous genes, N66 and N44, in the pearl sac transciptome of *Pinctada maxima* and *Pinctada margaritifera* allografts and xenografts (standard bp sizes: 766, 500, 300, 150, 50). The N66 *P. maxima* species-specific primer set (N66 Fmax and N66 R2 primers) and a N44 *P. margaritifera* species-specific primer set (N44 Fmarg and N44 R primers) were each applied to 10 *P. maxima* allografts (Ss; 3a and 3b), 10 *P. margaritifera* allografts (Bb; 3a and 3b), 10 xenografts where *P. maxima* was the donor oyster (Bs; 3a and 3b) and 10 xenografts where *P. margaritifera* was the donor oyster (Sb; 3a and 3b).