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Déterminisme génétique des caractères d'intérêt perlicole de l'huître perlière *Pinctada margaritifera*

Carole Blay

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THÈSE DE DOCTORAT

de l'Université de recherche Paris Sciences et Lettres
PSL Research University

Préparée à l'École Pratique des Hautes Études

Déterminisme génétique des caractères d'intérêt perlicole de l'huître
perlière *Pinctada margaritifera*

École doctorale de l'EPHE – ED 472

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*« Dans la vie, rien n'est à craindre, tout est à comprendre »
Marie Curie*

A la mémoire d'Antoine de Léocour et Vincent Delory

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Chapitre 1

Introduction générale

1. INTRODUCTION GENERALE

La perliculture en Polynésie Française, basée sur l'exploitation de l'huître perlière *Pinctada margaritifera*, s'est considérablement développée au cours de ces trente dernières années pour devenir une ressource indispensable à l'économie de la Polynésie française (seconde ressource après le tourisme). L'huître perlière, *P. margaritifera*, en Polynésie française, est aux premières étapes de programme de sélection génétique. Cette espèce étant exploitée pour sa capacité à produire des perles de culture, l'étude du déterminisme génétique des caractères d'intérêt perlicole, leurs transmissions, expressions, dans ce modèle particulier impliquant deux animaux, représente un enjeu important pour orienter les programmes de sélection génétique afin d'augmenter la qualité de production. C'est dans ce contexte que s'est déroulé le travail de thèse que je vais vous présenter.

L'objectif de ce travail de thèse était d'obtenir une estimation de la composante génétique et de l'héritabilité des traits phénotypiques de la perle pour développer des populations issues d'écloserie de *Pinctada margaritifera*, comme modèle d'espèces de mollusques marins produisant des perles de culture. Dans ma thèse, après une approche globale sur les traits phénotypiques de la perle et une étude de l'impact du site de culture sur des familles issues d'écloserie, j'évalue le rôle de l'huître donneuse et de l'huître receveuse au niveau de l'organisme chimérique (i.e. allogreffe) tant au niveau du phénotype « taille » que du phénotype « qualité de la perle », afin d'envisager des stratégies de sélection. Puis je décris les paramètres d'héritabilité des caractères d'intérêts perlicoles ainsi que l'expression de certains gènes cibles de biominéralisation, en fonction des relations parents descendants sur plusieurs familles issues d'écloserie.

Dans l'introduction générale qui suit, je commence par présenter une vue d'ensemble des différents organismes produisant des perles. J'aborde ensuite l'histoire de la perliculture en Polynésie, puis je détaille l'acte de greffe. Je poursuis cette introduction par une description systématique et biologique du modèle d'étude, *P. margaritifera*, puis je décris les aspects de biominéralisation chez l'huître perlière.

Ensuite, je présente la contribution que peut apporter l'estimation des paramètres génétiques notamment dans une dynamique de sélection de lignées en aquaculture. Puis, je situe mon travail dans le contexte des études précédemment menées sur ce modèle et identifie les questions restées en suspens ou nécessitant d'être davantage explorées. Je termine enfin en présentant la structure de la thèse.

1.1. VUE D'ENSEMBLE DE LA CULTURE DE PERLE

MOLLUSQUES PRODUCTEURS DE PERLES

Bien que tous les mollusques possédant une coquille peuvent produire des perles (Strack 2006 ; Webster 1994), seules quelques familles au sein desquelles quelques genres en produisent réellement. Pour les espèces marines, des perles ont été trouvées naturellement dans 17 genres de 11 familles de mollusques, 11 genres de 8 familles de gastéropodes et un genre de céphalopode (*Nautilus*) (Strack 2006). Concernant les perles d'eau douce, 43 genres issus de seulement de 2 familles produisent des perles (Strack 2006). Les mollusques produisant des perles peuvent être divisés en deux groupes distincts ; ceux produisant de la nacre et ceux n'en produisant pas. La nacre est définie comme un carbonate de calcium CaCO_3 cristallisé sous la forme de cristaux d'aragonite. Les perles non nacrées sont des concrétions calcaires. Elles peuvent également être séparées en perles dites « naturelles » ou « fines » correspondant à celles produites sans intervention humaine et les perles de culture produites via une intervention humaine avec implantation d'un greffon, elles peuvent être ou non nucléées.

Mollusques produisant des perles fines non nacrées

D'après Strack (2006), au moins 5 familles de gastéropodes (*Strombidae*, *Cassidae*, *Muricidae*, *Fascioliariidae* et *Volutidae*) et 7 familles de bivalves (*Arcidae*, *Pectinidae*, *Spondylidae*, *Placunidae*, *Ostreaide*, *Tridacnidae* et *Veneridae*) peuvent produire des perles sans nacre. Les perles issues de ces espèces sont généralement des perles naturelles qui ont une faible valeur marchande et peu de couleur. Cependant quelques perles produites par les conques (famille *Strombidae*) et les volutes (famille *Volutidae*) (Matlins 2002 ; Strack 2006) sont très colorées et convoitées.

Mollusques produisant des perles nacrées

Les perles fines nacrées ont été découvertes pour la première fois accidentellement par un homme cherchant de la nourriture au temps de la préhistoire (Dakin 1913 ; kunz and Stevenson 1908 ; Ward 1995). Par la suite les perles ont été associées aux activités culturelles et religieuses (Strack 2006). Les coquilles associées aux perles ont été utilisées pour la décoration à travers l'histoire de l'homme. L'utilisation de perles pour la décoration a commencé au 5^{ème} siècle avant JC (Kunz and Stevenson 1908). C'est seulement durant l'ère romaine que les perles sont devenues un bijou très précieux (Ward 1995). L'« âge de la perle » a vraiment commencé au 16^{ème} siècle, même si au 18^{ème} siècle il y a eu un changement de la perle vers le diamant, la perle a repris la première position au 19^{ème} siècle quand une nouvelle huître perlière a été découverte et que la production de perle de culture a commencé (Strack 2006).

Les perles naturelles sont extrêmement rares, et leurs fréquences sont dépendantes de l'espèce concernée ; le ratio est de 1 : 500 (*P. radiata*) dans le golf persique, 1 : 5000 (*P. maxima*) dans la mer Sulu, 1 : 15000 (*P. margaritifera*) en Polynésie française (Strack 2006). La plupart des perles fines (i.e. naturelles) sont de forme irrégulière dite baroque.

En eau douce, les perles nacrées sont produites seulement par 2 familles de bivalves : Margaritiferidae et Unionidae (Strack 2006). Trois espèces de la famille des Unionidae sont connues et cultivées pour la production de perle : *Cristaria plicata*, *Hyriopsis cummingii* et *H. schlegeli* (Strack 2006 ; Wang and Wu 1994).

Au niveau de la perliculture marine, au moins trois familles de gastéropodes (Haliotidae, Trochidae et Turbinidae) et 4 familles de bivalves (Mytilidae, Malleidae, Pinnidae et Pteriidae) produisent des perles de nacre (Strack 2006). Cependant seulement 2 familles sont cultivées pour la production de perle : Haliotidae chez les gastéropodes et Pteriidae chez les bivalves dont deux genres *Pinctada* et *Pteria*. Le genre *Pinctada* est celui sur lequel les développements aquacoles sont principalement concentrés. Au

niveau de ce genre, les perles marines sont cultivées dans la région Indo pacifique ; de la mer rouge à l’océan pacifique. Le Japon est connu pour ses perles Akoya produites par *Pinctada fucata* ; l’Australie et l’Indonésie pour les perles produites à partir de *P. maxima* et les îles du pacifique et plus particulièrement la Polynésie Française pour les perles produites par *P. margaritifera* (Southgate 2008). *Pinctada margaritifera* (notre modèle d’étude) contribue approximativement à 1% du volume total de perle produite mais représente 17% de la valeur marchande totale. Les premières innovations sur la production de perle de culture ont été introduites par les chinois au cours du 5^{ème} siècle. Plus tard des essais ont été conduits par William Sarville-Kent sur *P. maxima* en 1890, suivi par Kokichi Mikimoto trois ans plus tard sur les perles hémisphériques (Simkiss and Wada 1980). En 1914, Mikimoto dépose un brevet sur la production de perle de culture ronde. Cette méthode (toujours d’actualité) utilise un nucléus enrobé et un greffon (un morceau de manteau) qui sont implantés à l’intérieur de la gonade d’une huître receveuse. Cette méthode, certes avec des améliorations, reste toujours la base de toute la perliculture moderne.

On estime en 2013, la valeur totale des perles de culture marine a approximativement 397 Millions US\$ (Müller 2013) et 150 Millions US\$ pour les perles d’eau douce (Anon 2007) (Tableau 1.1).

Tableau 1.1 : Production des perles de cultures marines et d'eau douce cultivées dans le monde (Source Müller 2013 et Anon 2007)

Espèces	Type perle	Pays producteurs	Valeur US\$ millions	Prod. (tonnes)	% de la valeur totale des perles
<i>Pinctada maxima</i>	White south sea or Silver/Gold pearls	Australia , Indonesia, Philippines, Myanmar	192	13	35%
<i>Pinctada fucata and martensi</i>	Akoya pearls	Japan (90%), China	111	25	20%
<i>Pinctada margaritifera</i>	Black or Tahitian pearls	French Polynesia (93%), Cook Island, Fiji	94	15	17%
<i>Freshwater Mussels Cristaria plicata, Hyriopsis cumingii and H. schlegeli</i>	Freshwater pearls	China, Japan	150	1500	27%

LA PERLICULTURE EN POLYNÉSIE

Perle : Histoire et origine

Dès l'arrivée des premiers hommes en Polynésie, les huîtres perlières ont été récoltées dans le but de fabriquer des bijoux d'ornement ou cérémoniels ainsi que des hameçons et leurres de pêche. A la fin du XVIIIe siècle, après les premiers contacts entre les Polynésiens et les explorateurs européens, les huîtres perlières sont pêchées pour alimenter le marché des grandes métropoles européennes (utilisées pour des objets de décorations ou boutons de nacre). La demande de la nacre étant de plus en plus élevée et les huîtres de plus en plus difficiles à trouver, le service de la pêche met en place en 1962 un système de sauvetage de la filière en imposant des quotas de production ainsi que des périodes de récolte. En parallèle, l'arrivée sur le marché du bouton en polyester diminuera la demande en nacre (Le Pennec 2010).

Depuis les années 1980, *P. margaritifera* est surtout utilisée pour ses capacités à produire des perles de culture. Les premiers essais de production de perles sont réalisés dès 1961 afin de trouver une alternative au déclin de l'exportation de coquilles. Dès 1977, 1601 concessions maritimes destinées au collectage de naissains, à leur élevage et à la greffe sont accordées aux perliculteurs de Polynésie française (Lemer and Planes 2012). Le développement de la filière perlicole et la multiplication des concessions maritimes en Polynésie dans les années 1990 inondent le marché mondial de perles de toutes qualités, entraînant la diminution du prix au gramme de la perle (Le Pennec 2010). Aujourd'hui l'activité perlicole est toujours

en crise, pourtant elle permet de faire vivre des milliers de personnes et représente la deuxième source de revenus de la Polynésie après le tourisme.

Production de la perle de culture

La perle de culture de Polynésie française provient d'une greffe « allogénique » et de l'élevage en milieu naturel de l'huître perlière *P. margaritifera* var. *cumingii*. La greffe allogénique est un acte chirurgical qui consiste à introduire dans la poche perlière (i.e. un diverticule de la gonade) d'une huître perlière dite « receveuse » une portion de manteau (greffon) d'une huître différente dite huître perlière « donneuse » et une bille de nacre (nucléus provenant d'une moule du Mississippi) (Figure 1.1). La poche perlière est un diverticule de tissu conjonctif de la glande reproductrice. Les greffons (d'environ 2mm²) sont minutieusement découpés dans la zone du manteau sécrétant la couche de nacre colorée et irisée du bord de la coquille. Dans la poche perlière, le greffon va se développer autour du nucléus en une couche épithéliale. Après environ 14 jours, le greffon englobe complètement le nucléus et forme alors le sac perlier (Kishore and Southgate 2016). Le sac perlier, qui conserve ses capacités minéralisatrices, continue de produire de la nacre. Après l'opération (i.e. greffe), une phase d'élevage d'environ 18 mois est nécessaire pour obtenir une perle d'épaisseur de nacre suffisante pour être commercialisée (supérieure à 0,8 mm, décret du Service de la Perliculture, n° 2005-42 APF du 4 février 2005).

Au moment de la récolte, si la qualité de la perle est jugée bonne, le greffeur peut introduire un nouveau nucléus pour produire une nouvelle perle plus grosse dans le même sac perlier vidé de la première perle produite. Cette opération est appelée « surgreffe », habituellement 2 à 3 surgreffes sont réalisées (Demmer *et al.* 2016). Au final, les perles sont classées en catégorie en fonction de leurs qualités, des défauts, et les perles dites « top gemme » représentent moins de 1% de la production. Ainsi, même si la méthode de greffe a été établie depuis 1900, il existe un réel potentiel à augmenter la qualité moyenne de la perle par une meilleure compréhension des facteurs influençant le processus de biominéralisation dans le développement de la perle comme la sécrétion de nacre ou encore sa qualité.

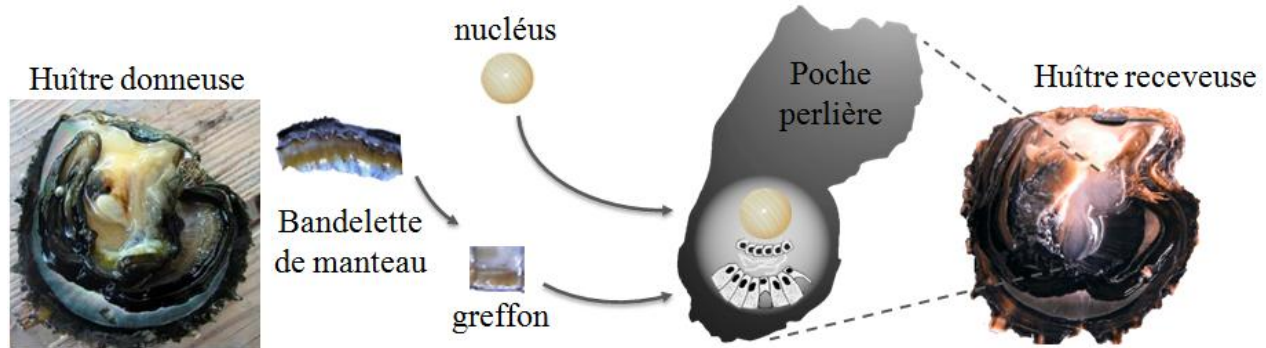


Figure 1.1 : Acte de greffe : Les perles de *P. margaritifera* de Polynésie française sont obtenues 18 mois après une greffe « allogénique » qui consiste à introduire dans une huître perlière receveuse une portion de manteau (greffon) d’une autre huître perlière (huître perlière donneuse) et une bille d’aragonite (nucléus) Schémas: d’après Joubert (2011).

1.2. PINCTADA MARGARITIFERA

TAXONOMIE ET DISTRIBUTION GÉOGRAPHIQUE

L’huître perlière *Pinctada margaritifera* (Linné, 1758) variété *cumingii* (Jameson, 1901), appelée communément « huître perlière à lèvres noires » ou « nacre » en Polynésie française, est un mollusque bivalve appartenant à la famille des Pteriidae et au genre *Pinctada* (Figure 1.2). Au total ce n’est pas moins de 100 espèces qui ont été décrites, mais aujourd’hui elles sont regroupées en 17 espèces (World register of Marine Species 2014).

Règne : Metazoa
 Enbranchement : Mollusca
 Classe : Bivalvia
 Sous-classe : Pteriomorpha
 Ordre : Pterioida
 Superfamille : Pterioidea
 Famille : Pteriidae
 Genre : *Pinctada*
 Espèce : *margaritifera* (Linné, 1758)
 Variété : *cumingii* (Jameson, 1901)



Figure 1.2 : L’huître perlière *Pinctada margaritifera*. Classification taxonomique de l’espèce *P. margaritifera* var. *cumingii* (gauche) et photo d’une huître perlière adulte (droite, © 2005 Francois CORNU)

La répartition de *Pinctada margaritifera* est assez large. Elle est présente dans l'écosystème corallien de l'Indo-Pacifique (zone intertropicale) depuis la Mer Rouge jusqu'au Pacifique oriental ou encore depuis le bas du Nord de l'Australie jusqu'au Sud du Japon (Le Pennec *et al.* 2010). *Pinctada margaritifera*, var. *cumingii* est très abondante en Polynésie française, notamment dans les archipels des Tuamotu et des Gambier où elle est exploitée pour la production de perles. Bien que l'espèce apparaisse polyphylétique entre les populations de l'ensemble de son espace de répartition, il semble que les populations de Polynésie française appartiennent à un même groupe monophylétique, malgré la très grande dispersion des lagons (Cunha *et al.* 2011; Lemer and Planes 2012). L'huître perlière se rencontre principalement à l'intérieur des lagons entre 0 et 50 m de profondeur, fixée par son byssus sur des substrats durs tels que les structures coralliennes (récifs internes ou pinacles).

ANATOMIE ET MORPHOLOGIE

L'anatomie de *Pinctada margaritifera*, est étudiée en détail dans l' « Atlas anatomique de l'huître perlière : *Pinctada margaritifera* » (Fougerouse et Herbaut 1994).

La coquille de *P. margaritifera* est formée de deux valves dissymétriques (la gauche étant plus convexe que la droite) dont la longueur peut atteindre jusqu'à 30 cm. Ces valves sont reliées entre elles par une charnière sans dent. La face externe de la coquille est généralement de couleur foncée, elle peut être grise, bleue, verte, rouge ou encore jaune au stade juvénile (jusqu'à environ 3 mois) et généralement noir ou rouge et rarement blanche (phénotype albinos) à l'âge adulte (Ky *et al.* 2017) (Figure 1.3). Sur la face interne, deux parties peuvent être distinguées : une partie noire à la périphérie de même nature que la face externe et une partie nacrée centrale. La bordure extérieure de la couche nacrée présente des irisations peacock, aubergines, vertes, jaunes, bleues ou grises (Ky *et al.* 2017) (Figure 1.3). L'édification de la coquille est un phénomène qui se produit continuellement pendant toute la vie de l'animal. Cependant, la vitesse de calcification évolue en fonction des saisons et semble être corrélée à l'activité métabolique ou à la nutrition de l'animal, ainsi qu'à la température du milieu (Joubert *et al.* 2014 ; Le Pabic *et al.* 2016).

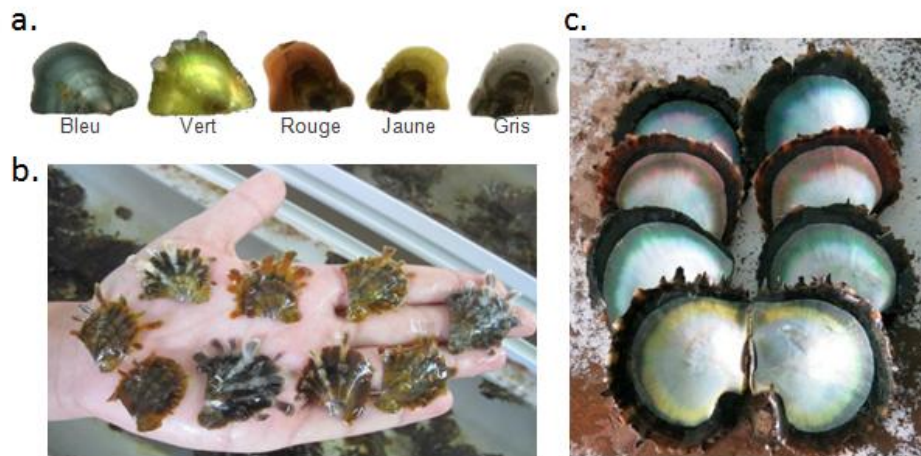


Figure 1.3 : Phénotype de la coquille *P. margaritifera*. a. face externe au stade juvénile (environ 50 jours). b. face externe au stade naissains (environ 120 jours). c. face interne au stade adulte (environ 18 mois) (© Ifremer)

Le manteau est l'organe responsable de la formation de la coquille. Ce fin tissu d'origine ectodermique est constitué de deux épithéliums (l'un interne, en contact avec l'hémolymphe, l'autre externe, en contact direct avec la coquille) séparés par un tissu conjonctif comprenant différents types cellulaires. La mise en place du périostracum va permettre la formation d'un compartiment isolé du milieu extérieur et va ainsi délimiter l'espace de minéralisation : l'espace extra-palléal. Des régions distinctes mais adjacentes de l'épithélium externe sont responsables de la formation des différentes couches de la coquille. En effet, ces cellules différenciées sécrètent un cocktail macromoléculaire spécifique capable d'induire la précipitation d'un polymorphe cristallin et/ou d'une microstructure particulière (Takeuchi and Endo 2006). La couleur noire du bourrelet externe a donné à *Pinctada margaritifera* le nom d' « huître perlière à lèvres noire » (Figure 1.4).

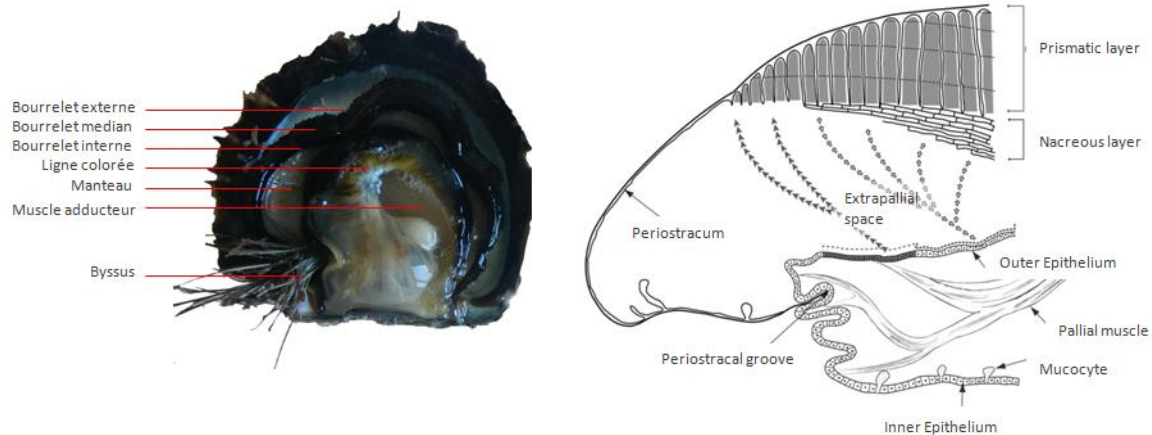


Figure 1.4 : Illustration du manteau. A gauche valve avec animal entier (© Blay), à droite physiologie de la sécrétion de la coquille nacré prismatique (D'après Marie 2008, schéma de droite).

Le cycle de développement de *P. margaritifera* est divisé en trois phases : la phase larvaire pélagique, la phase juvénile et la phase adulte benthique (Figure 1.5). *P. margaritifera* est un bivalve hermaphrodite protandre. En effet, à la première maturité, toutes les huîtres sont de sexe mâle puis le sexe femelle apparaît progressivement à partir d'environ 2 ans pour atteindre un équilibre avec un sex-ratio près de 1:1 pour les populations d'individus âgés de plus de 8 ans (Chávez-Villalba *et al.* 2011). L'expulsion des gamètes des organes génitaux semble être provoquée par des stimuli extérieurs tels que les variations de température. On estime que les femelles émettraient entre 40 et 50 millions d'ovules par ponte, les mâles quant à eux, pourraient libérer entre 400 et 4000 millions de spermatozoïdes. Une fois la fécondation externe effectuée, les divisions embryonnaires débutent donnant des larves trochophores puis véligères qui mènent une vie planctonique. Le premier stade de vie larvaire, larve-D, est atteint au bout de 20-24h. La larve a alors développé une pré-coquille (prodissoconque) d'une taille moyenne de 80 μm (Doroudi and Southgate 2003). La larve véligère se caractérise par la présence d'un organe bilobé cilié, le vélum, qui lui permet de nager et de se nourrir par filtration. Douze jours après la fécondation, l'umbo devient plus proéminent et la larve est dite « umbonée » et mesure 140 μm . A la fin du cycle larvaire pélagique, les larves font en moyenne 230 μm et développent un pied et un organe sensoriel sous la forme d'une tache pigmentaire appelée « œil » qui marque l'entrée dans le stade pédivéligère ou oeuillé. A ce stade, la larve

alterne alors des phases de nage et de reptation sur différents substrats grâce au pied. La durée de la phase larvaire en éclosure est en moyenne de 21 jours avant fixation (Doroudi and Southgate 2003). Cependant elle varie en milieu naturel entre 15 et 30 jours en fonction des conditions environnementales (Thomas *et al.* 2011).

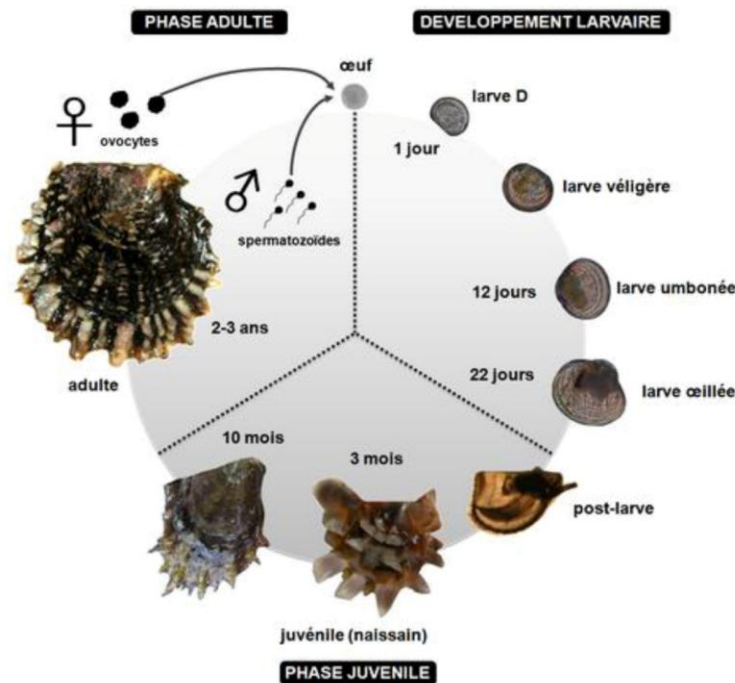


Figure 1.5 : Cycle de développement de l'huître perlière *P. margaritifera*. Après une fécondation externe, le développement de *P. margaritifera* se divise en 3 phases : la phase larvaire pélagique durant 3 semaines, la phase juvénile marquée par la métamorphose et la fixation au substrat, et la phase adulte benthique. (Schéma : Linard 2010, d'après Thomas 2009)

Après la métamorphose, la larve qui s'est transformée en « post-larve » (250 μm) va se fixer au substrat par des filaments du byssus, eux-mêmes guidés par le pied. Le vélum a disparu au profit des branchies et une nouvelle coquille se forme : la dissoconque. A ce moment, les post-larves sont appelées « juvéniles » ou « naissains ». La croissance des nacres est ensuite très rapide au cours des 3 premières années (10-12 cm), suivie d'un ralentissement, jusqu'à atteindre une taille maximale en moyenne de 15-18 cm selon les atolls (Pouvreau and Prasil 2001). La longévité ou l'espérance de vie d'une huître perlière du stock naturel est encore peu connue, cependant Gervis et Sims (1992) estiment que *P. margaritifera* peut vivre jusqu'à 30 ans.

1.3. FORMATION DE LA PERLE

BIOMINÉRALISATION

La biominéralisation est un processus largement répandu au sein du monde vivant, et les principes de biominéralisation sont extrêmement variés, à divers points de vue. En effet, les exemples pouvant illustrer ce phénomène sont très nombreux et diversifiés, tant du point de vue du type du biominéral formé et de sa fonction, que du point de vue des espèces exploitant ce processus, puisqu'on y trouve des représentants dans chacune des trois branches de l'arbre du vivant : Bacteria, Archea et Eukarya (Lecointre and Le Guyader 2007). Lowenstam et Weiner (1989) ont démontré chez les mollusques pas moins de 21 types de minéraux impliqués dans plus de 17 fonctions biologiques différentes. Notons parmi les biominéralisations amorphes, la présence de carbonate de calcium, de phosphate de calcium, de silice et parmi les minéraux cristallins, la calcite, l'aragonite, la vaterite, mais aussi la ferrihydrite, la magnétite, l'opale ou la goethite (Marie 2008). Une des fonctions majeures des structures biominéralisées est de constituer un support mécanique à diverses fonctions biologiques, telles que le maintien ou la motion (squelette des vertébrés) ou encore la préhension ou le broyage de la nourriture (dents, lanterne d'Aristote des oursins ...). Mais la biominéralisation offre bien plus qu'un support structural. Cette construction de la nature joue un rôle dans une variété de fonctions biologiques d'importance telles que : la protection contre les prédateurs (coquilles des mollusques), l'isolement par rapport au milieu extérieur (la coquille d'œuf), la perception de la gravité (statolithes), ou encore le stockage d'ions minéraux ... (Lowenstam and Weiner 1989).

La formation de la coquille des mollusques est l'aboutissement de la mise en place d'un processus de biominéralisation contrôlé par ces organismes. Les processus de biominéralisation mis en jeu se décomposent en une série d'étapes successives qui se déroulent au sein de compartiments distincts de natures diverses (manteau, espace extrapalléal, périostracum et coquille). Ces différents compartiments ont des rôles bien spécifiques, et agissent en synergie pour élaborer la coquille. Aujourd'hui, on sait que les différents composants de la coquille s'organisent en une architecture bien précise dont l'organisation

répond à une exigence structurale et fonctionnelle (Furuhashi *et al.* 2009). La coquille des mollusques possède une structure caractéristique multicouche, composée de carbonate de calcium sous la forme de calcite et/ou aragonite, comprenant une fraction variable (0,01% à 5%) de matière organique (Weiner 1986 ; Marin *et al.* 2012). Les polymorphes ont la même formule chimique mais diffèrent de par leur structure cristalline. Ils présentent de ce fait des propriétés physiques et chimiques différentes (Morse *et al.* 2007). La calcite et l'aragonite, respectivement de formes rhomboédrique et orthorhombique du carbonate de calcium (CaCO_3) sont retrouvées dans des zones bien distinctes des coquilles nacropismatiques de certains bivalves, tels que *P. margaritifera*.

L'élément central de cette mécanique est le manteau (le tissu minéralisateur), dont les cellules de l'épithélium externe (du côté de la coquille) qui sont le siège d'une activité cellulaire intense aboutissant à la synthèse des composants de la matrice organique (Gong *et al.* 2008; Jackson *et al.* 2010; Liu *et al.* 2011). La matrice organique est composée de protéines, glucoprotéines, lipides et polysaccharides, et est sécrétée par les cellules de l'épithélium externe. Bien que cette matrice ne représente que 0.1 à 5% de la composition totale de la coquille (en poids sec, Weiner 1986), elle est essentielle car elle interagit avec la surface des cristaux en orientant sa nucléation, et en contrôlant le polymorphisme soit en aragonite ou calcitique (Falini *et al.* 1996).

La formation de la perle qu'elle soit naturelle ou de culture est un processus complexe qui est toujours un sujet de recherches d'actualité. Les perles sont le résultat de la capacité des huîtres à biominéraliser via le manteau (Taylor and Strack 2008). Au niveau de la formation de la perle après l'acte de greffe, l'épithélium externe monostatif du greffon au contact du nucléus va se multiplier, et totalement englober le nucléus pour former un sac perlier en 14 jours chez *P. margaritifera* (Kishore and Southgate 2016). Ce sac perlier est composé d'une seule couche épithéliale qui conserve la fonction physiologique minéralisatrice du greffon dont il est issu et dépose des couches de nacre autour du nucléus. C'est le point de départ de la formation de la perle. Kishore et Southgate (2016) ont estimé que les premiers dépôts nacriers se font environ 30 jours après la greffe, ce qui confirme de précédentes études montrant que les

dépôts d'aragonite se font aux alentours de 15 jours après le développement du sac perlier (Kawakami 1952 ; Machii and Nakahara 1967) (Figure 1.6).

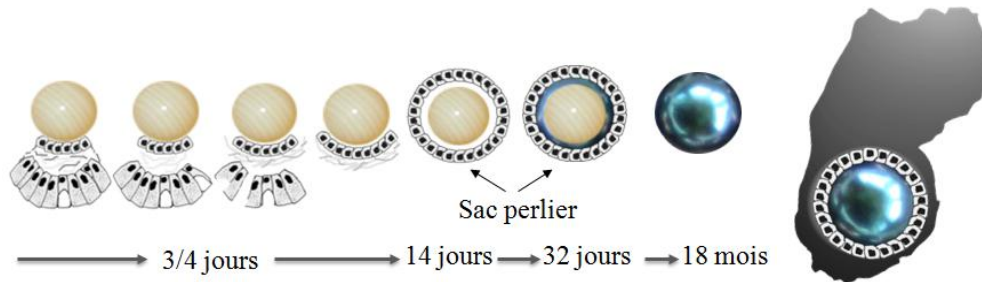


Figure 1.6: Mise en place du sac perlier et minéralisation de la perle. L'épithélium externe monostratifié du greffon au contact du nucléus va se multiplier, et totalement entourer le nucléus pour former un sac perlier au bout d'environ 18 jours. Le sac perlier est à l'origine du processus de biominéralisation qui aboutit au dépôt des couches de nacre à la surface du nucléus (à partir du 32^{ème} jour environ), et à la formation de la perle, récoltée au bout de 18 mois. (Schémas: modifié d'après Joubert (2011)).

PHENOTYPES DE LA PERLE

Dans cette thèse, les perles ont été qualifiées par 6 principaux facteurs à savoir : la taille (décomposée en poids et épaisseur de dépôt perlier), le grade comprenant les défauts de surface et le lustre, la forme, la présence de cerclage et enfin la couleur avec sa pigmentation principale ainsi que sa foncitude (Ky *et al.* 2013) (Figure 1.7). La relative importance de chacun dépend essentiellement des producteurs, bijoutiers et marchés visés.

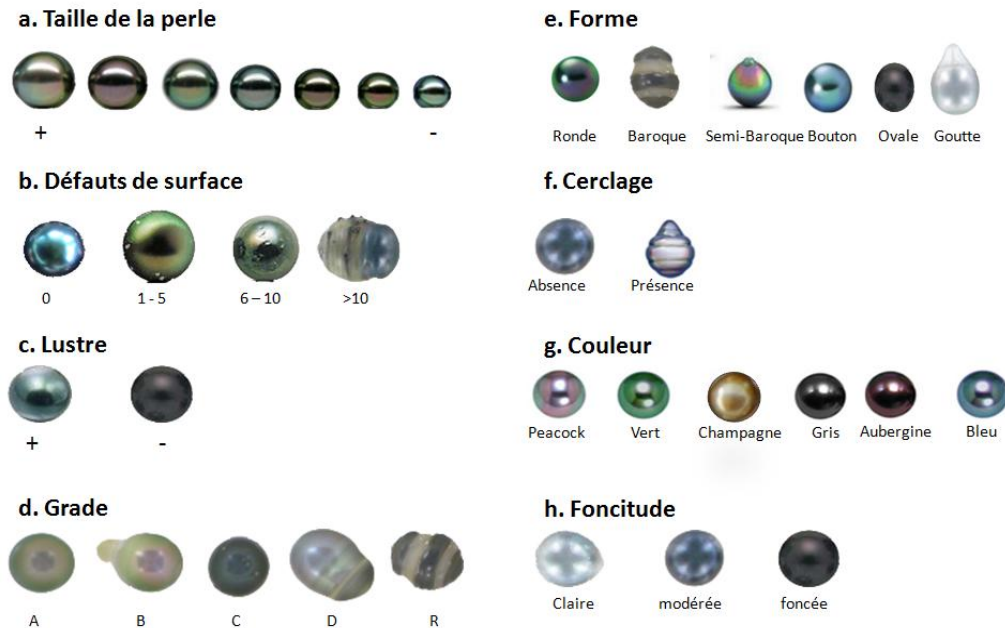


Figure 1.7 : Phénotypes des perles de *P. margaritifera*. a : la taille des perles. b : les catégories de défauts c : le lustre d : le grade e : les formes. f : cerclage. g : la couleur. h : la foncitude (© Ifremer)

La taille se mesure en millimètre arrondi à l'unité inférieure, et varie généralement entre 8 et 16 mm. Le poids se mesure en gramme (Blay *et al.* 2014).

Le lustre correspond à la réflexion de la lumière sur la surface de la perle plus ou moins parfaite (éclat ou brillance). Une perle hautement lustrée signifie une haute réflexion (effet miroir) tandis qu'une perle avec un faible lustre apparaît terne, mate ou encore calcaire. Le lustre est un critère très important car généralement les clients achèteront préférentiellement une perle baroque lustrée plutôt qu'une perle ronde mat (Matlins 2002). Ce critère de classification est hautement subjectif en raison d'une perception variable de chacun (Strack 2006).

La surface de la perle peut présenter diverses imperfections telles que : des piqûres, des rayures, des stries, des frisures, des creux, des bourrelets, des sillons, des dépôts organiques, des dépôts de calcite ou des zones de dévitalisation.

Le grade ou encore classification de la perle de culture de Tahiti dépend de 2 critères : l'état de la surface, qui s'évalue en fonction de l'importance de la surface lisse (sans imperfection) et du lustre. Le grade de la perle se codifie ainsi en 6 catégories : Parfaite (Top Gemme), A (10% d'imperfections légères concentrées et très bon lustre), B (30% d'imperfections légères concentrées et tolérance de deux imperfections profondes et bon lustre), C (60% d'imperfections légères concentrées et tolérance de 10% d'imperfections profondes, lustre moyen), D (plus de 60% d'imperfections légères et 20% de profondes, lustre faible). Sont définies comme rebuts des perles présentant l'un des critères suivants : plus de 20% de zones dévitalisées, plus de 20% de dépôts organiques, plus de 20% de dépôts de calcite, le nucléus visible, des piqûres profondes ou une épaisseur de la couche perlière inférieure à 0,8mm.

Les formes adoptées par les perles peuvent être très variables, mais sont regroupées en trois catégories de base à savoir les sphériques, les baroques et symétriques ; r : ronde, semi-ronde, b : semi-baroque et baroque et o : goutte (ou poire ou ovale), bouton respectivement. La présence de cerclage est également un critère avec présence ou absence de cercle quel que soit la forme d'origine. Les perles de Tahiti ont une proportion relativement importante de perles dites cerclées correspondant à une rainure concentrique à la surface de la perle. Les perles rondes sont les plus commercialisables du à leur rareté (Matlins 2002).

Enfin, la couleur de la perle est considérée comme un critère additionnel à sa classification. Il est possible que la perle possède une ou plusieurs couleurs secondaires. Les perles de Tahiti diffèrent de manière importante des autres productions de perles dans le monde par leurs couleurs naturellement foncées, à dominante gris sombre. Par ailleurs, les fameuses perles noires de Tahiti offrent une palette de couleurs naturelles très variées, aux reflets verts, peacock, aubergines, bleus, roses, cuivres, bronzes, dorés, argents ... qui les rendent toutes uniques.

De nombreux facteurs semblent influencer la qualité de la perle, il en résulte une variabilité importante aux niveaux des différents phénotypes de la perle avec des qualités très hétérogènes d'une récolte et d'une

ferme à l'autre. On estime en moyenne que 43% des perles récoltées sont commercialisables, et seulement 3% sont de qualité parfaite (Top Gemme).

1.4. CONTRIBUTION DE LA SELECTION GENETIQUE

Une fois que le cycle de vie en captivité d'une espèce d'aquaculture est maîtrisé (on parle alors de domestication), il est alors envisageable de travailler à sélectionner sur la base de phénotypes plus recherchés afin d'améliorer les productions. La sélection est un processus par lequel, la diversité, présente dans la population, expose les différences entre individus à un tri et conduit à un différentiel de reproduction. Les individus possédant le phénotype le mieux adapté dans un environnement donné sont sélectionnés et les allèles qu'ils portent se répandent dans la population sur les générations suivantes. La sélection génétique n'est possible que (i) s'il existe une variation populationnelle de la valeur des caractères, (ii) si cette variation affecte des caractères liés directement ou indirectement à la valeur sélective des individus et (iii) si les modalités des caractères sont hérissables.

L'homme a sélectionné de façon artificielle aussi bien dans le monde végétal qu'animal, soit de façon à éliminer les caractères négatifs par exemple en éliminant les individus malades ou faibles, soit de façon positive en gardant par exemple les individus grandissant plus vite ou produisant plus de viande ou de lait. Dans le monde aquacole, cette sélection orientée est devenue essentielle, même si les programmes de sélection sont assez récents comparé au monde terrestre. On retrouve ainsi des exemples de programme de sélection en aquaculture marine, principalement sur des poissons avec des espèces telles que la carpe (Bakos 1979; Wohlfarth *et al.* 1980), le tilapia (Eknath *et al.* 1993), le saumon (Friars 1993), la truite (Gjedrem 1992) et quelques autres espèces (Gjedrem and Rye 2016). Chez les bivalves, les études concernant les réponses à la sélection sont positives et encourageantes. Des études passées ont montré une variation génétique pour la croissance (aux stades de développement larvaire et/ou juvénile) pour la moule *Mytilus edulis* (Innes and Haley 1977; Newkirk *et al.* 1981; Mallet *et al.* 1986; Strömngren and Nielsen 1989), les huîtres *Crassostrea Gigas* and *C. virginica* (Lannan 1972; Losee 1978; Newkirk 1978 ; Evans

and Langdon 2006; Degremont *et al.* 2007; Swan *et al.* 2007), les clam *Mercenaria mercenaria* (Rawson and Hilbish 1990, 1991; Hadley *et al.* 1991; Hilbish *et al.* 1993), *Argopecten irradians* (Zheng *et al.* 2006), l'abalone *Haliotis asinina* (Lucas *et al.* 2006), la moule perlière *Hyriopsis cumingi* (Jin *et al.* 2012) et enfin les huîtres perlières *Pinctada fucata martensii* (Wada 1986 ; Wada and Komaru 1996 ; He *et al.* 2008 ; Wang *et al.* 2010), *Pinctada maxima* (Kvingedal *et al.* 2010 ; Jerry *et al.* 2012). Mais à l'heure actuelle, les travaux d'amélioration génétique sur l'huître perlière *P. margaritifera* ne font que commencer car le cycle biologique n'est maîtrisé que depuis peu.

Bien que la domestication en aquaculture soit jeune, le potentiel d'exploiter le haut niveau de diversité génétique existant en utilisant le stock sauvage est un avantage significatif lorsque l'on veut développer un nouveau programme de sélection génétique. Au sein d'une population, l'importance de la diversité génétique naturelle est associée à une meilleure santé générale et résistance/adaptabilité aux pressions de l'environnement fluctuant (Reed and Frankham 2003) et est donc favorable pour toute intervention en aquaculture. En plus de cela, une réponse soutenue à l'effort de sélection est dépendante de la présence suffisante de diversité génétique au sein d'une population et permettra également de continuer la sélection si les objectifs du programme changent au cours du temps (Davis and Hetzel 2000).

En Polynésie française, la perliculture repose essentiellement sur la collecte naturelle du naissain sur des supports artificiels contrairement à d'autres espèces comme chez *P. maxima* ou *P. fucata* où la production de nacres en écloserie s'est développée récemment et constitue l'essentiel de l'apport en huîtres. Afin de prévenir d'une dérive génétique, des méthodes ont été développées chez *P. fucata*, l'huître perlière japonaise (Yu and Chu 2006) et *Crassostrea virginica* (Yu and Guo 2006) utilisant quelques centaines de géniteurs, des stimulations contrôlées et des pontes synchronisées, promouvant le succès reproducteur pour la plupart des individus impliqués et réduisant les conséquences de l'expression du fardeau génétique.

LA VARIABILITE PHENOTYPIQUE

Les caractéristiques morphologiques d'une population peuvent se décrire par des valeurs de variances et de moyennes. La sélection fait varier la moyenne et/ou la variance des caractères de la population de différentes façons. Elle peut être directionnelle, stabilisatrice ou disruptive. Pour une distribution normale d'un trait, la sélection directionnelle favorise des phénotypes différents de la moyenne de la distribution et donc déplace la moyenne du trait dans une direction ou dans l'autre, pouvant diminuer la variance (Price *et al.* 1988). Le phénomène de sélection stabilisatrice élimine les phénotypes les plus divergents réduisant ainsi l'hétérogénéité, donc la variance du trait tout en gardant une moyenne identique (Zhang and Hill 2007). La sélection disruptive quant à elle, favorise les phénotypes extrêmes au détriment des phénotypes moyens, provoquant jusqu'à une distribution bimodale, ce qui augmente la variance du trait sans nécessairement changer sa moyenne (Rueffler *et al.* 2006).

L'expression d'un caractère ou phénotype, se traduit chez tout individu par une performance dont la mesure est généralement notée **P** (pour Performances ou valeur phénotypique). Elle est la somme de 2 facteurs. Le premier est génétique **G** (effet combiné de tous les effets génétiques), le second est dû au milieu et aux influences qu'il exerce sur les performances appelé **E** pour environnement. Contrairement aux effets de l'environnement, seule la valeur génétique d'un animal pour un caractère donné se transmet en moyenne à sa descendance. La valeur phénotypique peut aussi se décomposer de la façon suivante :

$$\mathbf{P} = \mathbf{G} + \mathbf{E} = (\mathbf{A} + \mathbf{D} + \mathbf{I}) + \mathbf{E}$$

Où **A** est la valeur d'amélioration ou valeur génétique additive c'est-à-dire la fraction transmissible à la descendance (la somme des effets moyens des gènes paternels et maternels), **D** est la déviation de dominance due à l'ensemble des effets de dominance entre gènes homologues et **I** est la déviation d'épistasie due aux interactions entre couples de gènes homologues.

L'HERITABILITE

La génétique quantitative permet d'explorer l'architecture génétique complexe des traits phénotypiques (Kruuk and Hadfield 2007). La fraction variable dans un trait phénotypique dont l'origine de transmission est génétique est appelée héritabilité (Falconer and Mackay 1996; Roff 1997; Lynch and Walsh 1998). Les méthodes de génétique quantitative permettent de déterminer la part de la variance phénotypique dite génétique par rapport à la part dite environnementale. L'héritabilité (h^2) exprime la proportion de la variation dans la population qui est attribuable à des différences génétiques entre les individus. Il est intéressant d'évaluer la capacité des parents à transmettre leurs gènes à leur descendance. L'héritabilité (h^2), dans un contexte d'amélioration génétique d'une souche, indique la part du progrès réel (phénotypique) qui est dû à la génétique. Dans une population et pour un caractère donné, l'héritabilité au sens large correspond à un paramètre génétique qui quantifie la part de la variabilité génétique ($V(G)$) dans la variabilité observée du caractère $V(P)$ soit

$$H^2 = \frac{V(G)}{V(P)} = \frac{\text{variance génétique}}{(\text{variance génétique additive} + \text{variance génétique non additive} + \text{variance environnementale})}$$

Et l'héritabilité au sens étroit correspond à quantifier la part cette fois de la variabilité de la génétique additive (Falconer 1989):

$$h^2 = \frac{V(A)}{V(P)} = \frac{\text{variance génétique additive}}{(\text{variance génétique additive} + \text{variance génétique non additive} + \text{variance environnementale})}$$

En somme pour un caractère, être héritable, c'est présenter une variation dans la population qui soit transmissible à la génération suivante. L'héritabilité indique également quelle est, en moyenne, la part de la supériorité phénotypique qui est d'origine génétique et donc transmise aux descendants. Elle est comprise entre 0 et 1. Elle permet de prédire si l'amélioration génétique par sélection sera efficace et si la sélection est une stratégie adaptée pour améliorer ce caractère dans une population donnée.

Comprendre comment le génotype influence le phénotype est essentiel que ce soit au sein des populations sauvages afin de comprendre l'évolution en milieu naturel mais aussi dans les populations domestiquées afin d'évaluer les programmes d'amélioration et de sélection (Villemereuil *et al.* 2013).

1.5. QUELS SONT LES DETERMINISMES DE LA FORMATION DE LA PERLE ?

CONTRIBUTIONS DE LA DONNEUSE ET DE LA RECEVEUSE SUR LA PERLE

D'un point de vue génétique, une greffe associe deux génotypes distincts dont leurs propres identités génétiques sont maintenues au travers de l'organisme greffé. Ce processus est très bien documenté dans le règne végétal, aussi bien au niveau du rôle du greffon, du porte greffe que des interactions greffon/ porte greffe. Le porte greffe est souvent choisi par rapport à sa compatibilité, son adaptation au sol, sa vigueur, sa forme, sa résistance aux maladies alors que le greffon sera choisi pour ce que l'on veut sélectionner comme par exemple le fruit ou la fleur que l'on souhaite. En effet la greffe est couramment employée dans le domaine de l'agronomie afin de manipuler les phénotypes du greffon (Warschefsky *et al.* 2016). Le porte greffe, lui est un élément crucial du fait de son rôle dans l'apport en eau et nutriment. Dans le système végétal, les caractéristiques du porte greffe semblent diriger la croissance végétative et l'ensemble de la croissance du système greffé dépend des conditions environnementales (Vazifeshenas *et al.* 2009).

Dans notre modèle d'étude, des similarités semblent exister avec le système de greffon/porte greffe comme suggéré par de précédentes études. En effet, il est généralement considéré que le manteau issu de l'hôte donneuse est le premier responsable de la biominéralisation des gènes pour la formation de la coquille comme de la perle (Farn 1986). Des évidences issues de génotypage du sac perlier et de la comparaison allélique à l'aide de microsatellites avec ceux de la donneuse et de la receveuse ont montré que l'ADN originaire de la donneuse et de la receveuse sont présents dans le sac perlier récolté (Arnaud-Haond *et al.* 2007). Cependant, la composition génétique précise du sac perlier n'a pas encore été

déterminée et le rôle des cellules du donneur, receveur et leur contribution au développement de la perle restent inconnus.

La présence de l'ADN du manteau de la donneuse dans le sac perlier et les traits phénotypiques reliés à la donneuse suggèrent que les cellules de l'huître donneuse de greffon sont activement impliquées dans le processus de biominéralisation de la perle de culture. Plusieurs protéines régulant la sécrétion de la matrice organique et nacrée ont été identifiées dans le manteau, mais le rôle de ces protéines dans la formation de la perle de culture reste à ce jour mal connu. Le tissu de l'huître donneuse de greffon pour la production de la perle est excisé de la zone palliale, zone dont il a été montré qu'elle ne sécrétait que la couche nacrée dans la formation de la coquille (Sudo *et al.* 1997 ; Takeuchi and Endo 2006 ; Taylor and Strack 2008). La matrice protéique responsable de la formation de la couche nacrée dans la coquille est centrale dans la formation de la perle (Bédouet *et al.* 2001, 2007). Les protéines constituant la matrice organique contrôlent le processus de biominéralisation : polymorphisme de CaCO_3 (calcite et aragonite) et les microstructures des couches de la coquille (Marin *et al.* 2007). Les recherches sur le processus sont essentiellement focalisées sur les protéines de la coquille. Depuis la publication de la première séquence complète amino-acide des protéines de la nacre en 1996 (Miyamoto *et al.* 1996), de nombreuses protéines ont été identifiées et contribueraient au mécanisme moléculaire soulignant le développement de la coquille et de la perle en jouant un rôle dans la nucléation, orientation ou encore la morphologie des cristaux (Gao *et al.* 2016) comme PIF (Zhao *et al.* 2014), MSI60 (Sudo *et al.* 1997), Pearlin (Suzuki and Nagasawa 2013 ; Miyashita *et al.* 2000 ; Montagnani *et al.* 2011). D'autres sont impliquées dans la couche prismatique (calcite) comme Aspein (Tsukamoto *et al.* 2004 ; Takeuchi *et al.* 2008), Prismaticin-14 (Suzuki *et al.* 2004 ; Suzuki and Nagasawa 2007), la famille des Shematin (Joubert *et al.* 2010 ; Marie *et al.* 2012) et une autre impliquée à la fois dans la couche calcitique et aragonitique Nacrein (Miyamoto *et al.* 1996 ; Suzuki and Nagasawa 2013). En quelques années (depuis 1996), le nombre de protéines connues comme associées aux coquilles de mollusques a crû de manière exponentielle, surtout avec l'utilisation de techniques combinées de protéomique et de transcriptomique (Montagnani *et al.* 2011 ; Huang *et al.*

2013 ; Suzuki and Nagasawa 2013 ; Miyamoto *et al.* 2013 ; Shi *et al.* 2013 ; Marie *et al.* 2012). Chez *P. margaritifera*, des travaux ont été réalisés sur le manteau (Joubert *et al.* 2010 ; Marie *et al.* 2012) et à ce jour 80 protéines de la matrice coquillière ont été découvertes. Une récente étude s'est intéressée au déterminisme de la couleur via l'étude de l'albinisme et l'expression différentielle des gènes correspondant mettant en avant 5 gènes possiblement impliqués dans la coloration de la coquille (SHEM4, MP8, KRMP, CHIT et SERP) (Lemer *et al.* 2015).

Malgré des études afin de déterminer le rôle précis de ces protéines de la matrice dans la formation de la coquille (Tsukamoto *et al.* 2004 ; Takeuchi and Endo 2006 ; Inoue *et al.* 2010), des zones d'ombre restent présentes quand à leur rôle exact dans le processus de formation de la coquille. De récentes recherches sur l'expression des protéines de la matrice dans le sac perlier ont examiné leur potentiel influence sur la formation de la perle (Inoue *et al.* 2010, 2011 ; Wang *et al.* 2009 ; Funabara *et al.* 2014). Wang *et al.* (2009) a montré une différence d'expression entre certaines protéines entre le tissu greffé (le manteau) comparé au sac perlier, sous entendant une implication de l'huître receveuse sur la biominéralisation notamment par les hémocytes qui ont la capacité de se lier aux ions calcium (Mount *et al.* 2004). Il est intéressant d'établir le lien entre l'expression des gènes de biominéralisation du greffon et du sac perlier avec la qualité de la perle afin d'approfondir nos connaissances sur la fonction de ces protéines. Mais également de déterminer qui, de l'huître donneuse ou receveuse, est responsable de l'expression des gènes en lien avec la biominéralisation et qui contribue à la formation de la perle.

Comprendre les fondements génétiques de la formation de la perle fournirait des connaissances solides pour élucider le processus biologique général et pourrait apporter des informations de valeur pour l'industrie de l'huître perlière. Par exemple, à travers la sélection génétique utilisant des huîtres qui possèdent des gènes particuliers en lien avec des traits de la perle pourrait améliorer sa qualité. En ce qui concerne l'huître perlière *P. fucata* l'un des bivalves les plus importants dans la production mondiale de perles (Southgate and Lucas 2011), les programmes de sélection ont commencé au Japon dans les années 1960, et ont permis d'améliorer la proportion de perle de qualité. Les paramètres génétiques ont été

estimés pour les traits de croissance coquillière (Wada 1984, 1986). En Chine, les programmes de sélection sur *P. fucata* sont plus récents (2002). Des lignées sélectionnées pour une croissance accélérée ont été créées via sélection massale et les paramètres d'héritabilité concernant les traits relatifs à la croissance ont été estimés (He *et al.* 2008 ; Wang *et al.* 2010 ; Hua-yang1a *et al.* 2011)). Récemment le génome a été séquencé permettant d'obtenir des informations sur les mécanismes moléculaires impliqués dans la biosynthèse des perles (Takeuchi *et al.* 2012). Une récente étude a permis d'associer des marqueurs SNP et gène de biominéralisation afin de faciliter la sélection par marqueurs (Zhan *et al.* 2016). Chez *P. maxima*, les programmes de sélection sont plus récents. Le génome de cette espèce n'a pas encore été séquencé. Les paramètres génétiques et les interactions génotype/ environnement ont été estimés pour la réponse à la sélection de la croissance (Kvingedal *et al.* 2010). Une étude a permis d'identifier des QLT en association aux traits de croissance de la coquille (Jones *et al.* 2014). Enfin, l'étude de Jerry *et al.* (2012) montre pour la première fois que les traits de la perle seraient héréditaires. En effet si les traits de qualité de la perle ont une base génétique alors la sélection génétique en faveur de ces traits permettrait d'augmenter substantiellement la proportion en perle de haute qualité.

Concernant *P. margaritifera*, l'intérêt de l'industrie perlicole pour la sélection génétique et l'amélioration des huîtres perlières est grandissant et assez récent. Les principales études se sont concentrées sur les aspects phénotypiques de la perle. En effet la qualité d'une perle est déterminée par un ensemble très complexe de facteurs intrinsèques et extrinsèques durant la longue phase de culture, dans laquelle l'huître donneuse de greffon, sauvage (Tayale *et al.* 2012) ou issue d'écloserie (Ky *et al.* 2013), joue un rôle primordial. Quelques études phénotypiques ont examiné la couleur des perles montrant une tendance pour la coloration de la perle à être influencée par la couleur de la donneuse chez *P. maxima*, *P. margaritifera* et *fucata martensii* (Alagarwami 1987 ; Wada and Komaru 1996 ; McGinty *et al.* 2012 ; Ky *et al.* 2016). Chez *P. margaritifera*, nos premiers résultats sur la sélection génétique familiale pour les caractères de qualité de la perle montrent l'existence d'une propension marquée au sein des familles à donner, à l'issue de la greffe, une perle d'une couleur plutôt qu'une autre (Ky *et al.* 2014), ou des variations significatives

inter-familiales au niveau du dépôt perlier (Blay *et al.* 2014). Mais aucune étude concernant les paramètres génétiques de la coquille ou de la perle n'a été réalisée.

CONTRIBUTION DE L'ENVIRONNEMENT SUR LA PERLE

Etonnement, l'impact de l'environnement sur la biominéralisation des perles est très peu étudié, et les études existantes se sont principalement focalisées sur l'influence de l'environnement après l'acte de greffe ou encore sur les huîtres perlières non greffées. Une étude a montré que la proportion de perle de haute qualité serait augmentée si les huîtres receveuses subissent un traitement à faible salinité pendant 14 jours après la greffe (Atsumi *et al.* 2014). Une autre étude a montré que les niveaux de température et de nourriture influencent la croissance coquillière et le niveau d'expression des gènes de biominéralisation du manteau chez *P. margaritifera* (Joubert *et al.* 2014). La température est considérée comme un facteur important. La saison privilégiée pour la récolte est la saison dite fraîche. En effet, une plus faible température ralentirait le métabolisme de l'huître et aurait un impact sur les couches finale de nacre de la perle, notamment sur le lustre de la perle (Alagarswami 1987 ; Menzel 1991). D'autres facteurs, tel que l'acte de greffe, de surgreffe (Ky *et al.* 2015; Demmer *et al.* 2016) et la saison au cours de laquelle cette greffe est réalisée constituent aussi des éléments à prendre en considération, pour optimiser le taux de maintien des greffes (Ky *et al.* 2014), ainsi que la forme des perles produites (Ky *et al.* 2015). L'environnement au sens large, ainsi que les interactions « génotype x environnement », jouent donc un rôle important dans la détermination de la qualité d'une perle (Ky *et al.* 2015). Une étude récente a montré qu'il n'y a ni d'effet de la concentration en microalgues sur les traits de qualité de la perle après 12 mois de culture, ni d'effet du pré conditionnement des huîtres receveuses sur le niveau d'expression de gènes dans le sac perlier ni sur les traits de qualité de la perle (Latchere *et al.* 2017).

De nombreux points sont encore inconnus, comme par exemple les effets intrinsèques de la donneuse (i.e. paramètres génétiques, âge, taille) ou les effets extrinsèques (i.e. des sites de culture) sur les phénotypes de la perle, ou encore sur les gènes de biominéralisation (Figure 1.8).

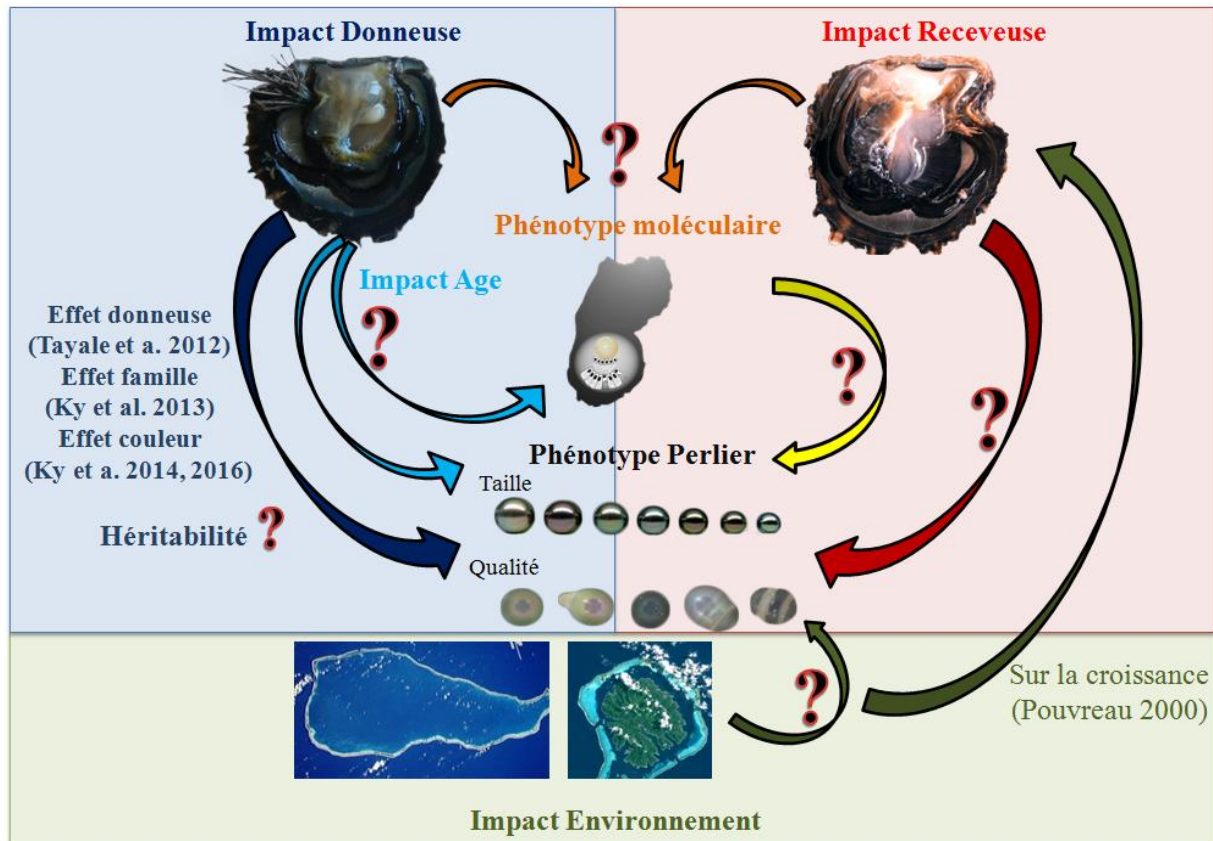


Figure 1.8 : Etat des lieux des impacts de la donneuse, de la receveuse et de l'environnement sur les phénotypes moléculaire et perlier chez *P. margaritifera*.

2. OBJECTIFS DE LA THESE ET STRUCTURE

Les recherches menées dans cette thèse s'inscrivent dans la continuité des travaux réalisés depuis 2011 sur l'amélioration de qualité de perle chez *P. margaritifera* via la sélection génétique. Comprendre le rôle de l'huître donneuse et receveuse sur la formation de la perle est l'objectif principal de cette thèse. La production de perle de culture est un processus biologique unique et complexe impliquant la contribution génétique de deux huîtres (la receveuse et la donneuse). Avant de commencer un programme de sélection, il est essentiel d'établir la contribution génétique de ces deux huîtres au niveau de l'expression des protéines liées à la biominéralisation et la variabilité des traits phénotypiques de perle.

Le premier axe de cette étude est une approche globale (Figure 1.9). Le manque de connaissance sur l'impact de l'environnement et le lien entre l'expression des gènes de biominéralisation et la qualité de la perle nous ont amenés à faire une étude préliminaire sur l'impact du site de culture sur les traits phénotypiques de la perle (**chapitre 2**), mais également d'étudier les gènes de biominéralisation impliqués dans les phénotypes qualité de la perle (**chapitre 3**).

Chapitre 2 : Quel est l'impact du site de culture sur la qualité des perles issues de donneuses de greffon produites en éclosérie ? Le chapitre 2 est présenté sous forme d'un article scientifique publié dans la revue *Estuarine, Coastal and Shelf Science*.

Chapitre 3 : Quel est le profil d'expression des gènes de biominéralisation suivant la qualité de la perle récoltée ? Le chapitre 3 est lui aussi présenté sous forme d'un article scientifique soumis dans *Marine biotechnology*.

Après cette approche globale, le second axe de cette thèse s'intéresse au rôle de la donneuse et de la receveuse sur le phénotype de la perle obtenue (Figure 1.9). Nous avons décidé d'étudier sous deux approches le phénotype taille de l'huître et ses conséquences sur le phénotype de la perle. Pour cela nous avons étudié l'influence de la taille de la donneuse à âge fixe (**Chapitre 4**) et âge variable (**Chapitre 5**) sur la production de perle afin de savoir si le potentiel de biominéralisation du greffon varie en fonction de la taille ou bien de l'âge de la donneuse :

Chapitre 4 : Quelle est la contribution relative du phénotype taille des huîtres donneuses et receveuses sur les phénotypes de la perle à âge fixe ? Le chapitre 4 est présenté sous la forme d'un article scientifique publié dans la revue *Scientific reports*.

Chapitre 5 : Quelle est l'influence de l'âge de l'huître donneuse de greffon sur les phénotypes de la perle produite et le potentiel de biominéralisation associé ? Ce chapitre est présenté sous la forme d'un article scientifique qui doit être soumis prochainement dans la revue *Plos one*.

Et enfin dans le dernier axe de cette thèse, nous avons estimé l'héritabilité et mesuré la contribution de la génétique dans la variation des traits phénotypiques de la perle ainsi que ceux de l'expression des gènes de biominéralisation du greffon et du sac perlier (**Chapitre 6**) (Figure 1.9). Ces valeurs sont essentielles afin de commencer un programme de sélection génétique. Je me suis servie de familles parent/descendant produites en éclosure et suivies en greffe expérimentale afin d'obtenir une matrice d'apparentement. J'ai intégré le pédigrée dans un modèle animal pour quantifier la contribution de la variance génétique additive aussi bien au niveau de l'expression des gènes de biominéralisation du greffon (au moment de la greffe), du sac perlier (au moment de la récolte) que dans les traits phénotypiques de la perle (produit de la greffe). Ces analyses ont permis de répondre à la question suivante : **quel est le potentiel évolutif de la population de *P. margaritifera* pour l'amélioration de la qualité de la perle ?** Ce chapitre est présenté sous la forme d'une publication soumise dans la revue *Molecular Ecology*.

Le **chapitre 7** fait la synthèse des principaux résultats obtenus au cours de cette thèse et résume les rôles de l'huître donneuse et receveuse sur le processus de la formation de la perle du phénotype aux gènes. Des nouvelles directions de recherche sont aussi abordées comme les perspectives d'un point de vue appliquées, le déterminisme fonctionnel des protéines, ou encore le déterminisme de la plasticité phénotypique (au niveau de la couleur).

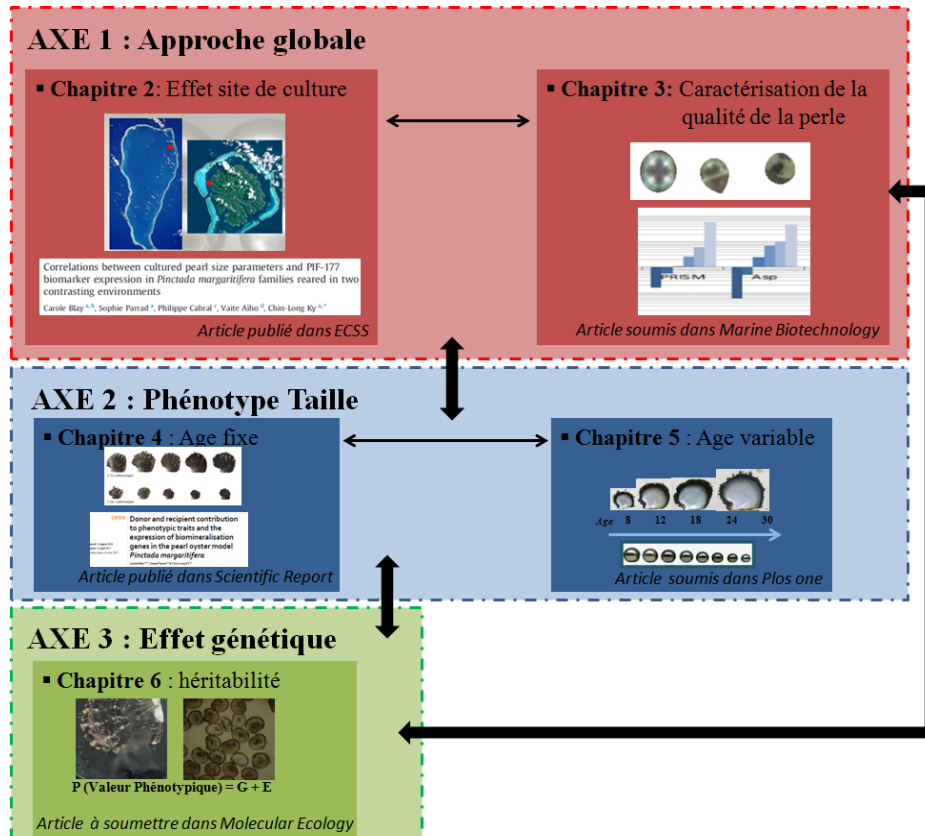


Figure 1.9 : Résumé graphique des axes de la thèse

En annexe, plusieurs publications auxquelles j'ai pu participer avant ou au cours de mes années de thèse sont présentées.

En annexe 1, cette étude s'intéresse aux relations entre la taille et les paramètres de qualité de la perle. Quelle est l'influence du poids/épaisseur de dépôt perlier sur le grade et la couleur de la perle chez *Pinctada margaritifera* ? Elle a été publiée dans *Aquaculture international*.

Blay C., Sham-Koua M., Vonau V., Tetumu R., Cabral P., Ky C.L., 2014, Influence of nacre deposition rate on cultured pearl grade and colour in the black-lipped pearl oyster *Pinctada margaritifera* using farmed donor families. *Aquaculture International* 22:937-953

En annexe 2, l'étude porte sur l'effet du site de culture (Rangiroa/ Tahaa) sur les traits grade, forme et couleur de la perle. Cette annexe est présentée sous la forme d'un article publié dans *Aquaculture Research*.

Ky CL, **Blay C**, Aiho V, Cabral P, Le Moullac G, Lo C., **2017**. Macro-geographical differences influenced by family-based expression on cultured pearl grade, shape and colour in the black-lip pearl oyster *Pinctada margaritifera* : a preliminary case study in French Polynesia. *Aquaculture Research*, 48(1), 270-282

En annexe 3, l'étude porte sur l'influence de l'âge sur le grade et la couleur de la perle via des expérimentations de greffes et surgreffes. Cette annexe est présentée sous la forme d'un article publié dans *Aquaculture Research*.

Ky CL, Demmer J, **Blay C**, Lo C, **2017**. Age-dependance of cultured pearl grade and colour in the black – lipped pearl oyster *Pinctada margaritifera*. *Aquaculture research* 48(3), 955-968.

L'annexe 4 présente mon curriculum vitae

Chapitre 2

Correlations between cultured pearl size parameters and PIF-177 biomarker expression in *Pinctada margaritifera* families reared in two contrasting environments

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Correlations between cultured pearl size parameters and PIF-177 biomarker expression in *Pinctada margaritifera* families reared in two contrasting environments

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1. ABSTRACT

The black-lipped pearl oyster, *Pinctada margaritifera*, produces the largest and most valuable coloured pearls in the world. Cultured pearl size remains one of the most important quality traits. Despite the great geographical area covered by pearl farms in the atolls of French Polynesia, little is known about the influence of grow-out site effects on pearl size attained. To explore the genetic and environmental impact on the size of pearls as well as the genetic x environment interaction, a uniform experimental graft was designed on two contrasting macro-geographical lagoons. Five biparental families of donor oysters were grafted and then reared at both sites. After 18 months of culture, phenotypic parameters corresponding to pearl size, i.e. nacre weight and thickness, were recorded among the harvested pearls. The expression of Pif-177 gene, a biomarker encoding protein in the aragonite nacreous layer, was analysed in the corresponding pearl sac. The results show a family effect for nacre weight and thickness on both sites, with family F058 producing the heaviest and thickest nacre, and F805 the lightest and thinnest. By contrast, inter-site comparison revealed no significant site effects for these two parameters. In addition, grow-out location did not modify the relative gene expression of Pif-177 in the pearl sac between donor families in either culture site. Both nacre weight and thickness were positively correlated with the level gene expression of Pif-177. These results suggest that pearl size parameters were not affected by the environment in the present study and this is supported by the relative gene expression of Pif-177 observed. This knowledge constitutes an initial step in the study of pearl size trait inheritance, which will be helpful in the near future for the diffusion of genetically selected donor oyster lines produced by hatchery systems throughout production sites.

Keywords: Pearl oyster; *Pinctada margaritifera*; Pearl size; PIF-177; Environment

2. INTRODUCTION

Pearl production is based on the aquaculture of the mollusc *Pinctada margaritifera* var. *cumingi* (also known as the black-lipped pearl oyster). Production and annual exportation of cultured pearls increased from 86 kg in 1980 to 16 tons in 2012 (7.7 million cultured pearls), representing nearly 65 million Euros in export value (customs statistics, Wane 2013). Today, French Polynesia boasts 487 pearl farms representing 7,772 hectares of maritime culture concession area, distributed among 25 atolls and islands with 79 pearl farms in the Gambier Archipelago, 43 in Society and 365 in Tuamutu (2013 DRMM statistics data) (Figure 2.1). The commercial value of cultured pearls depends on different parameters, including pearl size (nacre thickness and weight), shape (which includes the presence or absence of “circles”), colour (body colour and overtone), darkness level, classification grade, lustre and surface quality. Pearl size remains one of the most important traits, with the largest pearls being the most valuable (with equal value for the other quality traits). In French Polynesia, the export of cultured pearls is strictly controlled: pearls must have a nacreous layer with a minimum thickness of 0.8 mm built up on the nucleus or else they must be destroyed.

The long-term development of the pearl industry in French Polynesia will rely on the development of private hatcheries that can guarantee production of selected spat, to improve commercially important traits such as pearl size. This trait, like many quantitative and qualitative traits, is controlled by genetics, environmental factors and their interactions (Falconer and Mackay 1996). Genotype by environment (G*E) interaction for pearl quality traits have been estimated on *P. maxima* reared at two commercial grow-out locations (Jerry *et al.* 2012). They showed that pearl size has a heritable basis on the donor oyster side. In addition the phenotypic correlation between recipient shell weight and size of pearls has been examined in *P. fucata* and a small positive relationship has been found whereby recipient oysters with heavier valves produced bigger pearls (Wada and Komaru 1996). It is therefore possible that an underlying genetic correlation exists between the two traits, whereby selection for faster growing oysters

might also improve at least the pearl size. Another major consideration for the pearl industry is the grow-out location effect, as many pearl farms are geographically distant and subject to the influence of different environmental conditions. Indeed, many studies show that the environment plays an essential role in somatic and shell growth. Shell growth rate is influenced by rearing environment, types of islands (atoll vs. island) for *P. margaritifera* (Pouvreau and Prasil 2001), and different salinities in the ocean for *P. maxima* (Kvingedal *et al.* 2008). In most pearl oyster, the main environmental factors affecting growth rate are water temperature and food availability. In *P. fucata martensii*, the shell growth rate could increase at locations with greater chlorophyll concentration and adequate water temperatures (20–26°C), resulting in a longer growing season (Tomaru *et al.* 2002).

Cultured pearls are the result of the biomineralization process of a piece of the mantle tissue (“*saibo*”) of a selected donor oyster, inserted together with a round bead of nacre (the “nucleus”) into the gonad of a recipient oyster. The outer mantle epithelial cells of the implanted mantle graft elongate and surround the nucleus, where a pearl sac is formed within 1 to 3 weeks after nucleus implantation (Ellis and Haws 1999). The graft deposits successive layers of different calcium carbonate polymorphs around the nucleus during the entire period of culture (18 to 24 months) (Wilbur 1964; Wataba 1988). The shell of *P. margaritifera* consists of two distinct structures: the inner nacreous layer composed of aragonite and the outer prismatic layer made of calcite. Shell formation is a highly controlled process involving multiple matrix proteins. In *P. fucata*, the mantle tissue was seen to be differentiated into two regions, the dorsal “nacreous-layer formation” region and the ventral “prismatic-layer forming” region, so as to produce these two clearly distinct shell layers (Takeuchi and Endo 2006). Nacre is formed by hundreds of thousands of aragonite crystals in a protein matrix, which overlap alternately on the nucleus, as observed by electronic microscopy (Zhang and Xu 2013). Recently, the number of genes identified as coding for molluscan shell matrix components has increased (Joubert *et al.* 2010; Montagnani *et al.* 2011; Huang *et al.* 2012; Suzuki and Nagasawa 2013; Miyamoto *et al.* 2013), revealing the wide variety of proteins implicated in the biomineralization process (Miyamoto *et al.* 2005; Suzuki *et al.* 2009). Marie *et al.* (2012) identified more

than 80 shell matrix proteins from *P. margaritifera* and *P. maxima* from the nacreous and prismatic layers, such as Pif-177, Nacrein, Pearlin, NUSP, and Shematin. They demonstrated that prisms and nacre are assembled from very different protein repertoires. The Pif-177 gene is an important component in the nacreous layer and takes part in the initiation of aragonite crystallization as well as subsequent stacking of aragonite tablets in the nacreous layer (Joubert *et al.* 2010; Zhao *et al.* 2014). Functional analyses using RNAi experiment against Pif expression showed that the nacreous layer became disordered, indicating that Pif plays a key role and could be used as biomarker in the aragonite nacreous layer formation (Suzuki *et al.* 2009).

In an effort to understand the genetic and environmental contributions to the expression of cultured pearl size from *P. margaritifera*, we studied the family effect (“family” means “full sib family” and with an inter family difference, we could plan to do a family selection)” of hatchery donor oysters on two macro-geographically contrasting grow-out site locations (Island vs. atoll), through: 1) the measurement of nacre weight and thickness phenotypes on harvested pearls, and 2) the gene expression of Pif-177 in the corresponding pearl sac. For this, a uniform experimental graft was conducted by using: 1) a single expert grafter; 2) standard nucleus size; 3) a common initial grafting site; 4) donor oyster families produced in a hatchery system; and 5) the same recipient oyster stock. This study provides insight into the potential for successful culture over a wide geographic area of *P. margaritifera* hatchery stocks selected for pearl size traits.

3. MATERIALS AND METHODS

3.1. FULL-SIB FAMILY PRODUCTION AND REARING

Five bi-parental *P. margaritifera* families (named F058, F612, F804, F805 and F806) were produced in the Ifremer hatchery system facilities (Vairao - Tahiti, French Polynesia, using wild female and male broodstock from Takapoto atoll (Tuamotu Archipelago, French Polynesia). The corresponding breeding and larval rearing procedures were conducted using the protocol developed by Ky *et al.* (2013). The five families produced in a hatchery at distinct period have different age at graft moment, oysters from F058 family have 5 years old, oysters from F612 have 4 years old and oysters from F804, F805 and F806 families have 2 years old.

The juveniles were detached when they reached an average size of 30 mm. They were then pierced and tied together onto a CTN (Cord Technical Nakasai) rearing system (Cabral *et al.* 1985), where they were left until the start of the grafting experiment. The CTN was protected using plastic mesh to prevent predation and transferred to the lagoon where it was suspended on long lines. The animals were maintained at a depth of around 6 m and cleaned every 4 months. Two months prior to nucleus implantation, oysters representing all five families were taken from the rearing station, detached, washed with a high pressure spray (to remove epibiota) and stored ready for use in the grafting procedure. Samples of the five donor families were randomly selected and transported by plane to Rangiroa atoll (Tuamotu Archipelago) to allow the oysters to adapt to local environmental conditions.

3.2. GROW-OUT SITE LOCATIONS: RANGIROA ATOLL AND TAHAA ISLAND

Rangiroa atoll is located in the north-western part of the Tuamotu Archipelago (15°07'S, 147°38'W), about 350 km northeast of Tahiti (Figure 2.1). The atoll has a flattened elliptic shape, is

80 km in length and ranges from 5 to 32 km in width. The lagoon surface is 1446 km². The site of culture is located at Avaturu village (Gauguin's pearl farm).

Tahaa Island is located in the Society Archipelago (16°37'S, 151°30'W), about 2230 km northwest of Tahiti (Figure 2.1). The island surface area is 88 km². The island shares its lagoon of 290 km² with Raiatea Island. The culture site was located at Love Here pearl farm.

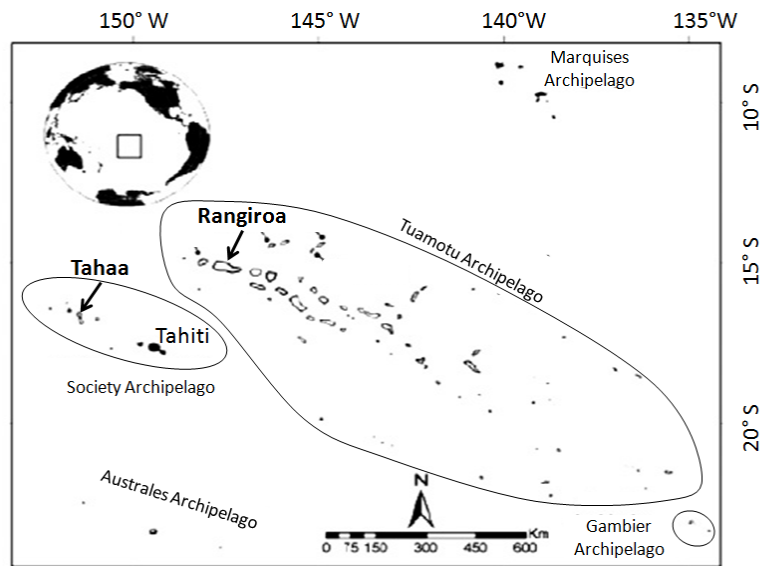


Figure 2.1 : Macro-geographical location of Rangiroa atoll (Tuamotu Archipelago) and Tahaa Island (Society Archipelago) in French Polynesia.

3.3. GRAFTING PROCEDURE AND SAMPLE HARVEST

All graft operations were performed by the same professional grafter in pearl farm production conditions (Gauguin's pearl farm, Rangiroa). A total of 50 donors (10 donors per family) were used to perform 1500 grafts (30 grafts per donor) over a 4-day period (Figure 2.2).

Recipient oysters from the wild were grafted using 2.4 BU nuclei (7.304 mm diameter; Nucleus Bio, Hyakusyo Co. Japan). Following implantation, the recipient oysters were placed in retention net bags and identified by labels according to the donor family and individual donor oyster. Recipient oysters were

checked 45 days post operation to estimate nucleus retention rates and mortalities. At this time, recipient oysters grafted from a single donor oyster were randomly split into two groups—one was kept at Rangiroa lagoon (574 oysters) and the second was transferred by plane to Tahaa Island (607 oysters). After 16 months of culture, the pearls were harvested. Some *keshi* (small non-nucleated pearls formed when an oyster rejects and expulses the implanted nucleus) were also harvested but these were not graded in the present study. At the same time, one recipient oyster that had been grafted from each donor was dissected to collect its pearl sac tissue. A total of 98 pearl sacs (50 from Rangiroa and 48 from Tahaa) were thus sampled and preserved in RNALATER®.

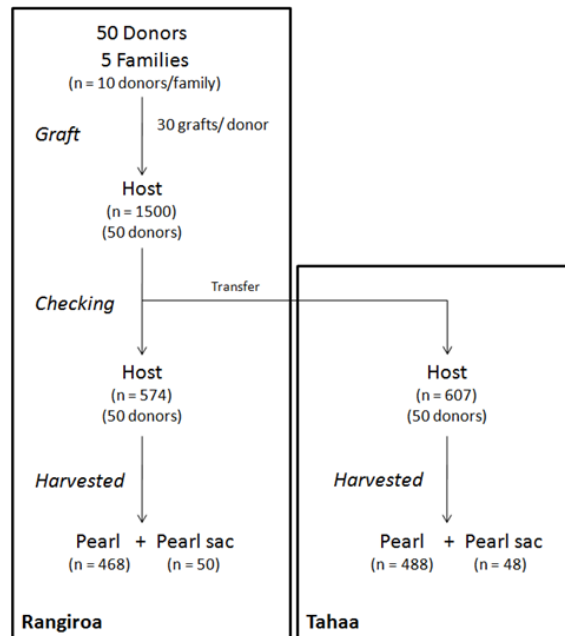


Figure 2.2 .: Experimental design of the grafting procedure and sample harvest. All the 1500 graft operations were performed in Rangiroa. After checking, recipient oysters grafted from each donor oyster were randomly split into two groups, one was left in Rangiroa and the second was transferred to Tahaa. Harvests were made in both sites.

3.4. MEASUREMENT OF PEARL SIZE PARAMETERS: NACRE WEIGHT AND THICKNESS

Cultured pearls were cleaned by ultrasonication in soapy water (hand washing) with a LEO 801 laboratory cleaner (2-L capacity, 80 W, 46 kHz).

The size of the cultured pearls was assessed by the measurement of two quantitative variables: nacre thickness and weight. Nacre thickness was measured using a digital micrometer (for diameter evaluation) (Ky *et al.* 2013).

$$\text{Nacre thickness} = \frac{\text{cultured pearl diameter} - \text{nucleus diameter}}{2}$$

Nacre weight was estimated using a digital balance (for weight evaluation)

$$\text{Nacre weight} = \text{cultured pearl weight} - \text{nucleus weight.}$$

3.5. PIF-177 GENE EXPRESSION IN THE PEARL SAC

Total cellular RNA was extracted using TRIzol® reagent (Life Technologies) from the harvested pearl sacs of *P. margaritifera*, according to the manufacturer's recommendations. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). The expression levels of the target gene Pif-177 were analysed by quantitative RT-PCR analysis and two reference genes. First, strand cDNA was synthesized from 500 ng total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and a mix of poly (dT) and random hexamer primers. Real-Time PCR amplifications were carried out on a Stratagene MX3000P. The amplification reaction contained 12.5 µL Brilliant II SYBR Green QPCR Master Mix (Stratagene), 10 µL cDNA template, and 2.5 µL of each primer (400 nM) in a final volume of 25 µL. Each run included a positive cDNA and a blank control for each primer pair. The run protocol was as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s. Lastly, the amplicon melting temperature curve was analysed using a melting curve program: 45–95°C with a heating rate of 0.1°C s⁻¹

and a continuous fluorescence measurement. All measurements were made in duplicate. All analyses were based on the Ct values of the PCR products. Relative gene expression levels were calculated using the delta–delta method normalized with two reference genes to compare the relative expression results (Livak and Schmittgen 2001) as follows: Relative expression $_{(\text{target gene, sample } x)} = 2^{-(\Delta\text{Ct}_{\text{sample, sample } x} - \Delta\text{Ct}_{\text{calibrator, sample } x})}$. Here, the ΔCt calibrator represents the mean of the ΔCt values obtained for the tested gene. The delta threshold cycle (ΔCt) is calculated by the difference in Ct for the target and reference genes. The relative stability of 18S and SAGE combination was confirmed using NormFinder (Anderson *et al.* 2004).

3.6. STATISTICAL ANALYSIS

Differences in nucleus retention and rejection rate, oyster mortalities, and harvested pearl and keshi rate among the five families in each grow-out location were evaluated using χ^2 tests.

For quantitative nacre weight and thickness data, the normality of distribution and the homogeneity of variance were tested and gene expression data was used with Shapiro–Wilk tests and F-tests.

Due to non-normality of nacre weight, thickness and Pif – 177 gene expression data, non-parametric ANOVA tests were used, the Scheirer–Ray–Hare (SHR) (Scheirer *et al.* 1976), to test for differences between families and site culture. If the overall test was significant, a Dunn procedure with a Bonferroni correction was performed among all pair of progenies. This procedure is an extension of the Kruskal–Wallis ranks test that allows the calculation of interaction effect.

Inter-culture site effect was evaluated for nacre weight and thickness at two levels, by considering: 1) the pooled value of the five families using χ^2 test, and 2) each family separately using Fisher’s exact test (Winer *et al.* 1991).

Spearman’s correlation coefficient was calculated to measure the strength of the relationship of pearl sac gene expression and the nacre weight and nacre thickness of the culture pearls harvested at the 5% alpha level. The gene expression values of each donor were associated with the corresponding nacre weight and thickness of each pearl having contributed to each donor pool. Therefore, the correlation was

tested using the 49 values of relative gene expression corresponding to Rangiroa donors and the 48 values of relative gene expression corresponding to Tahaa donors.

In all cases, the differences were considered statistically significant when p values were lower than 0.05. All analyses were performed using XLSTAT (version 2009.4.02) and R software (version 3.1.2).

4. RESULTS

4.1. NUCLEUS RETENTION AND REJECTION, HARVESTED SAMPLES AND OYSTER MORTALITIES / PREDATION RATES

The 1500 grafted pearl oysters showed 79% ($N = 1181$) nucleus retention, 15% ($N = 228$) nucleus rejection and 6% ($N = 91$) mortality and/or predation at 45 days post grafting. No family effect was recorded for nucleus retention ($p = 0.598$). By contrast, both nucleus rejection and oyster mortality / predation showed a significant donor family effect: $\text{Khi}^2 = 11.461$, $\text{df} = 4$, $p = 0.022$.

After 18 months of culture, 956 cultured pearls were harvested: 468 in Rangiroa atoll and 488 in Tahaa Island. No significant donor family or site effects were detected for the number of harvested pearls. The number of keshi harvested was 3% ($N = 15$) for the Rangiroa site and 6% ($N = 35$) for the Tahaa site. A significant site effect was detected for keshi harvested: $\text{Khi}^2 = 14.82$, $\text{df} = 2$, $p = 0.004$. At harvest time, mortality / predation during the entire culture period was evaluated on both sites, with Rangiroa showing 16% ($N = 91$) and Tahaa showing 10% ($N=61$). A significant site effect was detected for mortality / predation ($\text{Khi}^2 = 14.82$, $\text{df} = 2$, $p = 0.004$).

4.2. CULTURED PEARL NACRE WEIGHT AND THICKNESS

The average nacre weight among the 956 culture pearls was 0.80 ± 0.317 g, with minimum and maximum values of 0.18 g and 2.92 g, respectively. Inter-culture site comparison revealed no significant effect for nacre weight (Table 1).

In Rangiroa atoll, the average nacre weight was 0.79 ± 0.291 g. A significant donor family effect was detected for nacre weight in Rangiroa ($H = 34.81$, $df = 4$, $p < 0.0001$). In Tahaa Island, the average nacre weight was 0.806 ± 0.340 g and a significant donor family effect was also recorded ($H = 33.77$, $df = 4$, $p < 0.0001$). Cultured pearls from the family F805 were the lightest in both culture sites, with a weight of 0.70 ± 0.265 g nacre on average. Samples from family F058 showed the greatest average nacre weight in both sites, with 0.93 ± 0.389 g for Tahaa and 0.90 ± 0.307 g for Rangiroa (Figure 2.3). No significant effect of culture site was seen for either family.

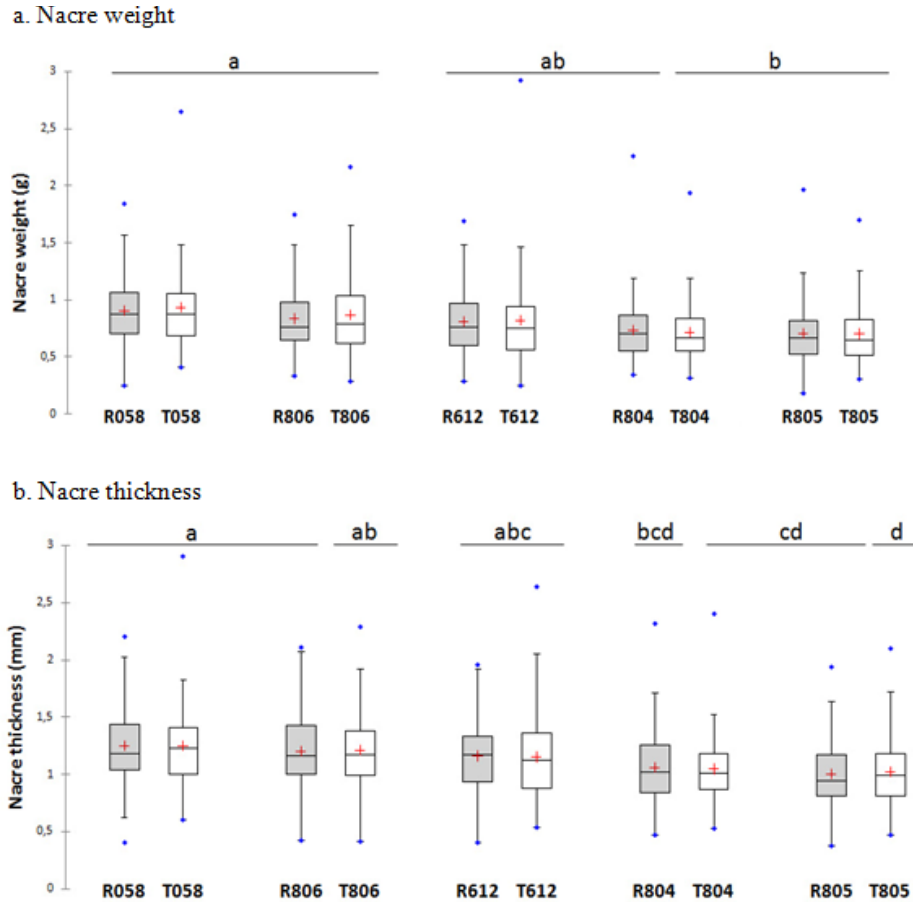


Figure 2.3 : . Box-plot showing: a. nacre weight (expressed in g.) and b. thickness of nacre (in mm) of cultured pearls produced using graft tissue from each of the *P. margaritifera* donor progenies. Each box-plot has the following 6 elements: 1) mean ("+" cross in the box-plot); 2) median (solid bar in the box-plot); 3) 25th to 75th percentile (rectangular box); 4) 1.5*interquartile range (non-outlier range of the box whiskers); 5) minimum and maximum values (extreme dots); and 6) outlier values (outside box whiskers). Box-plots in grey represent data from Rangiroa atoll and box-plots in white represent data from Tahaa Island. Statistical differences between families are indicated by a letter

The average nacre thickness among the 956 culture pearls was 1.13 ± 0.334 mm, with minimum and maximum values of 0.37 mm and 2.90 mm, respectively. Inter-culture site comparison revealed no significant effect for nacre thickness (Table 2.1).

Tableau 2.1 : Family site and family x site effects on nacre weight, thickness and Pif-177 gene expression (Scheirer–Ray–Hare non parametric ANOVA test).

Factor	Nacre weight	Nacre thickness	Pif-177 expression
Family	<0.0001	<0.0001	0.0003
Site	ns	ns	ns
Family * Site	ns	ns	ns

In Rangiroa atoll, the average nacre thickness was 1.13 ± 0.316 mm. A significant donor family effect was detected in Rangiroa for nacre thickness ($H = 41.24$, $df = 4$, $p < 0.0001$). In Tahaa Island, the average of nacre thickness was 1.13 ± 0.351 mm. Intra-site comparison showed a significant donor family effect ($H = 34.93$, $df = 4$, $p < 0.0001$). Cultured pearls from family F805 had the thinnest nacre in both culture sites (1.00 ± 0.281 mm and 1.02 ± 0.282 mm). Samples from the F058 family showed the greatest average nacre thickness in both sites with 1.24 ± 379 mm for Tahaa and 1.25 ± 0.324 mm for Rangiroa (Figure 2.3).

Among the five families, no significant culture site effect was observed for nacre thickness.

Nacre weight was strongly correlated with nacre thickness ($Rho = 0.963$; $p < 0.0001$) (Table 2.2).

Tableau 2.2 : Correlation matrix between relative Pif-177 gene expression and nacre weight and thickness. The Spearman's coefficient correlation (in italics) is presented on the right and the level of significance (in bold) on the left of the matrix.

	Pif-177 gene expression	Nacre weight	Nacre thickness
Pif-177 gene expression	1	<i>0.259</i>	<i>0.283</i>
Nacre weight	0.010	1	<i>0.963</i>
Nacre thickness	0.005	<0.0001	1

Correlation was significant at $p < 0.05$ ($n = 97$)

4.3. PIF-177 GENE RELATIVE EXPRESSION IN THE PEARL SAC

Within the same donor family, the comparison of the relative expression of the Pif-177 gene showed that it was not significantly different between the culture sites (Figure 2.3). By contrast, the comparison between the families revealed a significant family effect for Pif-177 relative expression in each of the sites: $p = 0.033$ ($H = 10.45$, $df = 4$, Rangiroa) and $p = 0.040$ ($H = 10.00$, $df = 4$, Tahaa). At the Rangiroa location, relative expression of the Pif-177 gene in family F058 showed the highest relative expression mean with 5.274 ± 6.198 compared with family F612 (1.889 ± 1.631), F804 (1.029 ± 1.734), F805 (1.044 ± 1.022) or F806 (1.625 ± 1.136). At the Tahaa location, family F058 showed the highest mean relative expression (5.479 ± 3.209) compared with family F612 (2.851 ± 2.438), F804 (1.328 ± 1.203), F805 (2.012 ± 2.189) or F806 (2.223 ± 1.753) (Figure 2.4). Pif-177 relative gene expression was positively correlated with nacre weight and thickness ($Rho = 0.259$; $p = 0.010$ and $Rho = 0.283$; $p = 0.005$, respectively) (Table 2.2).

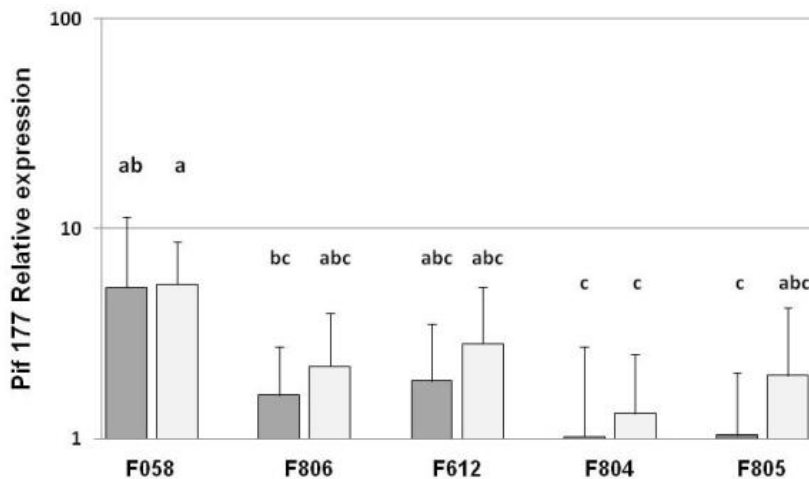


Figure 2.4 : Pif-177 gene relative expression performed on *P. margaritifera* pearl sacs ($N = 10$) each grow-out location. Histograms in dark grey represent data from the Rangiroa location and histograms in light grey represent data from Tahaa Island. Statistical differences between grow-out locations are indicated by letters.

5. DISCUSSION

5.1. MACRO-GEOGRAPHICAL SITE EFFECTS

Macro-geographical site effects (environment effect) of Tahaa Island and Rangiroa atoll did not seem to influence the size attained by cultured pearls in *P. margaritifera* in the present study. Indeed, no site effect was recorded for: 1) the nacre weight and thickness from harvested oysters that were grafted with saibo from the same donor family, and 2) the corresponding Pif-177 gene relative expression in the pearl sac. This is a surprising result as the differences between the contrasting lagoons of Tahaa Island and Rangiroa atoll involve multiple and complex factors, such as physical water parameters, during the entire oyster culture period, which often includes different seasonal variations (Ky *et al.* 2015). The similarity could be explained by the fact that these two distinct culture sites could present the same seasonal food availability and low seasonal temperature variation. A study observed a strong dependency between the level of particulate organic matter concentration and the atoll/island latitude in Polynesian lagoons (Charpy *et al.* 1997). In our study, two sites have an intermediate latitude location in French Polynesia, and have the same seasonal environmental parameter variation with low variation in temperature and food availability. The annual average water temperature between the two contrasting site is very close; 28.1°C (27.4 to 29.1°C) for Rangiroa atoll and 27.9°C (26.8°C to 28.8°C) for Tahaa island according to measurement between 1998 to 2001 (Ifremer sources). In fact, chlorophyll concentration and temperature variation are known to influence shell growth and also nacre deposition onto the nucleus; both processes result from the biomineralisation activities of the mantle tissue. Some studies demonstrated significant influence of culture site in *P. margaritifera* shell growth reared in the Cook Island, French Polynesia and the Great Barrier Reef lagoon (Australia) (Sims 1994; Pouvreau and Prasil 2001; Yukihiro *et al.* 2006 respectively). In *P. margaritifera*, the shell growth is influenced by two major factors microalgal concentration and temperature, both of which regulate the expression of most of the shell matrix protein genes implicated in the biomineralisation process (Joubert *et al.* 2014). The two sites (atoll vs. island) were not contrasting as

we think, and it would be better to choose several types of environment with a more important latitude difference and inherently different microalgal concentration and water temperature.

5.2. PEARL SIZE PARAMETERS

Cultured pearl size differed significantly between families where a significant family (genetic) effect was observed, whatever the grow-out sites. Indeed, the average nacre thickness and weight produced from extreme families differed by approximately 15% and 18%, respectively, between the highest and lowest families (F058 was the heaviest and F805 the lightest). The rates (15% and 18%) were similar to those found in a duplicated experimental graft using wild *P. margaritifera* donors (Tayale *et al.* 2012), and hatchery-produced families (Ky *et al.* 2013), where significant family effect was observed. This result confirms the significant genetic role the implanted mantle graft plays in the biomineralization process of a cultured pearl. Given the differences in nacreous deposition for the five families observed in the present study and the importance of cultured pearl size for the value of a pearl, there may be advantages to the industry of using donor pearl oysters that exhibit rapid nacreous deposition (Blay *et al.* 2014). Nevertheless, any correlation between pearl size and other pearl quality traits must also be considered. Indeed, nacre weight and thickness were correlated with pearl darkness levels, with the palest pearls being the smallest (lowest thickness and weight) (Tayale *et al.* 2012; Blay *et al.* 2014). Here, this tendency is confirmed when comparing F058 (the family with fastest nacre deposition) and F805. Our results showed that the palest pearls (low darkness level) had the thinnest layer of nacre and weighed the least. Indeed, F058 also showed significantly less pearls of pale coloration (low darkness level) than F805 (data not shown). In our study, we equally showed that pearl from F806 is significantly thicker and heavier than pearls from F805, two donor families with the same age. Furthermore three families with three different ages (F058, F612 and F806) have not shown significant difference for the nacre weight and thickness. We can conclude that genetic capacities of the donor family oyster predominate on the donor family age because the nacre thickness and weight seems to be not dependant of the family age. In addition, a study

of comparison between the graft-surgreffe and the duplicated graft experiments highlighted the indirect role played by the youth of the recipient oysters, which must be optimized for optimal pearl quality realization and the complex interplay between the donor and the recipient. Indeed, the pearl sac depends on the recipient oyster for the supply of nutrients throughout the period of pearl formation. A strong host oyster can provide sufficient nutrition and, potentially, a more suitable environment for the pearl sac, resulting in the greater vigour of the pearl sac, promoting nacre secretion rates (Yukihira *et al.* 1998; Ky *et al.* 2015).

5.3. PIF-177 GENE EXPRESSION

In addition, Pif-177 gene expression level in the pearl sac of *P. margaritifera*: 1) differed significantly between families, and 2) was at the same level in an intra-family comparison scale, regardless of the grow-out site. Family F058 revealed the higher Pif-177 gene expression and confirmed the phenotypic observation on the corresponding harvested pearls, the heaviest and the thickest samples. Consequently, correlations of Pif-177 gene expression with nacre weight and thickness were observed (data on Table 2). Thus, the Pif-177 gene could constitute an interesting molecular tool for genetic selection in *P. margaritifera*, targeting high nacreous deposition rate (Blay *et al.* 2014). This is linked to the calcium metabolism in the epithelial tissue around the pearl (biomineralization process). The pearl sac is formed during the first 1 to 3 weeks after nucleus implantation. The genome of the donor oyster is still present and active in the pearl sac tissue during the entire period of culture and at harvest time (Arnaud-Haond *et al.* 2007). In *P. fucata* and *P. maxima*, genes originating from the donor oyster were expressed for at least 18 months after implantation in the pearl sac (Masaoka *et al.* 2013; McGinty *et al.* 2010, 2012). Correlations between pearl size and gene expression might need to be factored into future breeding programs for *P. margaritifera*. In addition, the quantitative genetics of these traits might be studied. Re ranking of family performance indicating that the best performing family in one location may not necessarily be the family

with the highest genetic merit when reared at the alternative site. If G x E effects are found to be equally pervasive when oysters are reared at other sites, site-specific breeding programs may need to be set up.

5.4. OYSTERS MORTALITY AND / OR PREDATION

Finally, our results reveal that at harvest time, mortality and / or predation was significantly different between the two grow-out locations studied, with significantly higher levels at Rangiroa atoll. Oyster mortalities during the entire culture process in the lagoon were mainly due to: 1) irreversible trauma after the surgical grafting act (checking at 45 days post graft), and 2) predation, which has become more problematic in oyster aquaculture in French Polynesia, because some animals modify their diet due to both the concentration and abundance of oysters (Ky *et al.* 2013). Our findings can be explained by the fact that oyster predators are more abundant in Rangiroa (DRMM pers. comm.). Indeed, Rangiroa atoll is internationally known for its high fish concentration.

6. CONCLUSIONS

For the first time, this study simultaneously evaluated two aspects of *P. margaritifera* culture in two contrasting grow-out locations: 1) the cultured pearl size through nacre weight and thickness measurements, and 2) the relative expression level of the Pif-177 gene that encodes proteins of the shell matrix in the pearl sac. Accordingly, the results using five donor families produced in a hatchery system showed that: 1) within each grow-out site, family effect was detected as previously reported by Ky *et al.* (2013), and 2) no grow-out site effect was observed for nacre weight and thickness. In addition, nacre weight and thickness were correlated with Pif-177 gene expression in the pearl sac, a gene coding for proteins involved in the biomineralization process.

Further studies, such as the quantitative genetics of pearl size, are needed to confirm the heritable basis of this trait from the *P. margaritifera* donor oyster and the response to shell growth selection in

several types of environment. It would also be beneficial to perform other experimental grafts using other grow-out sites and simultaneously measure some lagoon parameters such as the food availability and temperature variations during the culture period. Overall, the use of multi-trait selection approaches, which take into account quantitative genetic control, environmental effects and associated correlations, may be the most effective strategy to improve pearl quality for the design of future breeding programs of *P. margaritifera*.

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Chapitre 3

Cultured pearl surface quality profiling by the shell matrix protein gene expression in the biomineralised pearl sac tissue of *Pinctada margaritifera*

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Cultured pearl surface quality profiling by the shell matrix protein gene expression in the biomineralised pearl sac tissue of *Pinctada margaritifera*

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1. ABSTRACT

Nucleated pearls are produced by molluscs of the *Pinctada* genus through the biomineralization activity of the pearl sac tissue within the recipient oyster. The pearl sac originates from graft tissue taken from the donor oyster mantle and its functioning is crucial in determining key factors that impact pearl quality surface characteristics. The specific role of related gene regulation during gem biogenesis was unknown, so we analysed the expression profiles of eight genes encoding nacreous (PIF, MSI60, PERL1) or prismatic (SHEM5, PRISM, ASP, SHEM9) shell matrix proteins or both (CALC1) in the pearl sac (N = 211) of *Pinctada margaritifera* during pearl biogenesis. The pearls and pearl sacs analysed were from a uniform experimental graft with sequential harvests at 3, 6 and 9 months post-grafting. Quality traits of the corresponding pearls were recorded: surface defects, surface deposits and overall quality grade. Results showed that: 1) the first three months of culture seem crucial for pearl quality surface determination, and 2) all the genes (*SHEM5*, *PRISM*, *ASP*, *SHEM9*) encoding proteins related to calcite layer formation were over-expressed in the pearl sacs that produced low pearl surface quality. Multivariate regression tree building clearly identified three genes implicated in pearl surface quality, *SHEM9*, *ASP* and *PIF*. *SHEM9* and *ASP* were clearly implicated in low pearl quality, whereas *PIF* was implicated in high quality. Results could be used as biomarkers for genetic improvement of *P. margaritifera* pearl quality and constitute a novel perspective to understanding the molecular mechanism of pearl formation.

Keywords: Biomineralization, Relative gene expression, Pearl sac, Biomarkers, Pearl quality, *Pinctada margaritifera*

2. INTRODUCTION

Biom mineralization refers to the processes by which organisms form minerals. It is an extremely widespread phenomenon, leading to a variety of biological structures such as teeth, bone, otoliths, spicules, shell and pearl (Lowenstam and Weiner 1989). In molluscs, although organic macromolecules, especially proteins, represent no more than 5% (w/w) of shell weight, they play key roles in nucleation, orientation, morphology, polymorphism and organization of the calcium carbonate crystallites of the shell (Belcher *et al.* 1996; Zhan *et al.* 2015). The pearl oyster, which has the unique ability to produce pearls, is an ideal model animal to study biom mineralization. Structurally, the pearl oyster shell consists of two distinct calcified microlaminates, the inner aragonite nacreous layer, which is similar to the nacreous layer of pearls, and the outer calcite prismatic layer (Marin *et al.* 2007). Although both calcite and aragonite are constructed with calcium carbonate, they display distinctly different configurations and characters (Zhan *et al.* 2015). The accumulation of calcium carbonate as calcite and aragonite crystals is thought to be regulated by proteins secreted from the mantle (Funabar 2014). To understand biom mineralization mechanisms, one must possess knowledge on the structures of the organic matrix components together with the entire process.

The production of pearl culture consists of three steps: 1) initial 2 years oyster grow out phase, 2) nucleus implantation and 3) about 2 years of culturing before harvest. Nucleus implantation is a delicate surgical operation where a trained technician inserts a round nucleus made of shell and a small piece of mantle (*saibo*) from the outer mantle of a donor mollusc into a diverticulum of the gonad of a recipient oyster (Southgate 2008). Around the nucleus, a pearl sac (PS) is formed by proliferation of the outer mantle epithelial cells of the mantle graft (Inoue *et al.* 2010), which secretes successive nacre layers onto the nucleus. The established pearl sac consists of mucous cells containing large acidophilic granules and epidermal cells (Liu *et al.* 2012) that secrete proteins resulting in cultured pearl formation, a highly controlled biom mineralisation process similar to development of inner shell regulated by the mantle (Zhan *et al.* 2015). The role of the pearl sac in nacreous layer biom mineralization is thought to mirror the role of

the oyster mantle that was grafted (Arnaud-Haond *et al.* 2007; Wang *et al.* 2009; Inoue *et al.* 2010). Tahitian cultured black pearl quality is determined according to a wide range of criteria, including pearl classification grade, surface quality and lustre, shape, colour (bodycolor and overtone), darkness level and size (Tayale *et al.* 2012; Ky *et al.* 2013). In French Polynesia, the production of cultured pearls of *Pinctada margaritifera* remains the most valuable export industry and is the second most important source of income after tourism (Ky *et al.* 2016). It has been estimated that only 5–10% of cultured pearls per harvest are of gem quality, but this small percentage accounts for about 95% of a farm's income (Ellis and Haws 1999). Producing cultured pearls of high quality with *P. margaritifera* is one of the major challenges for the pearl industry in French Polynesia, making the process of biomineralization of the nacreous layer of great economic interest. The regulation of shell matrix proteins in biomineralization during the pearl culture and the impact of this process on pearl quality are therefore priority research topics.

Many studies have focused on oyster shell formation because the nacreous layer of shells is structurally similar to the nacreous layer of pearls. Since the report of the first nacre-shell protein nacrein in 1996 (Miyamoto *et al.* 1996), dozens of shell matrix proteins have been found to contribute to the molecular mechanism underlying the development of shell and pearl, playing important roles in crystal nucleation, orientation, polymorph morphology (Gao *et al.* 2016). Some genes are involved in the formation of nacreous aragonite, such as *PIF* (Zhao *et al.* 2014), *MSI60* (Sudo *et al.* 1997) and *Pearlin* (Suzuki and Nagasawa 2013; Miyashita *et al.* 2000; Montagnani *et al.* 2011). Other genes involved in prismatic calcite include *Aspein* (Tsukamoto *et al.* 2004; Takeuchi *et al.* 2008), *Prismalin-14* (Susuki *et al.* 2004; Suzuki and Nagasawa 2007) and the *Shematin* family (Joubert *et al.* 2010; Marie *et al.* 2012). One gene, *Nacrein*, has been found in both the nacreous and prismatic layers of the shell (Miyamoto *et al.* 1996; Susuki and Nagasawa 2013). The role of this matrix of proteins in crystal formation has been examined (Addadi 1985; Belcher *et al.* 1996; Falini *et al.* 1996; Levi *et al.* 1998; Weiner 1975). Although other proteins have been isolated as matrix proteins from the prismatic and nacreous layers, it is not clear how these two layers are formed in molluscan shells (Funabara *et al.* 2014).

The functioning of the pearl sac is crucial in determining key factors that impact pearl quality surface characteristics. Detailed knowledge of the variation in gene expression of protein constructing the matrix in the pearl sac during the biogenesis of the gems is an essential step toward a better understanding of pearl formation process that can help us towards the objective of improving overall pearl quality. In this study, we monitored the gene expression of 8 matrix proteins in the pearl sac by using quantitative RT-PCR, according to: 1) duration of culture, at 3, 6 and 9 months post grafting operation, and 2) cultured pearl surface quality traits, which were surface defects, grades and surface deposits (no nacreous deposit). Through these analyses, we obtained new insights into the determination of *P. margaritifera* pearl quality based on a molecular approach. These results allowed us to build a model based on expression of the eight genes, and to make predictions about a quality pathway. This study set out to explore the status of functional genes, especially those regulating pearl formation, to see whether these are constant or variable according to pearl surface quality and duration of the culture period. Results of this study may contribute to the development of adapted grafting methods and aquaculture processes that will better take into account gene expression regulation pathways and the role they play in the determination of surface quality.

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL ANIMAL AND TISSUE SAMPLING

Pearl oysters were cultured in the lagoon of Mangareva for in situ experiments (Regahiga pearl farm, Gambier Archipelago, French Polynesia). A total of 600 grafts (40 donors) were performed under standard production conditions over a 2-day period, using 1.8 BU nuclei (5.45 mm diameter; Imai Seikaku Co. Ltd., Japan). All grafts were performed by a single expert grafter so as to avoid any grafter effect (described in Ky *et al.* 2015) on pearl quality traits. Details of the grafting process are given in Blay *et al.* (2017).

The experiment was monitored over time to evaluate changes in the gene expression of the protein matrix. Two recipient oysters were harvested for each donor after 3, 6, and 9 months of culture. Pearls and pearl sac tissue were collected at the same time. At the time of pearl harvest and in order to minimize the mixture of recipient tissues, the pearl sacs were excised from host oysters by removing the outer layers with a surgical blade until only a thin (< 0.5 mm) layer of tissue surrounding the pearls remained, and immediately transferred into 2.0 ml tubes with RNAlater® where they were preserved until RNA extraction (McGinty *et al.* 2012). A total of 80 pearl sacs were sampled every 3 months, giving a total of 240 samples over 9 months of culture.

3.2. EVALUATION OF PEARL SURFACE QUALITY

Once harvested, cultured pearls were cleaned by ultrasonication in soapy water with a LEO 801 laboratory cleaner (2-L capacity, 80 W, 46 kHz). They were rinsed in distilled water. Some *keshi* (small non-nucleated nacre deposits) were also harvested but these were not graded in the present study.

Surface defects, grades and surface deposits (no nacreous deposit) of the cultured pearls were evaluated. Visible surface defects on the sampled pearls, including pits, bumps and scratches, were counted visually (without a magnifier) and each cultured pearl was then classified into one of the four categories: “0” (no defects), “1” (1 to 5 defect(s)), “2” (6 to 10 defects) and “3” (up to 10 defects). Cultured pearl grade was also determined for each pearl according to the official Tahitian classification (Journal Officiel 2001 n° 30, 26 July 2001) from the most to the least valuable quality: A, B, C, D and “Rejects” (*rebut*). Briefly, the five grades are based on surface purity and lustre, from A (cultured pearls showing no surface defects or small defects confined to less than 10% of their surface and having very good lustre) to D (cultured pearls showing many highly visible defects over more than two thirds of their surface and having poor lustre) and “Rejects” (cultured pearls that have too many defects to be graded). “Rejects” are discarded and ultimately destroyed. Grade descriptions are illustrated in Blay *et al.* (2014). Surface deposits or other surface flaws involving the nacreous layer or not were classified into 5 categories: 0 (no deposit), + (10% deposit coverage), ++ (25% of deposit), +++ (50% of deposit) and ++++ (complete coverage by deposit).

The culture pearl quality description is illustrated in Figure 3.1. To ensure homogeneity in parameter assessment, all evaluations were made visually (without a jeweller’s loupe) by two operators working together and cross checking.

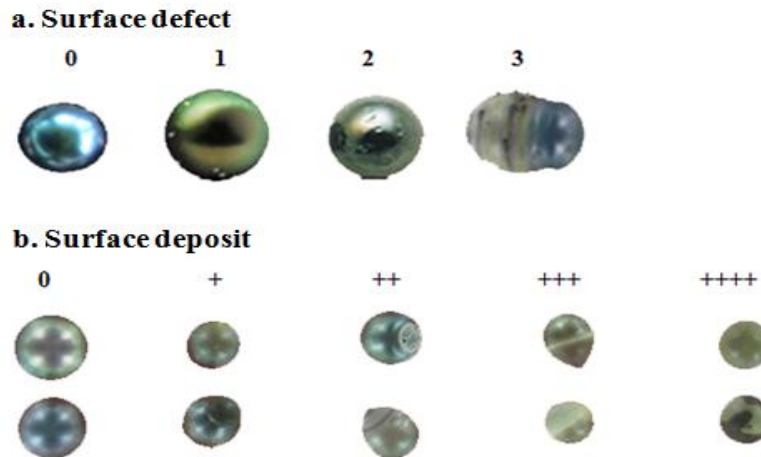


Figure 3.1 : Cultured pearl description. a. surface defect levels “0”: no defects, “1”: 1 to 5 defect(s), “2”: 6 to 10 defects and “3”: more than 10 defects. b. Surface deposits (“0” no deposits to “++++” in which deposits cover the whole surface)

3.3. GENE EXPRESSION

We analysed pearl sac tissues to compare relative gene expression by screening three aragonite-related genes (*Pif-177*, *MSI60*, *Perline*), for calcite-related genes (*Aspein*, *Shematin*, *Prismalin*) and one gene implicated in both layers (*Nacrein*) (Blay *et al.* 2017). After removing the RNAlater by pipetting and absorption, total cellular RNA was extracted from the individual pearl sac samples (80 pearl sacs sampled every 3 months, giving a total of $n = 240$ pearl sac tissue samples over 9 months) using Trizol® reagent (Life Technologies) according to the manufacturer’s recommendations. Dnase treatment, cDNA synthesis and Real-Time PCR amplifications were performed as described in Blay *et al.* (2017).

All measurements were made in duplicate and all analyses were based on the Ct values of the PCR products. Relative gene expression levels were calculated using the delta–delta method, normalized with two reference genes SAGE and GAPDH (Blay *et al.* 2017), to compare the relative expression results (Livak 2001) as follows: Relative expression $_{(\text{target gene, sample } x)} = 2^{-(\Delta\text{Ct sample, sample } x - \Delta\text{Ct calibrator, sample } x)} = 2^{-\Delta\Delta\text{Ct}}$.

3.4. STATISTICAL ANALYSIS

Differences in pearl surface defects, grade and surface deposit rates between the three harvest times were evaluated using a χ^2 test.

Relative expression analysis. Normality and homoscedasticity of gene relative expression data were checked using Shapiro–Wilk and Bartlett’s tests. One-way ANOVA was performed followed by Tukey’s multiple comparison tests to determine expression differences of candidate marker genes among the different harvest times or in relation to the pearl quality categories considered.

Decision tree. We adapted and performed a multivariate regression tree (MRT), widely used in the domain of ecology for modelling species-environment relationships (De’Ath 2002), to evaluate the hierarchical importance of the effect of relative gene expression on the pearl quality surface pathway. Divisions in the MRT were determined by cross-validation. We performed these analyses with the *rpart* package v4.1-10 (Therneau *et al.* 2015) using the default parameters of the *rpart* function.

All the statistical analyses were performed using R version 3.3.2, an environment and language for statistical computing.

4. RESULTS

Of the 600 grafted oysters, 55 rejected the implanted nucleus, 26 died and 15 were lost during the course of the experiment (likely due to natural predation). A total of 464 pearls and 40 keshi were harvested over the 12-month experimental period. Overall, we analysed a total of 211 pearls and pearl sacs over the 9 months, with 64 at 3 months, 77 at 6 months and 70 at 9 months (tissues from the last point at 12 months were of too poor a quality to be used in the analyses). The cultured pearl quality traits are described in Table 1. Results on expression levels of the matrix protein genes are presented in the next three sections according to 1) the different stages of culture, 2) pearl quality grade, and 3) the amount of pearl surface deposits, including a predictive model of pearl quality characteristics according to expression levels.

4.1. RELATIVE GENE EXPRESSION LEVEL OF MATRIX PROTEINS AT DIFFERENT STAGES OF PEARL CULTURE

Relative gene expression in the pearl sac of the panel of genes coding proteins implied in calcite or aragonite layers at three stage of the pearl formation is illustrated in Figure 3.2. The relative gene expressions of *PIF*, *MSI60* and *PERL1* were significantly much higher at month 3 of culture than at months 6 or 9 ($p = 0.006$, $p < 0.0001$ and $p < 0.0001$ respectively). For all other genes, the relative expression level was not significantly different between the different samples made over time after the graft.

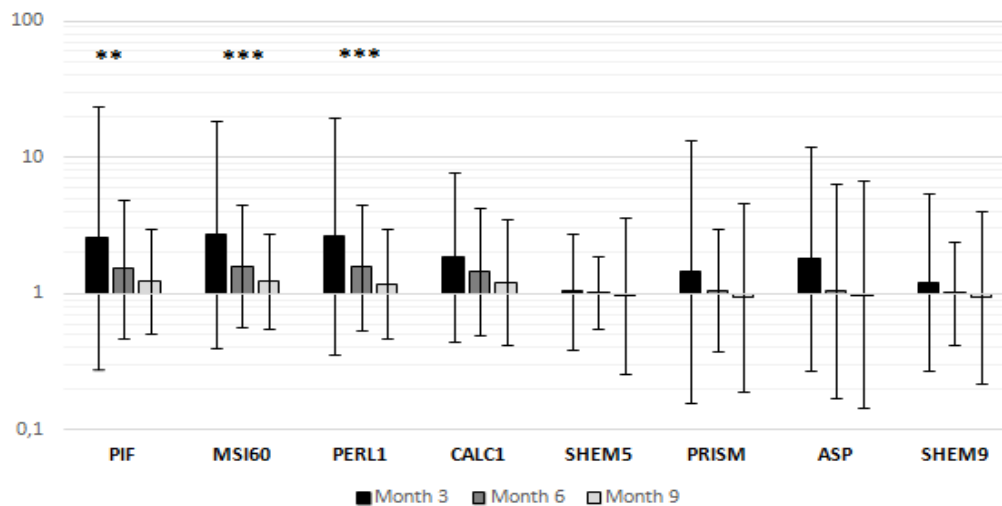


Figure 3.2 : Relative expression levels of matrix proteins in the pearl sac at different stages of pearl development. Histograms in dark grey represent data for all pearls at 3 months and histograms in light grey represent data at 9 months. Y axes are in the logarithmic scale. Error bars indicate standard deviations. Statistical differences between the phenotypes are indicated by asterisks: * for $0.05 < p < 0.005$, ** for $0.005 < p < 0.0005$, *** for $p < 0.0005$.

Analyzing kinetics data over the 9 months following the graft, we did not find any preferential harvest time when focusing on changes in surface defects. After 3 months of culture, the pearl surface defects were distributed as follows: “0” 4.7%, $n = 3$; “1” 31.2%, $n = 20$; “2” 25.0%, $n = 16$ and “3” 39.1%, $n = 25$. After 9 months of culture, the distribution was similar: “0” 4.3%, $n = 3$; “1” 40.0%, $n = 28$; “2” 22.9%, $n = 16$ and “3” 32.8%, $n = 23$ (Table 3.1).

Data analysis showed a highly significant difference in pearl surface deposits between harvest times ($p < 0.0001$). After 3 months, pearls possessed significantly more surface deposits than at 6 and 9 month pearls. For example, at the 3 month sampling, 18.8% of the pearls were “++++” while only 5.2% and 2.8% fell into the “++++” category at 6 and 9 months, respectively. In the 3-month sample, pearls without deposits accounted for 46.9% of the collection, against 76.6 and 82.9% after 6 and 9 months of culture, respectively (Table 3.1).

Tableau 3.1 : Cultured pearl quality traits from the experimental graft. Percentages of cultured pearls (and number in brackets) at each harvest time (3 months, 6 months and 9 months of culture) among the following variables are presented: 1) Surface defect classes (“0” = 0 defects, “1” = 1–5 defects, “2” = 6–10, and “3” = >10 defects); 2) Classification grade (A, B, C, D and reject: R); and 3) Surface deposit (“0” without deposit, “+” to “++++” small deposits to complete coverage)

	Surface defect				Grade					Surface deposit				
	0	1	2	3	A	B	C	D	R	0	+	++	+++	++++
Month 3	4.7 (3)	31.2 (20)	25.0 (16)	39.1 (25)	11.0 (7)	14.1 (9)	18.7 (12)	18.7 (12)	37.5 (24)	46.9 (30)	15.6 (10)	6.2 (4)	12.5 (8)	18.8 (12)
Month 6	2.6 (2)	48.0 (37)	16.9 (13)	32.5 (25)	11.7 (9)	35.0 (27)	20.8 (16)	18.2 (14)	14.3 (11)	76.6 (59)	10.4 (8)	1.3 (1)	6.5 (5)	5.2 (4)
Month 9	4.3 (3)	40.0 (28)	22.9 (16)	32.8 (23)	10.0 (7)	20.0 (14)	42.9 (30)	17.1 (12)	10.0 (7)	82.9 (58)	10.0 (7)	2.9 (2)	1.4 (1)	2.8 (2)

Data analysis showed a highly significant difference in pearl surface deposits between harvest times ($p < 0.0001$). There were also significantly more *rebut* among the pearls harvested at 3 months after grafting than among those collected after 6 and 9 months. After 3 months of culture, 37.5% of the pearl fell into the “R” category while only 14.3% and 10.0% were classified as “R” after 6 and 9 months of culture, respectively (Table 3.1).

4.2. IMPACT OF BIOMINERALIZATION GENE EXPRESSION LEVEL ON PEARL GRADE

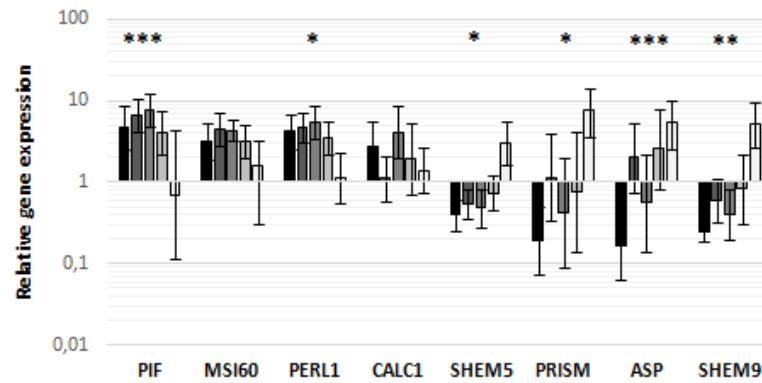
After 3 months of culture, only *CALC1* and *MSI60* were not significantly different among the different pearl grades. In fact, two genes encoding aragonite proteins, *PIF* and *PERL1*, had higher relative

gene expression in pearl sacs of A, B and C grade pearls (fold change > 4) compared to pearl classified as “R” (rejects) (fold change = 0.7 and 1.1 respectively) ($p < 0.0001$ and $p = 0.002$, respectively). Inversely, *SHEM5*, *PRISM*, *ASP* and *SHEM9*, which encode proteins playing a role in calcite formation, had higher gene expression levels for rebut pearls (fold change = 3.0, 7.5, 5.4, 5.2, respectively) than for grade “A” pearls (fold change < 0.4) (Figure 3.3).

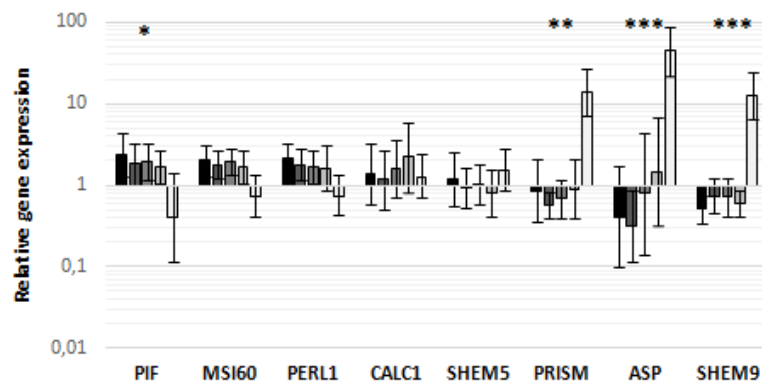
At 6 months, *PIF* expressions were significantly different between the grade categories of the pearls ($p = 0.01$). Data analysis showed lower expression of this gene for “R” pearls. *PRISM*, *ASP* and *SHEM9* showed significantly higher fold changes (13.7, 44.4, 12.5 respectively) for rebut pearls compared to other pearls, which had fold changes < 1 for *PRISM* and *SHEM9* and < 1.5 for *ASP* ($p = 0.02$, $p < 0.001$ and $p = 0.0006$ respectively).

After 9 months of culture, *PRISM*, *ASP* and *SHEM9* expression levels were significantly different among the pearl grades ($p = 0.003$, $p = 0.0001$, $p = 0.005$ respectively). *PRISM*, *ASP* and *SHEM9* had higher expression levels for rebut pearls (fold change = 53.3, 89.9, 13.5 respectively) than for A, B and C pearls (fold change < 1) (Figure 3.3). Gene expression profiles over the first 9 months of culture for the different surface defect and surface deposit categories are given in Figure S1 and Figure S2, respectively.

a.3 months



b.6 months



c.9 months

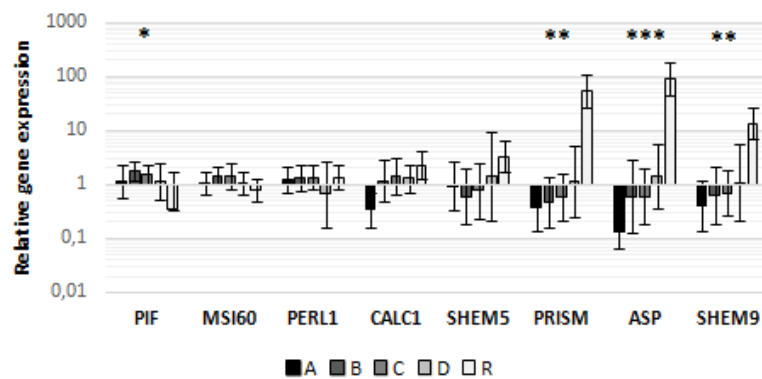


Figure 3.3 : Relative expression of 8 biominerzalization genes in the pearl sac of *P. margaritifera*: a. at 3 months, b. 6 months and c. 9 months. Histograms in dark grey show data for “A” grade pearls and light grey histograms show data for the “R” grade pearls (rejects). Y axes are in the logarithmic scale. Error bars indicate standard deviations. Statistical differences between the phenotypes are indicated by asterisks: * for $0.05 < p < 0.005$, ** for $0.005 < p < 0.0005$, *** for $p < 0.0005$.

4.3. PREDICTIVE MODEL OF CULTURED PEARL SURFACE DEPOSITS

To determine the major gene expression level explaining, and thus affecting, pearl surface quality, we performed three multivariate regression trees, as shown in Figure 3.4. From the MRT model obtained for surface deposit, it appeared that, among the eight input variables, the most important factors/predictors were relative expression levels of four genes: *SHEM9*, *ASP*, *CALCI* and *PIF*. The first regression tree split is based on the relative gene expression level of *SHEM9*. When this level is higher than 9.571, following the MRT to the left, a second split then occurs based on the relative gene expression level of *PIF*. Samples showing *PIF* expression level greater or equal to 0.314 are on “+++” deposit pathways (50% of the total “+++” pearls harvested), whereas samples showing a level lower than this critical value are on overall calcite pearl pathways (67% of “++++” pearls harvested). If the expression level of *SHEM9* is lower than 9.571 then, following the regression tree to the right, a second split is based on the relative gene expression level of *ASP*. Samples showing an *ASP* expression level lower than 6.958 are on the “without deposit” pathway (93% of the total pearls without deposits harvested), whereas samples showing an expression level greater or equal to 6.958 are on pathways with more surface defects. Finally, the third and the last split of the regression three is based on the *CALCI* expression level, and differentiates samples without deposit and samples with “+” low deposit.

From the MRT model obtained for surface defects, it appeared that the most important factors/predictors were relative expression levels of 3 genes: *SHEM9*, *ASP* and *PIF*. The first regression tree split is based on the relative gene expression level of *SHEM9*. When this level is higher than 9.459, following the MRT to the right, samples are on pathways of more than 10 defects with a 0% error. If the expression level were < 9.459, a second split then occurs based on the relative gene expression level of *ASP*. Samples showing *ASP* expression levels lower than 0.241 are on “1” surface defect (less than 5 defects) pathways.

From the MRT model obtained for classification grade, it appeared that the most important factors/predictors were relative expression levels of 4 genes: *SHEM9*, *ASP*, *PRISM* and *PIF*.

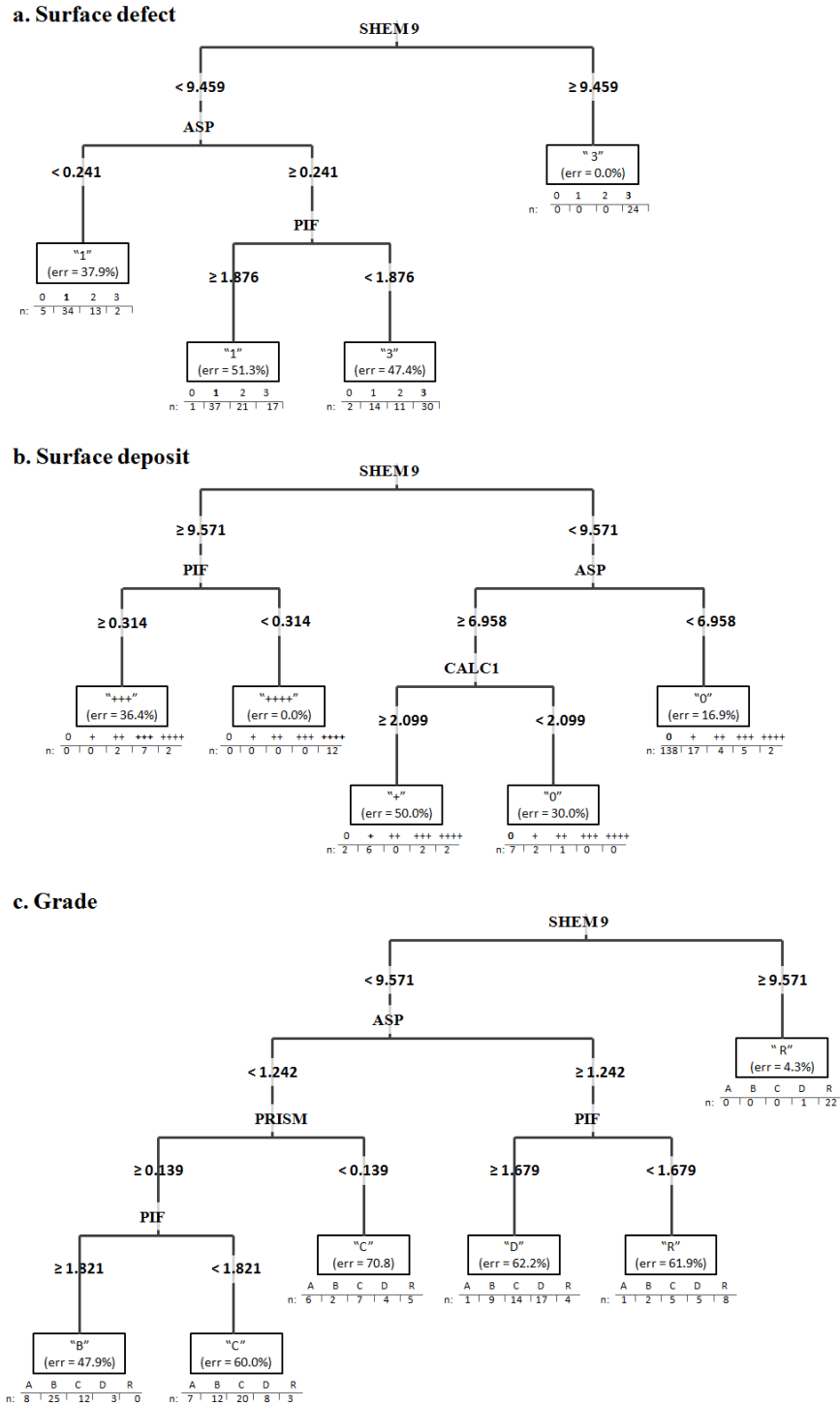


Figure 3.4 : Multivariate regression trees of pearl quality categories (n = 211). Candidate predictor/explanatory variables are the relative expression ratio of the eight potential marker genes, and the response variables are a. the four sub-clusters of surface defect without to more than 10 defect on surface pearl, b. the five sub-clusters of surface deposit, without to overall deposit on surface pearl, and c. the five sub-clusters of grade classification, “A” to “R” (reject) pearls.

The first regression tree split is based on the relative gene expression level of *SHEM9*. When this level is higher or equal than 9.571, following the MRT to the right, samples are on Rebut pathways with a 4.3% error. If the expression level is lower than 9.571, subsequent splits occur based on the relative gene expression level of *ASP* and then *PRISM* and *PIF*. Samples showing an *ASP* expression level lower than 1.242 are on “B” and “C” grade pathways while with an *ASP* expression level greater or equal to 1.242, samples are on D or R grade pathways. Thus, following these MRT model of *P. margaritifera* pearl surface quality, we can associate pearls with the particular pathways by analysing the expression level of three common genes (i.e., *SHEM9*, *ASP* and *PIF*), which relate to surface defect, surface deposit and grade.

5. DISCUSSION

For the first time, cultured pearl biogenesis was followed over the first nine months of *P. margaritifera* pearl culture examining both pearl phenotype and pearl sac molecular parameters. Results showed that the three first months of pearl oyster culture is primordial in determining pearl surface quality. In addition, three genes encoding proteins involved in the biomineralization process (*SHEM9*, *ASP* and *PIF*) were implicated in the surface quality signature of the cultured pearl.

5.1. BIOGENESIS OF THE QUALITY OF CULTURED PEARL SURFACES

The pearl oyster shell typically consists of an outermost organic layer known as the periostracum, and calcium carbonate oriented in two distinct microlaminates: the outer calcite prismatic layer and the inner aragonite nacreous layer (Zhu *et al.* 2015). In previous studies, which essentially investigate the first day after the graft (less than 80 days monitored), the first deposit on the nucleus was aragonitic (Cuif *et al.* 2008; Liu *et al.* 2012) or calcitic (Ma *et al.* 2007) prismatic layer and this was followed by the nacreous layer, which started to develop on top of the prismatic layer. One study described two consecutive stages in the whole process of pearl formation (for the first 35 days), starting with an irregular calcium carbonate

deposition on the bare nucleus (Liu *et al.* 2012). Calcium carbonate deposition then becomes increasingly regular until the mature nacreous layer has been formed on the nucleus.

In our study, the level of surface deposit on pearls decreased over the culture period. At three months, 54% of culture pearls harvested had some deposits vs. 17% at 9 months. Concerning grade classification results (which relies on lustre and surface defects), we showed that the rate of rejects declines over time, with 37.5% at the 3-month harvest vs. 10% at the 9-month harvest. Meanwhile, pearl surface defects, such as pits, bumps and scratches, had a steady proportion after 3, 6 or 9 months of culture (about 35%). Our study suggests that prismatic layer formation takes place during the first 3 months after nucleus implantation and that aragonite layer formation had not yet started for all pearls by this point. Surface defects appeared after the grafting procedure and remained throughout the culture period, while surface deposits appeared after grafting procedure and could then be covered over during the by a nacreous layer. Surface defects were thus formed early and could be the consequence of haemocyte accumulation after the grafting. A previous study showed that accumulation of haemocytes during pearl sac development may result in malformation of the pearl sac, which is likely to result in reduced pearl quality (Kishore and Southgate 2014), and according to our results could not be corrected during the culture period. To avoid surface defects on the pearl and improve pearl quality, careful graft preparation and surgery are essential parameters (Southgate and Lucas 2011). One of our previous study showed that the grade is correlated with pearl nacre thickness, with Grade A pearls having the thickest and heaviest nacre on average, and thickness increasing over time of culture (Blay *et al.* 2014). The thickness of nacreous deposit is essential to improve the quality of the pearl surface. After examining the evolution of the pearl surface quality over the culture period, it is essential to understand the role of the matrix proteins in the pearl formation.

5.2. THE ROLE OF THE MATRIX PROTEINS IN THE FORMATION OF PEARL SURFACE QUALITY

The mechanisms of the matrix proteins, which are fundamental for pearl quality formation, are not well known. The role of these proteins is a crucial question that still needs to be elucidated in pearl oyster because of its important implications for pearl quality and, therefore, the success of the pearl oyster aquaculture. Here we selected eight genes as potential markers of the quality pearl pathway based on the literature (Marie *et al.* 2012; Joubert *et al.* 2010). These genes have already been shown to play a role in shell formation through the biomineralization process (Suzuki *et al.* 2009, 2013; Miyamoto *et al.* 2005, 2013; Montagnani *et al.* 2010; Huang *et al.* 2012; Shie *et al.* 2013).

This study cannot answer the question of what kinds of factors affect the gene expression patterns of individual pearl sacs, but we studied the evolution of gene expression in pearl sacs over the pearl culture period and the impact of this expression on pearl quality surface. Through a multivariate regression tree, we identified three genes whose expression level correlated with the pearl surface quality pathway and which can thus be considered as major quality determinants. The three genes implicated in the surface quality pathway are *SHEM9*, *ASP* and *PIF*. Interestingly, the relative gene expression level of *SHEM9* (< 9.5) and *ASP* (<1) appear crucial in lowering surface deposits, surface defects and creating a pathway that will lead to a good grade. The low expression of these two genes can inhibit surface deposits and lead to top quality pearls. These findings suggest that inhibition of the prismatic layer in pearl formation should decrease the proportion of low quality pearls by diminishing the presence of calcite and pearl surface defects, which increases the grade classification. RNA interference (RNAi) is a technique that suppresses the expression of specific gene by neutralizing targeted messenger RNA molecules. This method would be useful to investigate functions of genes and their impact on cultured pearl surface quality. For example, an RNAi experiment could be performed using double-stranded RNA (dsRNA) of a calcitic gene by injecting it into the pearl sac and examining the pearl surface quality. This technique has been widely used to investigate functions of uncharacterized genes (Fire *et al.* 1998) and has been

effectively applied in bivalves (Suzuki *et al.* 2009; Fang *et al.* 2011; Yan *et al.* 2014; Funabara *et al.* 2014; Owens and Malham 2015).

Moreover, our results indicate that in the first 6 months of the pearl formation, the three “aragonitic-layer-forming” genes were significantly less expressed for the pearls wholly covered by surface deposits. During the first 3 months of the pearl culture, the three aragonitic-layer-forming genes were also significantly less expressed for pearls with more than six surface defects. Moreover, the levels of expression for these aragonitic-layer-forming genes decreased with time of culture. In the later periods (after 3 months), their expression levels were relatively low. Studies have observed that MSI60, a matrix protein in the nacreous layer, has several characteristic domains that constitute the baseline of the nacreous layer (Sudo *et al.* 1997; Inoue *et al.* 2011). Pif, an acidic protein isolated from the nacreous layer, has been reported to regulate the formation of this layer (Suzuki *et al.* 2009). Pearlin, which is mainly present in the nacreous layer, induces formation of aragonite crystals when Pearlin is fixed to the substrate (Suzuki and Nagasawa 2013). These results show that the three genes contributing to the formation of the aragonitic layer are high expressed at the beginning of pearl biogenesis. In Blay *et al.* (2017), we showed that nacreous deposition is not linear during the pearl formation process and that the highest deposition rate was in the first 3 months. After 6 months, almost 70% of the final nacre thickness had already been reached. For early stage aragonite formed in the 15 days after grafting, the deposition did not appear to be accurately controlled by the organic matrix (Liu *et al.* 2012). Two studies on *P. margaritifera* showed that the pearl sac development required between 12 (Cochennec-Laureau *et al.* 2010) and 14 (Kishore and Southgate 2014) days, which is a shorter period than that required in *P. maxima* (Scoones 1996) or 65 days in *P. fucata* (Wada 1968; Achari 1982). All of these results indicate the importance of the earliest stages of the culture process.

Another interesting finding was the relatively high level of *PRISM*, *ASP* and *SHEM9* expression in cases of complete deposit coverage of the pearl, reject-grade pearls or high levels of surface defects during pearl formation and the relatively low expression of these genes in cases of pearl without deposits or surface defects and belonging to grades A or B. Shematrixin is a family of glycine-rich shell matrix proteins

known to be present in the prismatic microstructure of several pearl oyster species (Gadner *et al.* 2011). Shematrins are framework proteins facilitating calcification of the prismatic microstructure (Yano *et al.* 2006). Aspein is involved in specific calcite formation in the prismatic layer of the shell and works on crystal formation in prisms (Tsukamoto *et al.* 2004; Takeuchi *et al.* 2008; Gao *et al.* 2016). Aspein would accelerate calcite formation (Takeuchi *et al.* 2008). In a similar way to Aspein, Prismaticin-14 is responsible for the prismatic layer of the shell but may act as a framework protein that mediates chitin and calcium carbonate crystals using its acidic and chitin-binding regions (Suzuki *et al.* 2004, 2007). These findings suggest when the pearl had surface deposits or defects, *Shematin* and *Aspein* and *Prismaticin 14* were expressed throughout the pearl culture period, but when the pearls were formed is without surface defects, these "prismatic-layer-forming" genes were not expressed. These results also suggest that surface deposits corresponded to a prismatic layer.

The findings reported in this study provide a more detailed understanding of gene expression of matrix proteins during the process of pearl biogenesis in *P. margaritifera* and provide a basis for future research towards developing improved pearl culture practice and pearl quality.

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AUTHORS CONTRIBUTIONS

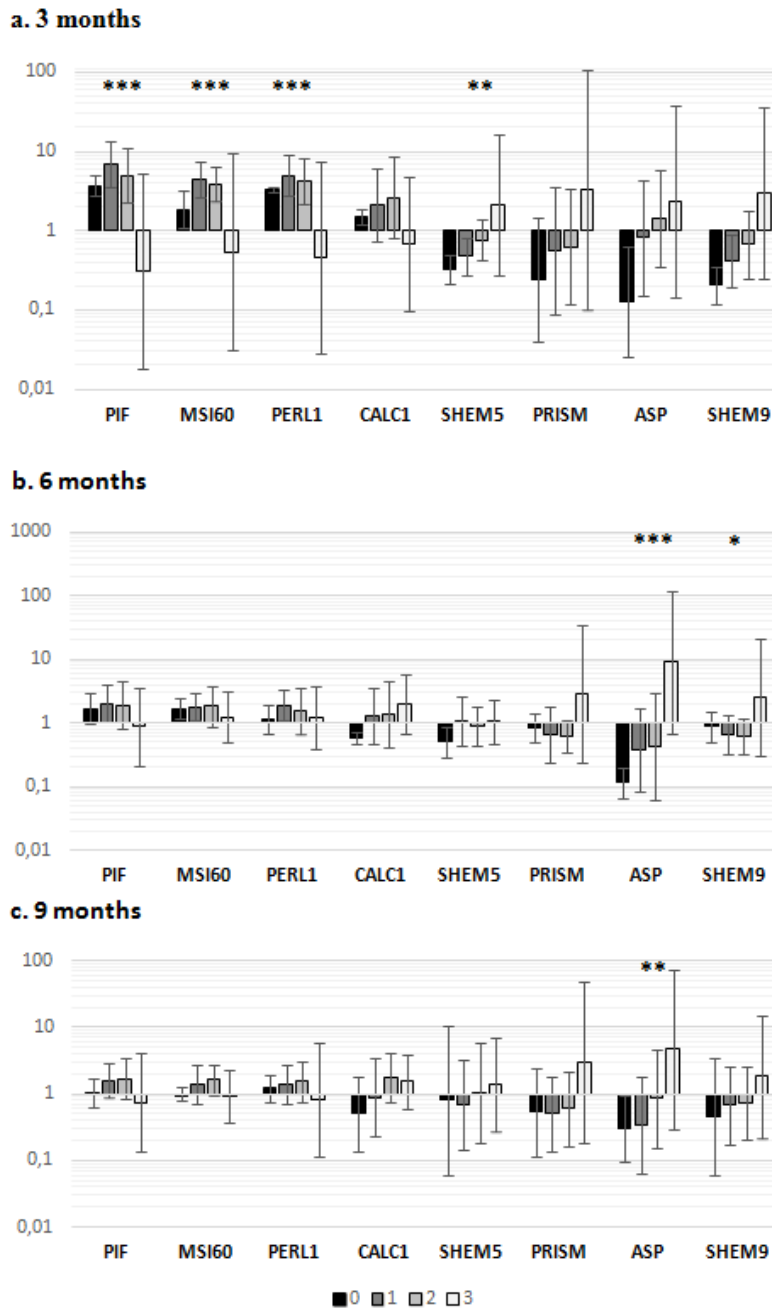
CB followed the graft experiments with the support of the pearl farm and performed the molecular analysis and drafted the manuscript. CLK designed the experiments and was CB's PhD supervisor, together with SP. SP and CLK helped to revise and enrich the manuscript. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

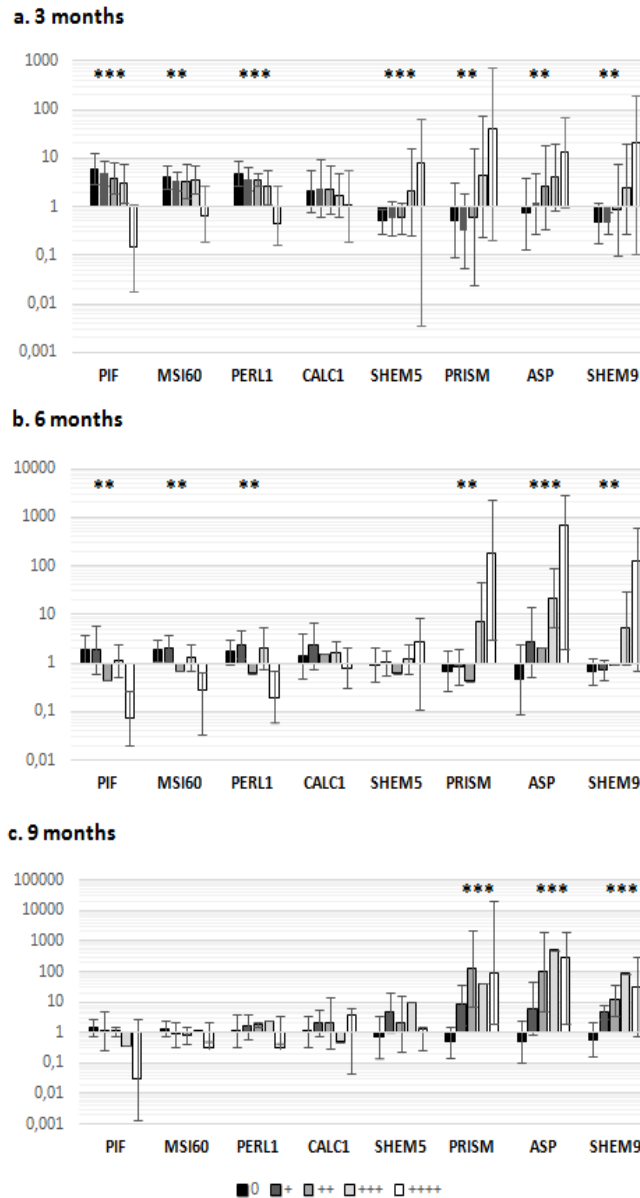
The authors declare no competing financial interests

6. SUPPLEMENTARY INFORMATION

Figure S1: Relative expression of 8 biomineralization genes in the pearl sac of *P. margaritifera*: **a.** at 3 months, **b.** 6 months and **c.** 9 months. Histograms in dark grey show data for pearls without surface defects and histograms in light grey show data with more than 10 defects. Y axes are in the logarithmic scale. Error bars indicate standard deviations. Statistical differences between the phenotypes are indicated by asterisks: * for $0.05 < p < 0.005$, ** for $0.005 < p < 0.0005$, *** for $p < 0.0005$.



FigureS2. Relative expression of 8 biomineralization genes in the pearl sac of *P. margaritifera*: **a.** at 3 months, **b.** 6 months and **c.** 9 months. Histograms in dark grey show data for pearls without surface deposits and light grey histograms show data for the pearls completely covered by surface deposits. Y axes are in the logarithmic scale. Error bars indicate standard deviations. Statistical differences between the phenotypes are indicated by asterisks: * for $0.05 < p < 0.005$, ** for $0.005 < p < 0.0005$, *** for $p < 0.0005$.



Chapitre 4

Donor and recipient contribution to phenotypic traits and the expression of biomineralisation genes in the pearl oyster model *Pinctada margaritifera*

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Donor and recipient contribution to phenotypic traits and the expression of biomineralisation genes in the pearl oyster model *Pinctada margaritifera*

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1. ABSTRACT

Grafting associates two distinct genotypes, each of which maintains its own genetic identity throughout the life of the grafted organism. Grafting technology is well documented in the plant kingdom, but much less so in animals. The pearl oyster, *Pinctada margaritifera*, produces valuable pearls as a result of the biomineralisation process of a mantle graft from a donor inserted together with a nucleus into the gonad of a recipient oyster. To explore the respective roles of donor and recipient in pearl formation, a uniform experimental graft was designed using donor and recipient oysters monitored for their growth traits. At the same time, phenotypic parameters corresponding to pearl size and quality traits were recorded. Phenotypic interaction analysis demonstrated: 1) a positive correlation between recipient shell biometric parameters and pearl size, 2) an individual donor effect on cultured pearl quality traits. Furthermore, the expressions of biomineralisation biomarkers encoding proteins in the aragonite or prismatic layer showed: 1) higher gene expression levels of aragonite-related genes in the large donor phenotype in the graft tissue, and 2) correlation of gene expression in the pearl sac tissue with pearl quality traits and recipient biometric parameters. These results emphasize that pearl size is mainly driven by the recipient and that pearl quality traits are mainly driven by the donor.

2. INTRODUCTION

From a genetic point of view, grafting creates a chimera of a single individual made up of genetic material issued from two (or more) distinct genomes, with each genome maintaining its own distinct genetic identity throughout the life of the grafted organism. Typically, grafting associates two tissues from different individuals, the recipient and graft, which form vascular connections and survive as a genetic chimera due to a unique symbiotic relationship (Mudge *et al.* 2009). The partners in a graft are known as the scion and root in plants and the donor and recipient in pearl oysters. The use of grafting technology in both plant cultivation and pearl production provides a means of improving quality in these industries. Grafting is a well-established practice that has many horticultural and biological uses. The numerous applications of plant grafting include vegetative propagation, avoidance of juvenility, cultivar change, creation of unusual growth forms, repair, size control, biotic and abiotic stress resistance and physiological improvement (Mudge *et al.* 2009). Some studies have examined the contribution of the scion; for example, a scion of a red-flowering rose grafted on a white rose stock will continue to produce red roses (Mudge *et al.* 2009). Grafting is commonly employed in agronomy to indirectly manipulate scion phenotype (Warschefsky *et al.* 2015). As for the host organism, rootstocks are commonly used to propagate selected scions to improve fruit tree tolerance to environmental stress, and to control tree size (Webster 2001; Tworkoski and Miller 2007). Roots anchor plants in the ground to provide water and nutrients from the soil and can serve as storage organs. The root system is a crucial component in coordinating response to a range of abiotic and biotic stressors (Hodge *et al.* 2009; Kuijken *et al.* 2015). The influence of rootstocks on yield productivity is being increasingly recognized to be as important as grafted scions. In the plant system, the genetic characteristics of the root appear to drive vegetative growth and the overall growth of the grafted system depending on environmental conditions (Vazifeshenas *et al.* 2009).

In the pearl oyster grafting process (*Pinctada* sp.), similarities to the scion–root system were suggested by studies on the contributions of the recipient and donor to the expression of the overall phenotype (including cultured pearl quality traits). In *Pinctada maxima*, results indicated low donor-related heritability of pearl size (Jerry *et al.* 2012), and xenografts between *P. maxima* and *P. margaritifera* demonstrated that individual donors significantly influence the color and the surface complexion of the pearl (McGinty *et al.* 2010). In *P. margaritifera*, one study demonstrated the differential influence of individual donors with black or red outer shell phenotypes, combined with green or yellow inner shell phenotypes, on pearl darkness level, color categories and luster (Ky *et al.* 2017). Another study examined the phenotypic correlation between recipient shell weight and size of pearls in *P. fucata*, revealing a limited positive relationship whereby recipient oysters with heavier shells produced bigger pearls (Wada and Komaru 1996). These results indicate potential underlying genetic correlations between the recipient or donor growth and pearl size, whereby selection for faster growing oysters might further improve pearl size.

In order to understand the rootstock–scion interaction, some reviews have investigated physiological and biochemical aspects (Martinez-ballesta *et al.* 2010), or hormonal signaling (Aloni *et al.* 2010). In pearl oysters, earlier studies have examined the influence of genetics, environmental factors and their interactions on pearl quantitative and qualitative traits (Falconer and Mackay 1996). A study in *P. maxima* indicated that characteristics of both donors and hosts were correlated with pearl traits (McDougall *et al.* 2016). In *P. margaritifera*, one such study demonstrated the influence of grow-out sites on cultured pearl quality traits over a broad geographic scale between archipelagos in the South Pacific (Ky *et al.* 2017). Another analyzed environmental influence on pearl size parameters in relation to the recipient oyster biometric parameters (Le Pabic *et al.* 2016). Although it is clear from these studies that both donor and recipient oysters are involved in pearl formation in *P. margaritifera*, the relative contributions have not yet been clearly identified. Despite much study on pearl oysters, there remains a knowledge gap about donor size influence on pearl phenotype.

In the present study, the goal was to improve our understanding of the mechanisms driving the phenotypic variability arising from the recipient and the donor. The working hypothesis was that the recipient oyster confers quantitative traits to the pearl while the individual donor oyster drives qualitative traits of the pearl. In other words, everything related to growth and transfer of energy would be driven by the recipient oyster, and everything related to the quality of the nacrein deposit would be driven by the donor of the graft. Within this framework we defined four specific experiments to determine respective donor and recipient effects. (1) We sought to discern whether the donor or recipient was the main driver of pearl growth rate by comparing different recipient-donor phenotype combinations. (2) We evaluated drivers of the pearl phenotype by comparing effects of different recipient-donor phenotype combinations on nacre deposit quality. (3) We measured biomineralisation potential through a comparison of quantitative gene expression in the mantle graft (donor only effect) and the pearl sac (donor + recipient effect). For this approach, a representative panel of eight genes encoding proteins involved in aragonite biomineralisation (Pif-177, MSI60 and Perline), calcite biomineralisation (Aspein, Shematin and Prismalin) or proteins implicated in both layers (Nacrein) was screened (Joubert *et al.* 2010; Marie *et al.* 2012; Xiang *et al.* 2013). Finally (4), we followed the kinetics of the recipient-donor influence on pearl formation by monitoring pearl size and quality parameters over 12 months. This information provides basic knowledge to help us to understand the donor-recipient correlations and their relative contributions in pearl oyster grafts.

3. RESULTS

Overall, a total of 289 cultured pearls were harvested and analyzed. Donor pearl oysters were divided into two distinct groups according to their shell size (dorso-ventral measurement): small donor oysters, denoted “QL”, and large ones, denoted “TL”. The distribution of QL and TL oysters are shown in Table 4.1. Results are presented in four sections relating to 1) the influence of donor and 2) recipient shell size effect on cultured pearl size and cultured pearl quality, 3) biomineralisation capabilities and 4) pearl development kinetics.

3.1. QL VS. TL AND INDIVIDUAL DONOR INFLUENCE ON PEARL PHENOTYPE

At grafting time, the donor oyster width, height and thickness were recorded, showing average measurements of 48.6 ± 5.2 mm, 48.6 ± 5.2 mm and 12.7 ± 1.4 mm, respectively, for the QL donor group. The TL donor group was significantly larger for each parameter ($p < 0.0001$) with 79.8 ± 6.3 mm for shell height, 79.9 ± 6.9 mm for shell width and 21.0 ± 2 mm for shell thickness.

Comparing differences in the nacre produced between the QL and TL donor groups, we found that the average nacre weight and thickness of the QL donor group were 0.38 ± 0.22 g and 0.79 ± 0.04 mm, respectively (N = 142). In the TL donor group, the average nacre weight and thickness were 0.34 ± 0.20 g and 0.70 ± 0.04 mm (N = 147). No significant effect of donor oyster shell size phenotype was detected for either nacre weight or thickness ($p = 0.058$ and $p = 0.065$, respectively) (Figure 4.1).

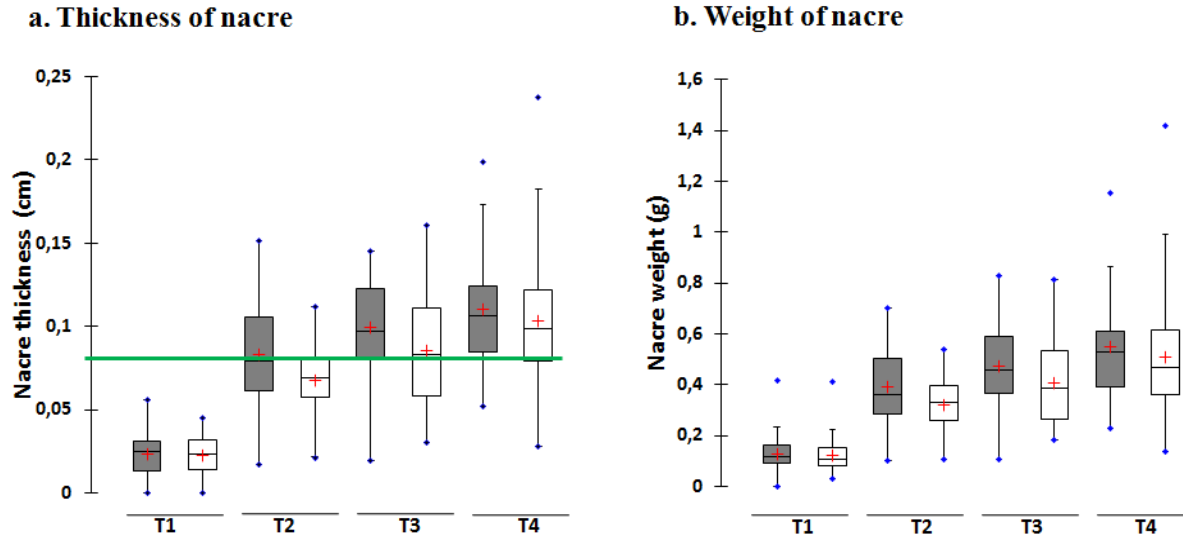


Figure 4.1: Box-plot showing: a. thickness of nacre (cm) and b. weight of nacre (g) on cultured pearls produced using graft tissue from each of the *P. margaritifera* donor phenotypes at each harvest time (T1 corresponds to 3 months of culture, T2 to 6 months, T3 to 9 months and T4 to 12 months). The box-plots in grey represent data for the QL donor phenotype and box-plots in white represent data for the TL donor phenotype. Each box-plot has the following 6 elements: 1) mean ("+" cross in the box-plot); 2) median (solid bar in the box-plot); 3) 25th to 75th percentile (rectangular box); 4) 1.5*interquartile range (non-outlier range of the box whiskers); 5) minimum and maximum values (extreme dots); and 6) outlier values (outside box whiskers). The green line on the nacre thickness box plot corresponds to the minimum nacre thickness necessary for pearl export outside French Polynesia (0.08 cm) (N = 289).

When effects of QL / TL donor phenotype were compared for subsequent pearl quality, however, no significant effect of donor oyster size group was observed for any pearl qualitative traits (darkness, luster, surface defects, grade, shape or presence of circle/s) except for color categories (Figure 4.2), where the QL phenotype seems to give more pearls of “other” colors and less peacock pearls than the TL donor phenotype.

A significant individual donor effect was detected for nacre thickness ($p = 0.045$), but no significant individual donor effect was shown for nacre weight ($p = 0.059$). Pearls from donor “Q21” (N = 7 pearls) showed the greatest average nacre thickness (1.16 ± 0.50 mm) and nacre weight (0.566 ± 0.298 g) compared with donor “Q17”, which represented the minimum (N = 7 pearls) (0.45 ± 0.32 mm and 0.215 ± 0.141 g, respectively). The influence of donor size and the evolution of this relationship through time on qualitative pearl traits at harvest are illustrated in Figure 4.2.

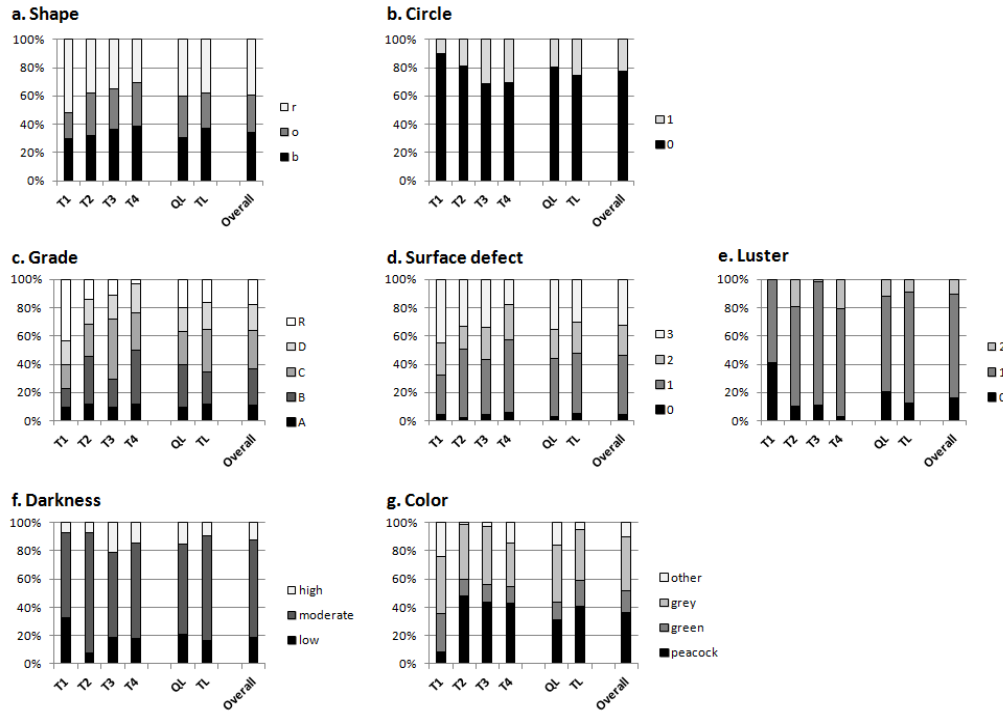


Figure 4.2 : Cultured pearl quality traits from the experimental graft distribution. Percentage of cultured pearls for each harvest time (T1 corresponds to 3 months of culture, T2 to 6 months, T3 to 9 months and T4 to 12 months), for each donor phenotype (groups QL and TL) and for the overall harvest among the following variables are presented: a. shape categories (“b” for baroque and semi baroque, “o” for oval and drop, “r” for round and semi round pearls), b. pearl circles (“0”= absence and “1”= presence), c. classification grade (“A” to “D” and Rejects), d. surface defect classes (“0” = 0 defects, “1” = 1–5 defects, “2” = 6–10, and “3” = >10 defects), e. luster levels (“0” = absence of luster, “1” = moderate luster, and “2” = high luster), f. darkness level (low, moderate and high darkness) and g. visual color categories (“peacock”, “green”, “grey” and “other”, corresponding to white and yellow pearls).

A significant individual donor effect was detected for darkness level ($p = 0.002$), color category ($p < 0.0001$), cultured pearl luster (matte or shiny / glossy / highly glossy) ($p = 0.0002$) and the surface defect classes ($p < 0.0001$). However, no significant difference was recorded between the individual donors for the absence / presence of circles ($p = 0.218$) or for the three shape categories ($p = 0.156$).

Tableau 4.1: *Pinctada margaritifera* juvenile (N = 1535) shell diameter summary data. Values below 3 cm correspond to the QL phenotype group (n = 393) and values above 4 cm correspond to the TL phenotype group (n = 292).

	QL	Medium	TL
N	393	850	292
Mean	2.51	3.60	4.68
(± SD)	(± 0.44)	(± 0.32)	(± 0.39)
Minimum	1.17	3.03	4.06
Maximum	3.01	4.04	5.82

3.2. RECIPIENT INFLUENCE ON PEARL PHENOTYPE

Nacre thickness and weight were both significantly correlated with recipient oyster shell biometric parameters (weight, height, thickness and width), Spearman rho coefficients ranged from 0.51 to 0.65 and 0.49 to 0.62, respectively. The highest significant positive correlations were found for nacre thickness and pearl weight with recipient pearl oyster weight (Rho = 0.65 and Rho = 0.62, respectively, $p < 0.0001$). In addition, there were significant positive correlations of nacre thickness and weight with shell width (+0.51 and +0.49, respectively, $p < 0.0001$) (Figure 4.3).

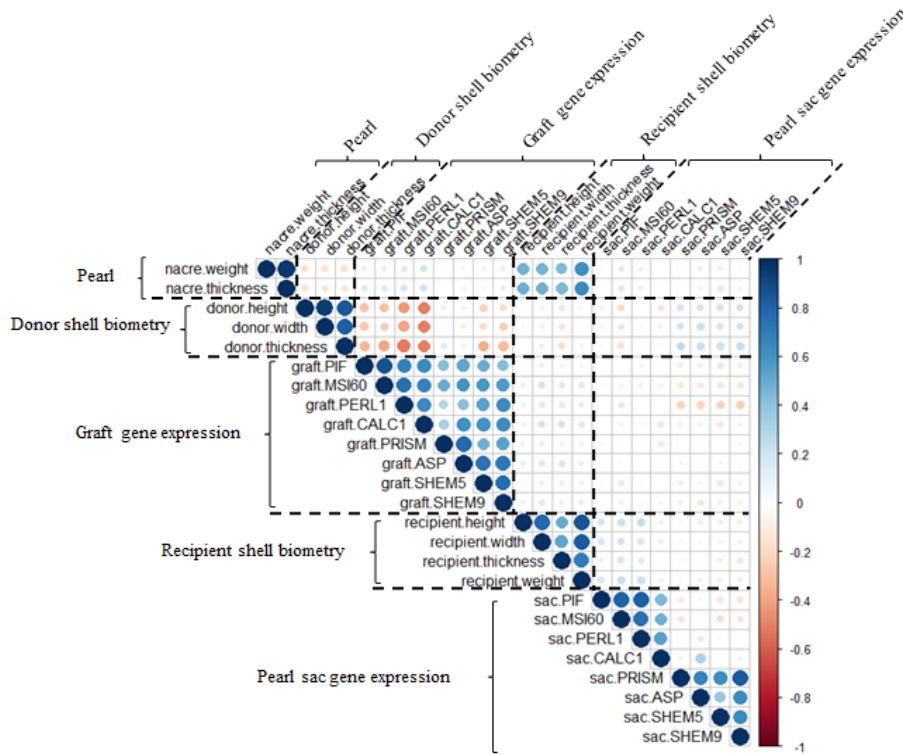


Figure 4.3 : Correlation matrix, made using corrplot, between *Pinctada margaritifera* donor and recipient shell biometric parameters (shell height, width, and thickness, and total oyster weight (soft tissue parts + shells)), pearl size (nacre weight and thickness), and the relative expression of the 8 biomineralisation genes in the mantle graft and pearl sac. The areas of circles show the value of corresponding Spearman correlation coefficients. Correlation values are presented in the upper panel in circle; positive correlations are displayed in blue and negative correlations in red. Color intensity (light to dark) and the size of the circle (small to big) are proportional to the correlation coefficients (0 to 1 for the positive coefficient and 0 to -1 for negative coefficient) where for example the correlation coefficients on the principal diagonal is equal to 1 (it was represented in dark blue and biggest size of circle). The legend on the right side of the correlogram shows the Spearman correlation coefficients with their corresponding colors.

3.3. MOLECULAR PHENOTYPE

The relative expression levels of the eight biomineralisation genes analyzed in the donor oyster mantle graft tissue and pearl sacs from the QL and TL groups are shown in Figure 4.4a. The comparison between the two groups of donors for the three aragonite-related genes, *Pif-177*, *MSI60* and *Perl1*, showed significant higher gene expression levels in the TL group ($p < 0.0001$) in the graft tissue compared with the QL group. *Shem5*, *Shem 9* and *Calc1* also showed significant higher gene expression levels in the TL group ($p < 0.0001$) compared with the QL group.

All biometric parameters of the donor oysters were significantly correlated with the relative expression level of seven biomineralisation genes in the graft ($p < 0.0001$); only *Prism* was not correlated. Nacre thickness and pearl weight were not correlated with the relative expression level in the graft of eight biomineralisation genes (Figure 4.3). Furthermore, the recipients shell height was correlated with the relative expression level of *Perl1* in the pearl sac (Rho = 0.236, $p = 0.0004$ for shell height) (Figure 4.3).

Among the eight candidate genes studied in the pearl sac at the three harvest times, the expressions of six were significantly affected by donor phenotype (Figure 4.4b). *MSI60* and *Calc1* gene expression levels were significantly higher for “TL” phenotypes compared with “QL” phenotypes ($p = 0.009$ and $p = 0.014$ respectively). *Prism*, *Asp*, *Shem5* and *Shem9* showed significantly greater expression in “QL” phenotypes compared with “TL” ones ($p = 0.043$, $p = 0.049$, $p = 0.004$ and $p = 0.009$, respectively).

No significant differences in relative expression of the panel of genes in the graft were found for pearl quality traits. In the pearl sac, however, significant effects were observed for the majority of the panel of genes, depending on grade, level of surface defects, luster and color (Supplementary Table S1).

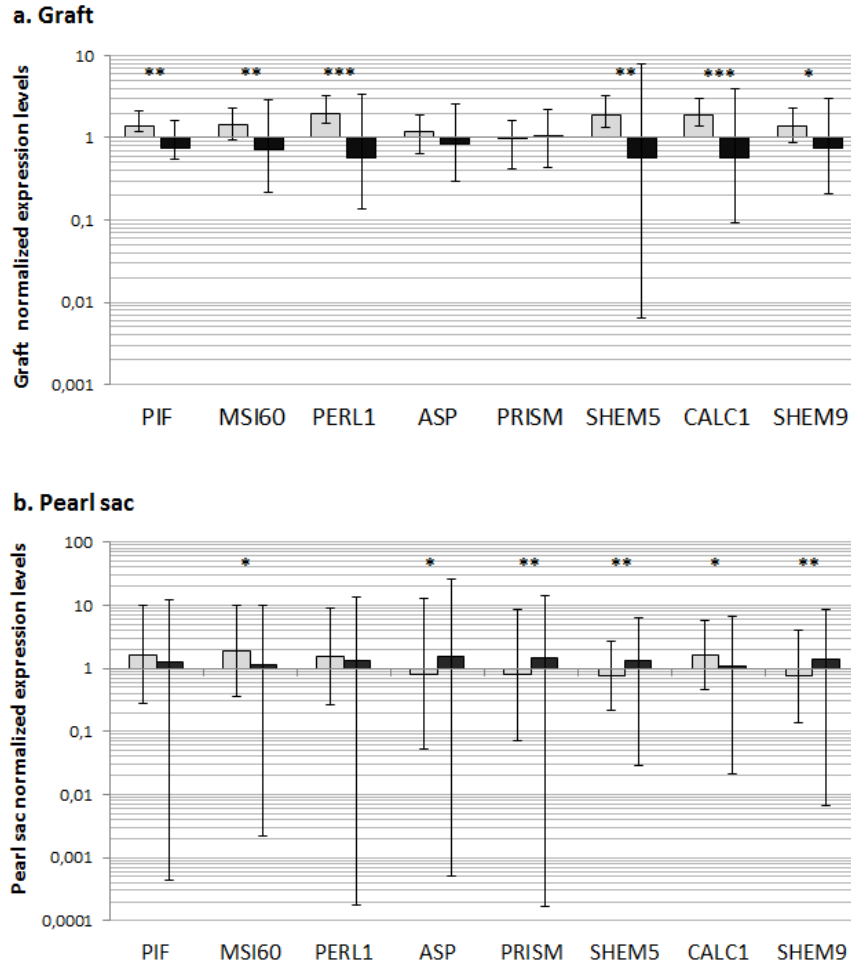


Figure 4.4 : Relative expression of 8 biomineralsation genes in *P. margaritifera* in: a. the mantle graft (N = 40) and b. the pearl sac (N = 217 with 108 QL and 109 TL). Histograms in light grey represent data for the TL donor phenotype and histograms in dark grey represent data for the QL donor phenotype. Y axes are in logarithmic scale. Error bars correspond to standard deviations. Statistical differences between the two phenotypes are indicated by asterisks: * for $0.05 < p < 0.005$, ** for $0.005 < p < 0.0005$, *** for $p < 0.0005$.

3.4. PEARL DEVELOPMENT KINETICS

Analyzing kinetics data over the 12 months of the study, we observed, as expected, a very highly significant difference in nacre thickness between harvest times ($p < 0.0001$). Mean nacre thicknesses at harvest times T1, T2, T3 and T4 were 0.02 ± 0.01 , 0.08 ± 0.03 , 0.09 ± 0.03 and 0.11 ± 0.03 cm, respectively. Nacre was significantly thinner at T1 than at T2, T3 and T4. T3 and T4 were not significantly different from one another. Pearl nacre weight was also highly significantly different

between harvest times ($p < 0.0001$). Mean pearl weights at harvest times T1, T2, T3 and T4 were 0.12 ± 0.07 , 0.35 ± 0.14 , 0.44 ± 0.17 and 0.54 ± 0.20 g, respectively. T1 pearls were significantly lighter in weight than T2, T3 and T4 ones. As with nacre thickness, T3 and T4 were not significantly different from one another (Figure 4.1). Measurements on recipient oysters showed highly significant differences between the four harvest times ($p < 0.0001$), as shown in Figure 4.5. Mean height (dorso-ventral measurement \pm SE) for recipient shell at T1, T2, T3 and T4 harvest times were 86.38 ± 5.79 , 91.42 ± 7.38 , 96.15 ± 7.27 and 102.07 ± 7.61 mm, respectively. Mean weight for recipient shell at T1, T2, T3 and T4 harvest times were 84.99 ± 19.34 , 110.38 ± 21.61 , 130.87 ± 24.75 and 144.57 ± 26.14 g, respectively.

Comparison between harvest times showed no significant difference between the proportions of the three shape categories ($p = 0.215$). After 3 months of culture, the pearl shapes were distributed as follows: Baroque 29.6%, N = 21; Round 52%, N = 37; Oval 18%, N = 13. After 12 months of culture, the distribution had remained similar: Baroque 38%, N = 26; Round 31%, N = 21; Oval 31%, N = 21.

Figure 4.2 shows the ratios of pearls with and without circles at each harvest time. A highly significant effect was recorded for the presence or absence of circles ($p = 0.005$) between each of the harvest times. The T3 and T4 harvest times yielded about three times more circled pearls than T1.

Data analysis showed a highly significant difference in pearl luster and grade between harvest times ($p < 0.0001$). T1 pearls were significantly more “matte” and less “glossy” than T2, T3 and T4 pearls. T1 had significantly less grade B pearls (12.7%) and more Reject pearls (43.7%) than the other harvest times T2 (34.2% and 13.9%, respectively), T3 (19.7% and 11.3%) and T4 (38.2% and 2.9%).

A highly significant difference in the darkness of pearls was observed between the different harvest times ($p = 0.0004$). T1 harvest time had significantly more lighter-toned pearls (32.4%) than the later harvest times T2 (7.6%), T3 (18.3%) and T4 (12.7%). T3 and T4 had significantly more dark pearls (21.1% and 17.7%, respectively) than T1 (7%) and T2 (7.6%) (Figure 4.2).

For cultured pearl surface defects, no significant harvest time effect was detected ($p = 0.067$) (Figure 4.2).

4. DISCUSSION

Our approach, based on studying the influence of donor and recipient phenotypes on resulting pearl and molecular phenotypes, has yielded important results for pearl oyster breeding strategy. Overall, the results show that it is mainly the recipients that influence the size of the pearl, while the individual donors influence pearl quality traits (except for pearl shape and the presence / absence of circles, which seem to be more influenced by graft surgical procedure).

In the present study, we showed that pearl size phenotype (nacre weight and thickness) was highly correlated with the biometric parameters of the recipient oyster, while those of the donor oyster (QL / TL groupings) had no impact. This positive correlation confirms results observed in *P. fucata* (Wada and Komaru 1996) and in *P. maxima* (McDougall *et al.* 2016). Furthermore we observe a significant positive correlation between the expression of the aragonite gene in the pearl sac (*Perl1*) and one recipient oyster biometric parameter (shell height). This result suggests that the recipient oyster affects the activities of the biomineralising tissues, which are mainly dependent on calcium metabolism in the epithelial tissue of the pearl sac (Wada 1972). The recipient oyster regulates the metabolism of the pearl sac by supplying nutrients during the period of culture and thus regulates the expression of the biomineralisation genes, especially those implicated in the aragonite layers (Joubert *et al.* 2014). High biomineralisation capabilities may have contributed to a greater nacre deposition, as already observed in *P. maxima* (Kono *et al.* 2000; Müller 1997; Strack 2006) and *P. fucata* (Gu *et al.* 2014). The recipient oyster can be compared to the rootstock of a grafted plant, with recipient oyster and rootstock each corresponding to a grafted organism. In the plant system, rootstocks are the driving component of production systems and studies have reported that they can influence tree growth, yield and fruit size, weight, and rind thickness (Autio 1991; Autio *et al.* 2003; Marini *et al.* 2002; Son and Kuden 2003; Al-Hinai and Roper 2004; Kurlus 2008; Incesu *et al.* 2013). However, it is not fully understood how the rootstock controls scion growth. The grafted organism seems to have a key role in growth owing to its nutritive function. Interestingly, the same principle applies in the animal system we studied. The positive relationship

between pearl size (nacre weight and thickness) and weight of the recipient is valuable information because it can serve as a basis for selective breeding strategy for fast growing recipient pearl oysters. For mollusk species, between 10% and 20% improvement in selected growth traits can be obtained per generation (Newkirk 1980; He *et al.* 2008).

A strong individual donor effect was observed on pearl quality traits, such as color (darkness and visual color categories) and quality parameters (luster, surface defects and grade). Pearl quality traits are influenced by a number of highly variable factors, including genetics, the environment and their interaction, as well as surgical technique and graft hygiene (Southgate 2008). A recent study in *P. maxima* found significant individual donor effects for color, nacre deposition, luster, shape and defects; however, each of these traits was grouped into broad categories in this previous study, making specific comparisons between the two studies difficult (McDougall *et al.* 2016). In plant models, research on rootstock–scion interaction demonstrated that yield and quality are conferred by the scion cultivar. Scion quality clearly affects final yield and fruit quality in grafted plants, but rootstock effects can also impact these characteristics (Tworkoski and Miller 2007; Davis *et al.* 2008).

Expression analysis in the pearl oyster graft, based on a panel of genes encoding proteins implicated in the shell biomineralisation process, indicated significant differences of gene expression level between the QL and TL donor groups for the three genes of the panel implicated in aragonite formation (*Pif-177*, *MSI60*, and *Perl1*). The protein genes *Pif-177* and *MSI60* regulate growth, nucleation and the organization of the aragonite crystal (Suzuki *et al.* 2009; Xiang *et al.* 2013; Joubert *et al.* 2014). Perlone is the protein equivalent of the N14 protein identified in *P. maxima* (Bédouet *et al.* 2007) and seems to be specifically involved in the formation of the nacreous layer and promotion of aragonite crystal nucleation (Kono *et al.* 2000). Aragonite-related gene expression levels were significantly higher in the graft tissue from the TL group than from the QL group. Some other genes (*Shematin 5 and 9* and *Nacrein*) showed significantly higher expression levels in the graft originating from the TL group. *Shematin 5 and 9* are implicated in the calcite mineralisation process, in which they facilitate the formation of calcite prisms (Yano *et al.* 2006). These results seem to show the opposite trend to the pearl phenotype observations,

since the TL and QL groups did not differ in nacre weight and thickness. The significantly higher expression levels of some genes in the TL group correspond to the biometric parameters of the donor oyster but not to the quantitative traits of the pearl. A study in *P. fucata* examined the differences of gene expression in large and small oysters. RNA-seq analysis revealed that the number of genes up-regulated in small oysters was greater compared to large oysters and could be explained by catch-up growth (Shi and He 2014). In the pearl sac analyses, we observed a relationship between relative gene expression in the pearl sac and grade, surface defects, luster and pearl color. The role of the pearl sac in nacreous layer biomineralisation is thought to mirror that of the oyster mantle. The gene-expression patterns of shell matrix proteins in pearl sacs were measured relative to the biomineralisation process of the nacreous layer (Liu *et al.* 2012). The genome of the individual donor oyster and its influence on the pearl biomineralisation process remain active in the pearl sac tissue during the entire pearl culture period right up until harvest time (Arnaud-Haond *et al.* 2007; McGinty *et al.* 2010, 2012; Masaoka *et al.* 2013), confirming the influence of individual donors on quality pearl traits.

Our examination of pearl formation over time (12 months of rearing) clearly showed that nacreous deposition is not linear. By the half-way point of the experiment (6 months), 69.7% of the final nacre thickness and 65% of the nacre weight had already been reached. At this point, nacre deposition slowed down. No previous study has examined the deposition of the nacreous layer on the pearl over culture time. However, some studies did examine the beginning stage (35 days) of nacreous layer deposition during pearl formation in *P. fucata* (Ma *et al.* 2007; Liu *et al.* 2012) and in *P. margaritifera* (Cuif *et al.* 2008). These found that the first CaCO₃ deposited on the nucleus was the aragonitic (Cuif *et al.* 2008) or calcitic (Ma *et al.* 2007) prismatic layer found in the cross section of the pearl, and that the nacreous layer then formed on top of this prismatic layer. The non-linear nacreous deposit seems to be influenced by the growth of the recipient oyster (whose biometric parameters are correlated with nacre weight and thickness). Shell growth is influenced by two major factors: microalgal concentration and temperature, both of which regulate the expression of most of the shell matrix protein genes implicated in the biomineralisation process (Joubert *et al.* 2014). However, recipient growth had a linear pattern over the 12

months of culture. Thus, the growth of the recipient seems to be an important driving factor of pearl growth but not the only influence implicated. The high influence of the recipient and the weak influence of the donor on pearl size at harvest suggest that their respective roles are not totally revealed here.

In our experiment, we also recorded that luster, grade and darkness of pearls improved with duration of culture, but we also noted an increase in the proportion of circled pearls. According to Wada (1999), who focused on surface color and pigmentation, the quality of nacreous pearls is determined by the ratio of the thickness of the lower prismatic layer to that of the upper nacreous layer. It was therefore logical that we found an improvement of quality grade over time, because nacre deposition increased with time of culture (Blay *et al.* 2014). Individual donor oysters influence quality traits such as color, luster, amount of surface defects and grade but do not affect shape or the presence of circles. The differences observed between quality traits underline the complexity of their genetic basis and that of the donor / recipient interaction.

The present study is the first to identify the relative impact of donor / recipient oyster size on pearl size and quality traits during culture time in the marine bivalve *P. margaritifera*. It was demonstrated that 1) pearl size characters (nacre weight and thickness) are influenced by the recipient oyster phenotype and 2) the major qualitative traits (color, darkness, luster, and incidence of surface defects) are influenced by individual donor oysters. The recipient oyster shell parameters are correlated with pearl size, with the largest recipient giving the largest pearls. Similarly to other examples in the living world, the recipient organism seems to have a more important role than expected. Donor / recipient combinations should therefore be selected carefully. For the pearl industry, the contribution of the recipient found here suggests it would be beneficial to develop breeding programs on growth of recipient oysters in order to obtain larger and thus more valuable pearls. In parallel, a breeding program could be developed for quality and color of donor oysters in order to obtain high quality pearls in desired colors. Furthermore breeding program could be based on pedigree. In fact, genealogy information is essential for genetic traceability. The importance of maintaining pedigree records could aim to increase effective population sizes and

minimise inbreeding to ensure long-term genetic gain, viability of aquaculture breeding programs and productivity by the development for example of a set of multiplex PCRs as in *C. gigas* oyster (Evans *et al.* 2004; Liu *et al.* 2017) or in the abalone *Haliotis midae* (Rhode *et al.* 2014). The present work could serve as a basis for future studies exploring the donor / recipient relationship in greater depth.

5. MATERIALS AND METHODS

5.1. ANIMALS

Wild *Pinctada margaritifera* were collected as spat using commercial collectors in the lagoon of Takaroa atoll (Tuamutu archipelago, French Polynesia) in March 2013. Nine months later (December 2013), 20 of these collectors were transferred to Ifremer facilities in Vairao on Tahiti Island (Society Archipelago, French Polynesia). Pearl oysters (N = 1535) were then removed from the collectors and divided into two distinct groups according to their shell size (dorso-ventral measurement): small “QL”, (N=393; 2.5 cm in means) and large “TL” (N = 292; 4.7 cm in means). The distribution and phenotypes of QL and TL oysters are shown in Table 4.1 and Figure 4.6. These oysters were then tied onto a CTN (Cord Technical Nakasai) rearing system (Cabral *et al.* 1985), where they were left to grow, protected using plastic mesh to prevent predation, until they reached a sufficient size to start the grafting experiment. These oysters were then transferred to Regahiga Pearl Farm (Mangareva island, Gambier archipelago, French Polynesia) to be used as donors. Prior to nucleus implantation and graft surgery, oysters from the QL and TL groups were collected from the rearing station, detached, washed with a high pressure spray (to remove epibiota) and stored until the grafting operation.

5.2. GRAFTING PROCEDURE

All grafts were performed under standard production conditions by a single expert at the Regahiga Pearl Farm so as to minimize the grafter effect on pearl quality traits described in Ky *et al.* (2015). The recipient oysters were issued from local natural spat collection in the Mangareva lagoon. A total of 40 donors (20 of the TL phenotype and 20 of the QL phenotype) were used to perform 600 grafts (20 grafts per donor for TL and 10 grafts per donor for QL) over a 2-day period, using 1.8 BU nuclei (5.45 mm diameter; Imai Seikaku Co. Ltd., Japan) (Figure 4.6). During the grafting process, 3 to 5 graft tissue pieces of each donor oyster were sampled, preserved in RNAlater® and stored at -80°C for subsequent RNA extraction. Recipient oysters were examined 45 days post grafting to estimate nucleus retention and mortality rates, as described in Ky *et al.* (2014).

5.3. MEASUREMENTS OF SHELL BIOMETRIC PARAMETERS AND PEARL GROWTH RATE

Prior to the grafting operation, shell height, width and thickness of the 40 donor oysters were measured using Vernier calipers. All recipient oysters were washed with a high pressure spray to remove epibionts and the following biometric measurements were taken: shell height, width, thickness, and total weight (soft tissue parts + shells) (to 0.01 g) (Figure 4.5) (Le Pabic *et al.* 2016).

Once harvested, cultured pearls were cleaned by ultrasonication in soapy water with a LEO 801 laboratory cleaner (2-L capacity, 80 W, 46 kHz); they were then rinsed in distilled water. Some *keshi* (small non-nucleated nacre deposits) were also harvested but these were not graded in the present study. The size of the cultured pearls was assessed by measuring nacre thickness and weight (Bay *et al.* 2014).

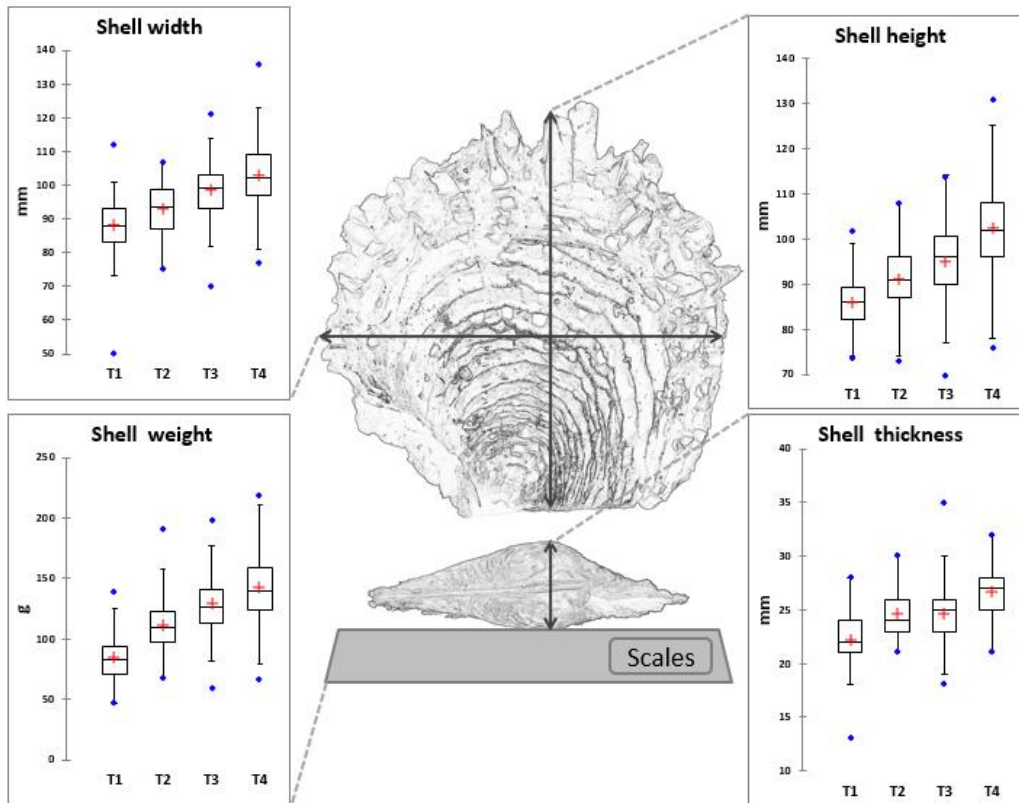


Figure 4.5 : *Pinctada margaritifera* recipient oyster shell biometric parameters measured at each harvest time (T1 corresponds to 3 months of culture, T2 to 6 months, T3 to 9 months and T4 to 12 months of rearing): shell width, height, thickness, and the total oyster weight (soft tissue parts + shells). Each box-plot has the following 6 elements: 1) mean ("+" cross in the box-plot); 2) median (solid bar in the box-plot); 3) 25th to 75th percentile (rectangular box); 4) 1.5*interquartile range (non-outlier range of the box whiskers); 5) minimum and maximum values (extreme dots); and 6) outlier values (outside box whiskers) (N = 289).

5.4. CULTURED PEARL QUALITY PARAMETER MEASUREMENT

Surface defects, luster and grade category of the cultured pearls were evaluated. Cultured pearl shape was characterized in two ways: the presence / absence of circle/s and the shape category ("b" for baroque and semi baroque, "o" for oval and drop, "r" for round and semi round pearls). Two kinds of color evaluation were made on the cultured pearls: the darkness of color and the visually-perceived color category (peacock, green, grey, other). Cultured pearl grade was determined for each sample according to the official Tahitian classification (Journal Officiel 2001 n° 30, 26 July 2001) from the most to least valuable quality: A, B, C, D and Rejects (*rebuts*). Briefly, the five grades are mostly based on surface purity and luster, from A (cultured pearls showing no surface defects or small defects confined to less than 10% of

their surface and having very good luster;) to D (cultured pearls showing many highly visible defects over more than two thirds of their surface and having poor luster) and Rejects (cultured pearls that have too many defects to be graded). The latter are consequently discarded and ultimately destroyed. Finally, surface defects and luster (components of cultured pearl grade) were determined separately so that they could be studied independently. Quality traits were evaluated as described in Ky *et al.* (2013). To ensure homogeneity in parameter assessment, all evaluations were made visually (without a jeweler's loupe) by the same operators.

5.5. BIOMINERALISATION GENE EXPRESSION ANALYSIS

Oyster shell and cultured pearls are formed by biomineralisation activities in two distinct tissues: the mantle of the recipient and the pearl sac in the gonad, formed from mantle tissue from the donor (Ellis and Haws 1999). Around the nucleus, a pearl sac is formed by proliferation of the outer mantle epithelial cells of the mantle graft which secretes successive nacre layers on the nucleus (Inoue *et al.* 2010; Kishore and Southgate 2014). Pearl sac consists of mucous cells containing large acidophilic granules and epidermal cells (Liu *et al.* 2012) that secrete proteins resulting into culture pearl formation, a highly controlled biomineralisation process similar to the development of inner shell regulated by the mantle (Zhan *et al.* 2015). The description of pearl sac collection is explained in the next section. Similarly to other bivalves, the shell of pearl oyster *P. margaritifera* consists of two polymorphs of calcium carbonate: the inner nacreous layer, which is composed of aragonite, and the outer prismatic layer, which is made of calcite (Sudo *et al.* 1997; Marin *et al.* 2004; Zhang and Zhang 2006). Shell formation is a highly controlled process involving multiple matrix proteins (Miyamoto *et al.* 2005; Suzuki *et al.* 2009; Marie *et al.* 2012). Recently, the number of genes identified as coding for molluscan shell matrix components has increased (Montagnani *et al.* 2011; Huang *et al.* 2012; Suzuki and Nagasawa 2013; Miyamoto *et al.* 2013; Shi *et al.* 2013). In order to identify the respective contributions of donor vs. recipient, we sampled tissues of the graft during the graft operation (*i.e.*, donor contribution) and tissues of the pearl sac during harvest (*i.e.*, recipient and donor contributions) to compare relative gene expression through screening aragonite-

related genes (*Pif-177*, *MSI60*, *Perline*), calcite-related genes (*Aspein*, *Shematin*, *Prismalin*) and one gene implicated in both layers (*Nacrein*) (Table 4.2). Two genes were used as housekeeping genes chosen based on their ubiquitous and constitutive expression pattern in *P. margaritifera* tissue: SAGE (SAGES: AGCCTAGTGTGGGGGTTGG/ SAGER: ACAGCGATGTACCCATTTCC) (called REF in Joubert *et al.* (2014)) and GAPDH (GAPDHS: AGGCTTGATGACCACTGTCC/ GAPDHR: AGCCATTCCCGTCAACTTC) (Lemer *et al.* 2015).

Tableau 4.2 : Set of forward and reverse primers used for the biomineralisation gene expression analysis in *Pinctada margaritifera*.

Primer name	Protein name	Function	GenBank Accession Numbers	Forward primer (5'-3')	Reverse primer (5'-3')
PIF	Pif-177	Aragonite formation	HE610401	AGATTGAGGGCATAGCA TGG	TGAGGCCGACTTTCTT GG
MSI60	MSI60	Aragonite formation	No accession number but described by B. Marie <i>et al.</i> 2012	TCAAGAGCAATGGTGCT AGG	GCAGAGCCCTTCAATA GACC
PERL1	Perline	Aragonite formation	DQ665305	TACCGGCTGTGTGCTA CTG	CACAGGGTGAATATC TGGAACC
ASP	Aspein	Calcite formation	No accession number but described by B. Marie <i>et al.</i> 2012	TGGAGGTGGAGGTATCG TTC	ACACCTGATACCCTGC TTGG
PRISM	Prismalin 14	Calcite formation	HE610393	CCGATACTCCCTATCT ACAATCG	CCTCCATAACCGAAAA TTGG
SHEM5	Shematin	Calcite formation	HE610376	GTCCGAAACCAAATCGT CTG	CTGTGGTGATGGTGAC TTCG
CALC1	Nacrein	Aragonite and calcite formation	HQ896199	CTCCATGCACAGACATG ACC	GCCAGTAATACGGACC TTGG
SHEM9	Shematin	Calcite formation	No accession number but described by B. Marie <i>et al.</i> 2012	TGGTGGCGTAAGTACAG GTG	GGAAACTAAGGCACG TCCAC

After removing the RNAlater by pipetting and absorption, total cellular RNA was extracted from the individual graft tissue (n = 40) or pearl sac samples (n = 240), using TRIzol® reagent (Life Technologies) according to the manufacturer's recommendations. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). Total RNA of each individual was then treated with DNase I using a DNA-free Kit (Ambion). First, strand cDNA was synthesized from 500 ng total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and a mix of poly (dT) and random

hexamer primers. Real-Time PCR amplifications were carried out on a Roche Light Cycler® 480. The amplification reaction contained 5 µL LC 480 SYBR Green I Mast (Roche), 4 µL cDNA template, and 1 µL of primer (1 µM), in a final volume of 10 µL. Each run included a positive cDNA and a blank control for each primer pair. The run protocol was as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s. Lastly, the amplicon melting temperature curve was analyzed using a melting curve program: 45–95°C with a heating rate of 0.1°C s⁻¹ and continuous fluorescence measurement. All measurements were made in duplicate and all analyses were based on the Ct values of the PCR products.

Relative gene expression levels were calculated using the delta–delta method, normalized with two reference genes, to compare the relative expression results (Livak *et al.* 2001) as follows: Relative expression $_{(\text{target gene, sample } x)} = 2^{-(\Delta\text{Ct}_{\text{sample, sample } x} - \Delta\text{Ct}_{\text{calibrator, sample } x})} = 2^{-\Delta\Delta\text{Ct}}$. Here, the ΔCt calibrator represents the mean of the ΔCt values obtained for the tested gene. The delta threshold cycle (ΔCt) is calculated by the difference in Ct for the target and reference genes. The relative stability of the GAPDH and SAGE combination was confirmed using NormFinder (Anderson *et al.* 2004). PCR efficiency (E) was estimated for each primer pair by determining the slopes of standard curves obtained from serial dilution analysis of a cDNA to ensure that E ranged from 90 to 110%. The primers used for amplification are listed in Table 4.2.

5.6. RECIPIENT AND DONOR INFLUENCE OVER KINETIC EVOLUTION

The experiment was monitored over time to evaluate changes in the recipient / donor influence as the graft became established within the recipient. Two recipient oysters were harvested for each donor after 3, 6, 9, and 12 months of culture. Biometric parameters were measured as described in the section ‘Measurements of shell biometric parameters and pearl growth rate’. Pearls and pearl sac tissue were collected at the same time. Measurement of pearl growth rate and associated quality trait phenotypes were measured as described in sections ‘Measurements of shell biometric parameters and pearl growth rate’ and ‘Cultured pearl quality parameter measurement’. At the time of pearl harvest and in order to minimise the

contamination of recipient tissues, we first cut the gonads from the host oysters. We then removed the gonad tissue with a surgical blade until only a thin (< 0.5 mm) layer of tissue surrounding the pearls remained. At this point, only the pearl sac and the pearl remain. Next, we made an incision in the pearl sac, removed the pearl, and transferred the pearl sac into a 2.0 ml tube with RNAlater® until RNA extraction (McGinty *et al.* 2012) and placed the pearl in a numbered box. While our technique does not completely remove the potential for gonad tissue contamination within pearl sac samples, it does significantly reduce the possibility for gonad contaminations. In addition, the potential for contamination is further reduced because the gonad itself cannot mineralise and thus poses very little risk, as confirmed by Wang *et al.* (2009) who found MSI60 was not expressed within the gonads of *P. Fucata*. A total of 80 pearl sacs were sampled every 3 months, giving a total of 240 pearl sac tissue samples over 9 months (tissues from the last point at 12 months were of too poor a quality to be used in the analyses). Biomineralisation potential for 40 grafts and 217 pearl sacs that contained pearls was screened via gene expression as described in section ‘Biomineralisation gene expression analysis’ (Figure 4.6).

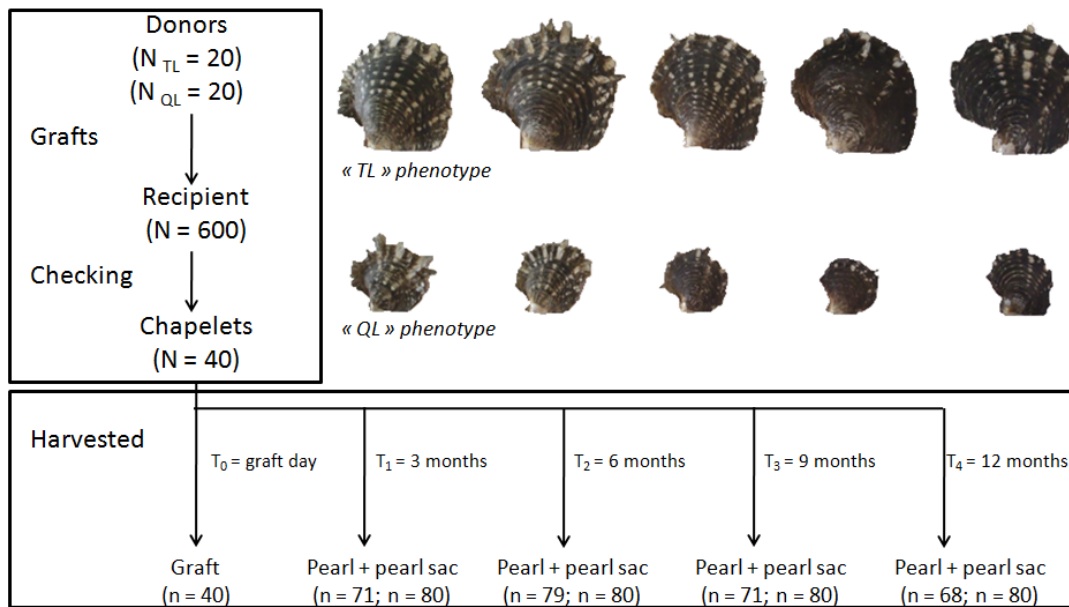


Figure 4.6 : Experimental graft design for *P. margaritifera* donors using QL and TL phenotypes as donors. The grafts (N = 600) were performed on Regahiga Pearl farm (Mangareva Island, Gambier archipelago) then nucleus rejection and receiver mortality were evaluated 45 days after the graft operation (checking). Two recipient oysters per donor were randomly harvested every three months of the experiment.

5.7. STATISTICAL ANALYSIS

The normality of the data distribution and homogeneity of variance were tested for pearl size, donor, recipient oyster biometric parameters and relative gene expression data using the Shapiro–Wilk test and Bartlett’s test. When necessary, transformations were used to adjust data to this distribution (logarithm or square roots). Group donor size (QL vs. TL) and time harvest were treated as fixed variables for statistical analysis, with donor treated as a random variable unless otherwise stated. A linear mixed effects model was used to assess the effect of the fixed and random variables on nacre weight, thickness and relative gene expression, and a generalized linear mixed effects model (family = binomial) was used for the presence or absence of circles and luster, both using packages lme4 and nlme (Bates *et al.* 2014) with the R© software. Qualitative classes based on cultured pearl surface defects, grade and darkness level were re-encoded into ordered categorical response variable. Scores from 0 to 4 were attributed to the different classes from the least to the most valuable. Analyses of ordered categorical response variables (pearl darkness, surface defects and grade) were analyzed using cumulative link mixed models implemented in the ordinal package (Christensen 2015) with the R© software. The significance of fixed variables was tested by comparing the fit of an all-inclusive model with that of a reduced model for each variable using a fisher test or chi-squared test. For the cultured pearl “color categories” and shape categories, donor and time of harvest effect were compared using χ^2 tests.

Spearman’s correlation coefficient was used to evaluate the correlations between nacre weight and thickness, and the four biometric measures of the recipient and donor oysters, and gene expression. We have adjusted the p-value with Bonferroni correction (level of significance = α/c , in which $\alpha=0.05$, and c = number of comparisons performed) to compare pairwise correlation in multiple comparison. The gene expression values of each graft donor and pearl sac were associated with the corresponding nacre weight and thickness of each pearl. The statistics and the visualization for the correlation matrix (Figure 4.3) were done with the R© software Hmisc and Corrplot package using the spearman method (Harell 2016; Wei 2016).

In all cases, the differences were considered statistically significant when p values were lower than 0.05. Statistical analyses were performed using XLSTAT (version 2009.4.02) and R© software (version 3.2.1).

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AUTHORS CONTRIBUTIONS

CB designed and carried out experiments at the pearl farm and the molecular genetic studies in the laboratory, analyzed the data and wrote the manuscript. CLK helped conceive and design the experiments. SP and CLK helped to draft and to revise the manuscript. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

6. SUPPLEMENTARY INFORMATION

Supplementary Table S1

Summary of statistical data between pearl quality traits and the relative expression of 8 selected genes implicated in the biomineralization processes in *P. margaritifera* mantle graft tissue and all pearl sacs. Statistical differences are indicated in bold after Bonferroni correction with the value of significance level ($p < 0.0004$) or “ns” for non significant values ($p > 0.0004$).

		Shape	Circle	Surface defect	Lustre	Grade	Darkness	Colour
Graft	PIF	ns	ns	ns	ns	ns	ns	ns
	MSI60	ns	ns	ns	ns	ns	ns	ns
	PERL1	ns	ns	ns	ns	ns	ns	0.002 ^{ns}
	ASP	ns	ns	ns	ns	ns	ns	ns
	PRISM	ns	ns	ns	ns	ns	ns	ns
	SHEM5	ns	ns	ns	ns	ns	ns	ns
	CALC1	ns	ns	ns	ns	ns	ns	ns
	SHEM9	ns	ns	ns	ns	ns	ns	ns
Pearl sac	PIF	ns	ns	<0.0001	<0.0001	<0.0001	0.019 ^{ns}	0.0002
	MSI60	ns	ns	<0.0001	0.001 ^{ns}	0.03 ^{ns}	ns	<0.0001
	PERL1	ns	ns	0.001 ^{ns}	0.001 ^{ns}	ns	ns	<0.0001
	ASP	0.013 ^{ns}	ns	<0.0001	<0.0001	<0.0001	0.002 ^{ns}	<0.0001
	PRISM	0.037 ^{ns}	ns	0.002 ^{ns}	<0.0001	<0.0001	0.007 ^{ns}	0.008 ^{ns}
	SHEM5	ns	0.043 ^{ns}	0.026 ^{ns}	<0.0001	0.01 ^{ns}	0.037 ^{ns}	ns
	CALC1	ns	ns	ns	0.014 ^{ns}	ns	0.002 ^{ns}	0.0003
	SHEM9	0.01 ^{ns}	ns	0.0002	<0.0001	<0.0001	0.013 ^{ns}	0.001 ^{ns}

Chapitre 5

Optimal age of the donor graft tissue in relation to cultured pearl phenotypes in the animal model, *Pinctada margaritifera*

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**Optimal age of the donor graft tissue in relation to cultured pearl phenotypes in the animal model,
Pinctada margaritifera.**

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1. ABSTRACT

Ageing is defined as the progressive loss of tissue and organ function over time. This study aims to evaluate the ageing effect on cultured pearl quality phenotypes (including size and quality traits) in the graft-recipient animal model: *Pinctada margaritifera*. For this, eight uniform experimental grafts were designed using two hatchery-produced pearl oyster families as donors, which were followed through time, between 7 and 30 months in age. For each age category, 20 donors were studied for each culture site giving a total of 2400 grafted oysters. Several phenotypic measurements were made: 1) donor growth performance from shell size records, 2) pearl size and corresponding quality traits, and 3) expression of some genes related to biomineralization processes on both the mantle graft and on pearl sac tissues. Results showed that: 1) donor age has an impact on pearl size, with grafts coming from the youngest donors yielding the biggest pearls; 2) grafts from donors between 12 and 18 months in age produced pearls of the highest quality (grade and surface quality), a result supported by an analysis where the level of expression for a panel of genes associated with biomineralization was greatest in donors within the 12 to 18 months age group. Donor age (between 12 and 18 month) with high potential for nacre deposition and high biomineralization potential could increase cultured pearl size and quality.

2. INTRODUCTION

The process of ageing affects living organisms, from single cell yeasts to multi cellular animals and plants. Most evolutionary biologists define ageing as the age-dependent or age-progressive decline in tissue function over time (Medawar 1955; Williams 1957; Rose 1991; Partridge and Barton 1996; Tatar 2001; Promislow and Bronikowski 2006; Bronikowski and Flatt 2010; Watson and Riha 2010; Fabian and Flatt 2011; Flatt 2012). Ageing processes have been studied for almost a century in humans *via* cellular cultures, with the assumption that human ageing stems from a cellular origin. In a graft context, it is tempting to speculate that donor age determines graft/ scion quality and further, the long-term function after transplantation. Cellular dynamism and more specifically cell age has been studied through numerous graft models. In human models, there is an upper age limit for the donor in many organ transplant centers. For example, some researchers reported that older livers had a higher rate of primary non-function, prolonged graft function recovery, and an increase in graft loss or mortality (Selzner *et al.* 2009; Valadao *et al.* 2009; Serrano *et al.* 2010; Wang *et al.* 2015). More recently, a study evaluated the impact of donor age on recipient survival in living donor liver transplants and found that younger donors exhibited significantly higher recipient survival (Kubota *et al.* 2017). In the plant kingdom, the relative effect of age on tree metabolism revealed age-mediated controls for tree growth, which are particularly important in the first years of its life (Mencuccini *et al.* 2005, 2006). Another study examined biological and environmental factors that control root dynamics and function through the effect of root ageing on grapevines (Comas *et al.* 2010).

Since the development of artificial pearl culture techniques in the early 1900's, the production of nucleated culture marine pearls has become a significant industry throughout Southeast Asia, northern Australia and Pacific Island nations (Southgate 2007). The marine pearl industry is primarily based on the culture of pearl oysters from the genus *Pinctada* spp or *Pteria* spp (Family: Pteriidae) (Gervis & Sims, 1992), with an estimated annual global value of over US\$397million (2013, Muller). The black-lipped

pearl oyster *Pinctada margaritifera*, mainly from French Polynesia, with minor productions in the Cook Islands, Fiji, the Marshall Islands and Micronesia, is valued for its ability to produce high quality colored pearls. From a genetic point of view, grafting associates two tissues from different individuals, the recipient and graft (two distinct genomes) that create a genetic chimera with each genome maintaining its own genetic identity throughout the grafted organism (Mudge *et al.* 2009). To initiate pearl formation in our model *P. margaritifera*, the gonad of a “recipient” oyster is surgically implanted with a spherical shell bead nucleus (made from the shell of a freshwater mussel from the Mississippi River) alongside a small graft (~4mm²) of mantle tissue cut from a donor oyster. The mantle tissue grows around the nucleus and becomes a “pearl sac”, which secretes successive nacre layers on the nucleus. This process results in the formation of a cultured pearl in about 15-20 months. Once a pearl has been harvested, it is then classified based on several factors that, in combination, will determine its quality and therefore its market value.

Pinctada margaritifera cultured pearls are still produced using wild populations due to the abundance of natural oyster resources and, until recently, the challenges associated with controlling the reproduction and early life stages *in vivo*. Rearing of the species over its entire life cycle, including artificial breeding, became possible through the domestication of *P. margaritifera*. This advance has allowed oyster age to be controlled, in contrast to current Polynesian pearl aquaculture methods which rely on wild spat collection where collected individuals are of undetermined age. To date, studies that examined pearl quality traits mainly focused on the genetics of the donor oyster. The influence of the donor on pearl quality traits has been definitively demonstrated using reciprocal xenografts between *P. maxima* and *P. margaritifera* in which donors were found to have a significant effect on both color and surface complexion (McGinty *et al.* 2010). Studies equally demonstrated a donor (Tayale *et al.* 2012) or family (Ky *et al.* 2013) effect on pearl quality traits (color, darkness, surface defect, lustre and grade). The influence of culture site (Island) on pearl quality traits has also been reported (Blay *et al.* 2016; Le Pabic *et al.* 2016; Ky *et al.* 2015; Jerry *et al.* 2012). A recent study evaluated the effect of donor shell size at a specific age on pearl quality traits (Blay *et al.* 2017) and found that the size of the donor shell does not impact the size or quality of the pearl. Despite existing knowledge about donor and recipient roles on pearl

quality traits, only one previous study examined the effects that the age of donor oyster might have on pearl grade, lustre, surface defect and color. Between 2 and 5 years old, the influence of donor age showed an improved pearl quality in favour of the younger donor (Ky *et al.* 2015). No study examines the impact of using a younger donor oyster (< 2 years old) and the age effect on pearl size.

The present study investigated the age effect (independent from shell size) of hatchery-produced donor *P. margaritifera*, on the culture pearl phenotypes and on the potential for biomineralization. For this, eight uniform experimental grafts were conducted in two culture sites (i.e. testing independence of local adaptation), with two different pearl oysters families aged from 7 to 30 months old. Several phenotypic measurements were made: 1) the shell biometric growth parameters of the donors, 2) the cultured pearl size and quality traits, and 3) the expression of eight gene encoding proteins involved in the biomineralization process in both the mantle graft of the donor and in the final pearl sac tissue. In the end, this study contributes to the optimization of the graft process by identifying the ideal age for the donor oyster which then improves pearl quality traits in hatchery pearl culture systems.

3. MATERIALS AND METHODS

3.1. ETHICS STATEMENT

The pearl oysters used in the current study are marine-cultured animals, and all of the experiments on pearl oysters were conducted following the institutional and national guidelines. No endangered or protected species is involved in the present study, and no specific permission is required for the location of the culture experiment.

3.2. ANIMALS EXPERIMENTAL DESIGN

Two bi-parental *P. margaritifera* families were produced at the Ifremer hatchery facilities (Vairao - Tahiti, French Polynesia), using female and male broodstock from Rangiroa Island (Tuamutu Archipelago, French Polynesia) for F_RGI family, and Mangareva Island (Gambier Archipelago, French Polynesia) for F_GMR family. Spawning was induced by thermal shock (Ky *et al.* 2015c). Artificial breeding, larval rearing and oyster culture procedures were conducted using the protocol developed by Ky *et al.* (2013).

Two months prior to nucleus implantation, oysters from the two families were taken from the rearing station, detached, washed with a high pressure spray (to remove epibiota) and stored ready for use in the grafting procedure. Individuals from the two families, used as donors, were randomly selected and transferred by plane to Rangiroa atoll for F_RGI family and Mangareva Island for F_GMR family to allow the oysters to adapt and grow in local environmental conditions before they were randomly selected for the grafting procedure.

3.3. EXPERIMENTAL DESIGN AND GRAFTING PROCEDURE

Donors from the F_RGI family were used at the Rangiroa atoll culture site at 12, 18, 24 and 30 months old (every 6 months). Twenty donors were randomly selected each time. F_GMR family was monitored at Mangareva island culture site at 7, 12, 18 and 24 months old. Twenty donors were also randomly selected each time. All grafts were performed under standard production conditions by a single expert from each pearl farm, Gauguin's pearl Farm (Rangiroa) and the Regahiga Pearl Farm (Gambier), to minimize the grafter effect on pearl quality phenotypes (Ky *et al.* 2015). The recipient oysters were issued from a local natural spat collection at each culture site. They were selected based on visible health status (color of the visceral mass and gills), shell size appearance and muscle resistance when the shells were pried open. A total of 8 different experimental grafts were performed with 80 donors for F_RGI and 120 donors for F_GMR family which were used to perform 1400 and 1000 grafts respectively (Table 5.1). The

difference between the number of grafts for the two locations is due to the number of saibo per donor used and the age of donor. At 45 days post-graft, recipient oysters were checked to estimate nucleus retention and oyster mortality rates as described in Ky *et al.* 2014.

Tableau 5.1 : Summary of experimental design with location, experiment name and age of donor description. *Graft*: date of grafted procedure, number of saibo per graft (20 donor oysters were used for each experiment), number of total grafted oysters. *Harvest*: date of harvest (18 months post grafting), percentage of pearls harvested and number of pearls and keshi. *Sample*: Number of graft tissues at graft time and number of pearl sac tissues at harvest time.

Location	Experiment name	Age of donor (month)	Graft			Harvest				Sample	
			Date	Number of saibo per donor	grafted oyster	Date	% Pearls	Pearls	Keshi	Graft tissue	Pearl sac tissue
Rangiroa	RGI12	12	sept-13	10	200	mar-15	31	62	10	19	20
	RGI18	18	mar-14	20	400	sept-15	23	93	8	20	20
	RGI24	24	sept-14	20	400	mar-16	29	115	12	20	19
	RGI30	30	mar-15	20	400	sept-16	33	130	4	20	19
Gambier	GMR7	7	oct-13	10	200	apr-15	63	125	10	0	19
	GMR12	12	mar-14	10	200	sept-15	44	88	10	18	0
	GMR18	18	sept-14	10	200	mar-16	80	159	3	19	19
	GMR24	24	mar-15	20	400	sept-16	79	314	8	20	19

After this check, recipient oysters that had retained their nuclei were fixed to chaplets after removing the net retention bags. Each chaplet was labelled according to the corresponding donor oyster for traceability. Furthermore, pearl oysters were regularly cleaned in order to remove biofouling (epibiota), which can hinder healthy oyster growth and pearl production.

3.4. CULTURED PEARL QUALITY PHENOTYPES

Pearl phenotype categories were recorded to characterize the quality of the pearl (Ky *et al.* 2013):

- Shape was characterized in two ways: the presence / absence of circle/s (shown by regular streaks or concave rings, whatever the shape category) and the shape category (“b” for baroque and semi baroque, “o” for oval and drop, “r” for round and semi round pearls).
- Color was evaluated through the darkness level (high, medium and low) and the visually perceived color category, which is conferred by bodycolour pigments and secondary colors (overtone). 5 major color categories were detected including green, grey, peacock, yellow and one named “other” including white, blue and aubergine pearls.
- Cultured pearl grade was determined for each sample according to the official A–D Tahitian classification (Journal Officiel 2001 n° 30, 26 July 2001) from the most to least valuable quality: A, B, C, D and Rejects (*rebuts*).
- Finally, surface defects and lustre (components of cultured pearl grade) were determined separately so that they could be studied independently. To ensure homogeneity in parameter assessment, all evaluations were made visually by the same operators.

3.5. BIOMINERALISATION GENE EXPRESSION PHENOTYPE

In order to identify the age effect on biomineralisation process, we sampled graft tissues (3 to 5 pieces) during the graft operation and pearl sac tissues during the harvest. Samples were preserved in RNAlater® and stored at –80°C for subsequent RNA extraction to evaluate relative gene expression of aragonite-related genes (*Pif-177*, *MSI60*, *Perline*), calcite-related genes (*Aspein*, *Shematin5*, *Prismalin*,

Shematin9 (only for pearl sac tissues)) and one gene implicated in both layers (*Nacrein*) (Blay et al 2017). Two genes were used as housekeeping genes chosen based on their ubiquitous and constitutive expression pattern in *P. margaritifera* tissue: SAGE (SAGES: AGCCTAGTGTGGGGGTTGG/ SAGER: ACAGCGATGTACCCATTTCC) (called REF in Joubert *et al.* 2014) and GAPDH (GAPDHS: AGGCTTGATGACCACTGTCC/ GAPDHR: AGCCATTCCCGTCAACTTC) (Lemer *et al.* 2015).

After removing the RNAlater® by pipetting and absorption, total cellular RNA was extracted from either the individual graft tissue or pearl sac samples, using TRIzol® reagent (Life Technologies) according to the manufacturer's recommendations. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). Total RNA for each individual was then treated with DNase I using a DNA-free Kit (Ambion). First, strand cDNA was synthesized from 500 ng total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and a mix of poly (dT) and random hexamer primers. Real-Time PCR amplifications were carried out on a Roche Light Cycler® 480. The amplification reaction contained 5 µL LC 480 SYBR Green I Mast (Roche), 4 µL cDNA template, and 1 µL of primer (1µM), in a final volume of 10 µL. Each run included a positive cDNA and a blank control for each primer pair. The run protocol was as follows: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s. Lastly, the amplicon melting temperature curve was analyzed using a melting curve program: 45–95°C with a heating rate of 0.1°C s⁻¹ and continuous fluorescence measurement. All measurements were made in duplicate and all analyses were based on the Ct values of the PCR products.

Relative gene expression levels were calculated using the delta–delta method, normalized with two reference genes, to compare the relative expression results as follows: Relative expression_(target gene, sample x) = $2^{-(\Delta Ct_{\text{sample } x} - \Delta Ct_{\text{calibrator, sample } x})} = 2^{-\Delta \Delta Ct}$ (Livak and Schmittgen 2001). Here, the ΔCt calibrator represents the mean of the ΔCt values obtained for the tested gene. The delta threshold cycle (ΔCt) is calculated by the difference in Ct for the target and reference genes. The relative stability of the GAPDH and REF1 combination was confirmed using NormFinder (Stability value for best combination)

(Anderson *et al.* 2004). PCR efficiency (E) was estimated for each primer pair by determining the slopes of standard curves obtained from a serial dilution analysis of a cDNA to ensure that E ranged from 90 to 110%. A total of 136 graft tissues and 135 pearl sac samples were used for the analyses. The graft from GMR7 and pearl sac sample from the GMR12 experiment were not sampled due to technical problems.

3.6. MEASUREMENTS OF SHELL BIOMETRIC PARAMETERS AND PEARL SIZE

Prior to the grafting operation, shell height, width and thickness of the 200 donor oysters were measured using Vernier calipers. At 18 months post-graft (for each experiment and location), the cultured pearls were harvested and placed into a compartmented box that allowed traceability between sample pearls, corresponding donor oysters family and corresponding experiments. Once harvested, cultured pearls were cleaned by ultrasonication in soapy water with a LEO 801 laboratory cleaner (2-L capacity, 80 W, 46 kHz) and then rinsed in distilled water. The size of the cultured pearls was assessed by measuring nacre thickness and weight (Blay *et al.* 2014).

3.7. STATISTICS

The normality of the data distribution and homogeneity of variance were tested for pearl size, donor oyster biometric parameters and relative gene expression levels using the Shapiro-Wilk test and Bartlett's test. When necessary, transformations were used to adjust data to the distribution (logarithm or square roots).

Group donor age was treated as a fixed variable. Firstly, an ANOVA test was performed to test age of donor effect on donor shell biometric parameters, cultured pearl weight, thickness, graft and pearl sac gene expression levels. If the overall test was significant, a Dunn procedure with a Bonferroni correction for multiple tests was performed among all pairs of age groups. Qualitative classes based on cultured pearl surface defects, lustre, grade, darkness and circles were re-encoded to give quantitative scores that would enable the mean value of age group to be obtained for each criterion, thus allowing them to be ranked. Scores from 0 to 4 were attributed to the different classes from the least to the most valuable (with grade,

surface defects, darkness and lustre). For each criterion, Kruskal-Wallis tests were then applied to compare the age and donor groups. For the cultured pearl “color categories” and shape categories, donors and times of harvest effect were compared using χ^2 tests.

In all cases, the differences were considered statistically significant when p values were lower than 0.05. Statistical analyses were performed using R© software (version 3.2.1).

4. RESULTS

A total of 1086 pearl issue from the 8 experimental grafts were analyzed (1086 pearls from 2400 grafted oysters). We studied the impact of the age of donor oyster on the growth shell, pearl size, pearl quality traits and gene expression in the graft and pearl sac tissue.

4.1. AGE EFFECT ON PEARL SIZE

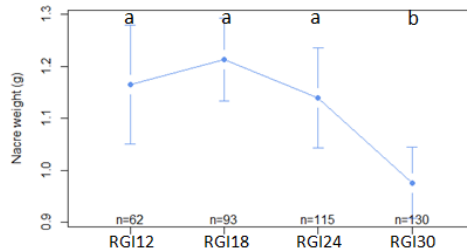
Results of pearl size in Rangiroa and Gambier for each donor age category are illustrated in Figure 5.1. A highly significant age effect was recorded for nacre thickness and weight in Rangiroa and Gambier ($p < 0.0001$).

In Rangiroa, pearls from experiments RGI12, RGI18 and RGI24 were significantly thicker ($p = 0.05$, $p < 0.0001$ and $p = 0.05$ respectively) and heavier ($p = 0.03$, $p = 0.0005$ and $p = 0.02$ respectively) than pearls from experiment RGI30. Pearls from experiment RGI18 showed the greatest average nacre thickness (1.52 ± 0.39 mm) and weight (1.21 ± 0.38 g) and experiment RGI30 showed the thinnest (1.27 ± 0.39 mm) and lightest (0.98 ± 0.39 g), representing a difference of 17% nacre deposit and 23 % nacre weight.

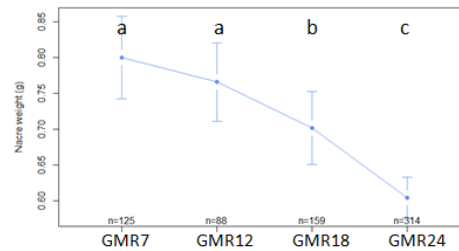
In Gambier, pearls from experiments GMR7, GMR12 were significantly thicker ($p = 0.003$ and $p = 0.03$ respectively) and heavier ($p = 0.01$ and $p = 0.04$ respectively) than pearls from experiments GMR18 and GMR24 ($p < 0.0001$). Moreover pearls from GMR18 were significantly thicker and heavier than pearl from GMR24 ($p = 0.005$ and $p = 0.01$ respectively). Pearls from experiment GMR7 showed the

greatest average nacre thickness (1.51 ± 0.47) and weight (0.80 ± 0.33) and GMR24 showed the thinnest (1.19 ± 0.40 mm) and lightest (0.60 ± 0.26) pearls corresponding to a difference of 21% nacre deposit and 25% nacre weight.

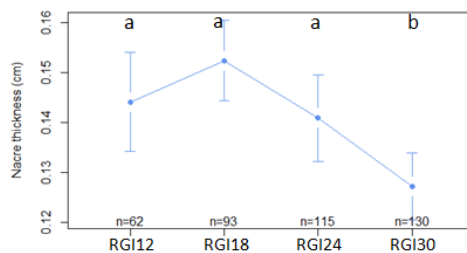
a. Nacre weight at Rangiroa



b. Nacre weight at Gambier



c. Nacre thickness at Rangiroa



d. Nacre thickness at Gambier

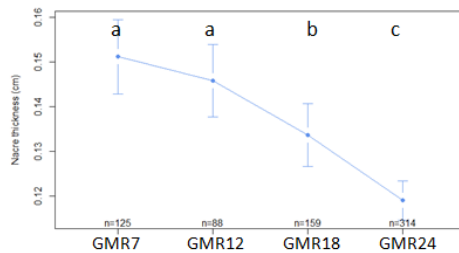


Figure 5.1 : Pearl size harvested for the two sites across four age classes. Mean nacre weight pearls in Rangiroa (a) and Gambier (b) and mean nacre thickness in Rangiroa (c) and Gambier (d) measured across four age classes. Error bars represent the confidence level at 95%. Letter indicates significant difference between the age group ($p < 0.05$).

No significant difference between sites was found between GMR12 and RGI12 while a significant site effect was found for oysters aged from 18 and 24 months. Pearls from GMR18 were significantly thinner than RGI18 ($p = 0.0005$) and pearls from GMR24 were significantly thinner than RGI24 ($p = 0.0017$).

4.2. AGE EFFECT ON PEARL QUALITY PHENOTYPES

An age effect of high significance was detected for a number of cultured pearl surface defects in Gambier and Rangiroa ($p = 0.01$ and $p = 0.001$ respectively). Cultured pearls from GMR24 and RGI24 presented the “best” surface quality, with 40% and 51% respectively of cultured pearls having less than 5

defects against GMR7 (24%) and RGI30 (22%). A large proportion of cultured pearls from GMR12 (48%) and RGI30 (48%) had up to 10 defects. Data analysis showed a significant age effect on cultured pearl grade ($p < 0.0001$). The pearls from GMR7 and RGI30 recorded the highest level of D-R pearls with a proportion of 49% and 57% respectively, while GMR18 and RGI24 recorded the highest level of A-B pearls with 35% and 46% respectively against 17% for GMR7 and 17% for RGI30. Cultured pearl quality trait distributions from each experimental graft are shown in Figure 5.2.

A low significant age effect was detected for shape categories in Rangiroa ($p = 0.05$) and Gambier ($p = 0.03$). No significant difference was recorded between the four different donor age experiments in Rangiroa for the absence or presence of circles, but a significant effect was recorded in Gambier ($p < 0.0001$). Pearls from GMR7 experiment were more circle than in other experiments with 62% of circles pearl compared to 31% of circles pearls, on average, in other experiments.

A significant age effect was recorded for darkness level: $p < 0.0001$. In Gambier, experiment GMR24 produced darker pearls where 31% of pearls were at the high darkness level, in contrast to only 9% recorded for GMR7 and GMR12. In Rangiroa, the darkest cultured pearls were found in experiment RGI30 which had 25% of the high darkness level whereas experiments RGI12, RGI18 and RGI24 produced around 9% of the high level of darkness.

A significant age effect was recorded for “color categories” ($p < 0.0001$). The different color proportions produced by the different experiments are illustrated in Figure 5.2. We equally observed a significant difference between the proportion of peacock and yellow color between the 2 sites. We found more yellow pearls in Rangiroa with on average 21% of the harvest against 4% in Gambier, and more peacock in Gambier with a proportion of 20% against 8% in Rangiroa.

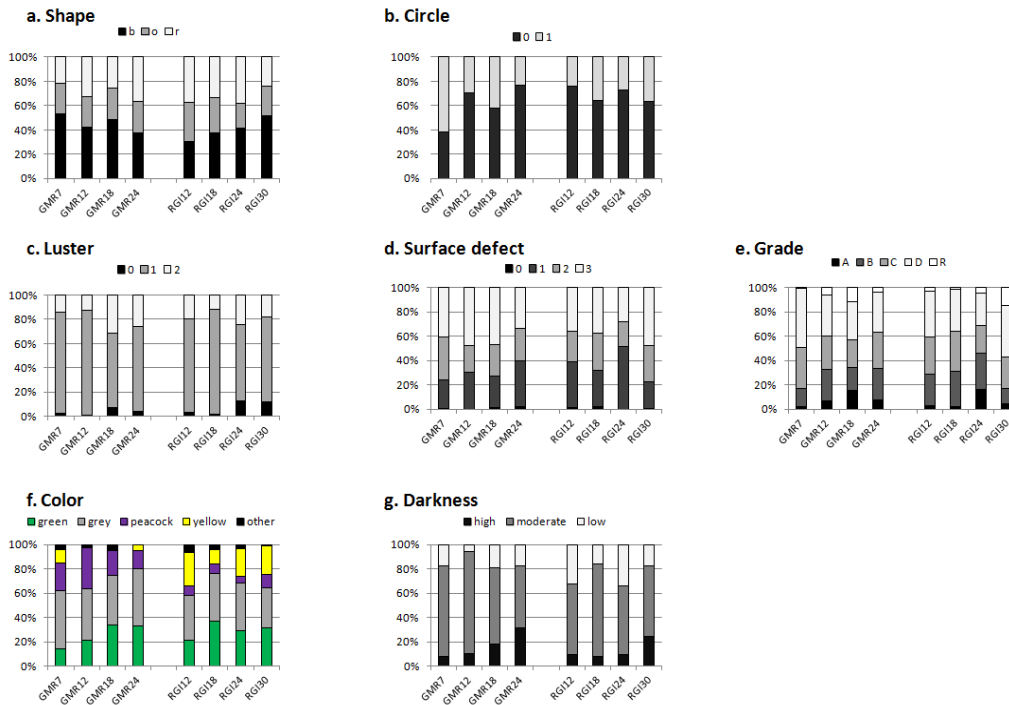


Figure 5.2 : Cultured pearl quality traits from the experimental graft distribution. Percentage of cultured pearls for each experiment in Gambier (GMR) and Rangiroa (RGI) for different donors in different age groups (7, 12, 18, 24 and 30 months old) with the following variables: a. shape categories (“b” for baroque and semi baroque, “o” for oval and drop, “r” for round and semi round pearls), b. pearl circles (“0”= absence and “1”= presence), c. luster levels (“0” = absence of luster, “1” = moderate luster, and “2” = high luster), d. surface defect classes (“0” = 0 defects, “1” = 1–5 defects, “2” = 6–10, and “3” = >10 defects), e. classification grade (“A” to “D” and Rejects), f. visual color categories (“green”, “grey”, “peacock”, “yellow” and “other”, corresponding to white, blue and aubergine pearls) and g. darkness level (low, moderate and high darkness).

4.3. AGE EFFECT ON BIOMINERALISATION PROCESS

Relative gene expressions for the panel of gene coding proteins implied in calcite and aragonite layers at Rangiroa and Gambier in the graft and pearl sac tissue were illustrated in Figure 5.3.

Concerning the graft tissue, the gene expression levels for the seven candidates varied at Rangiroa and were significantly different among the three donor ages (12, 18 and 24 months). PIF, MSI60, PERL1 and CALC1 gene expression levels were significantly higher for the 12 month old donors (2.36, 1.88, 3.40 and 2.30 respectively), compared to the 24 month old donors which had a fold change inferior to 0.6 for the four genes ($p < 0.0001$).

At Gambier, the relative expressions of 7 genes that encode for the biomineralization matrix proteins in the graft were significantly different between the donor age categories ($p < 0.0001$). PIF177, MSI60, PERL1, CALC1, ASP and PRISM14 gene expression levels were significantly higher for GMR18 compared with GMR24 which had a fold change inferior to 0.6 for all genes ($p < 0.0001$).

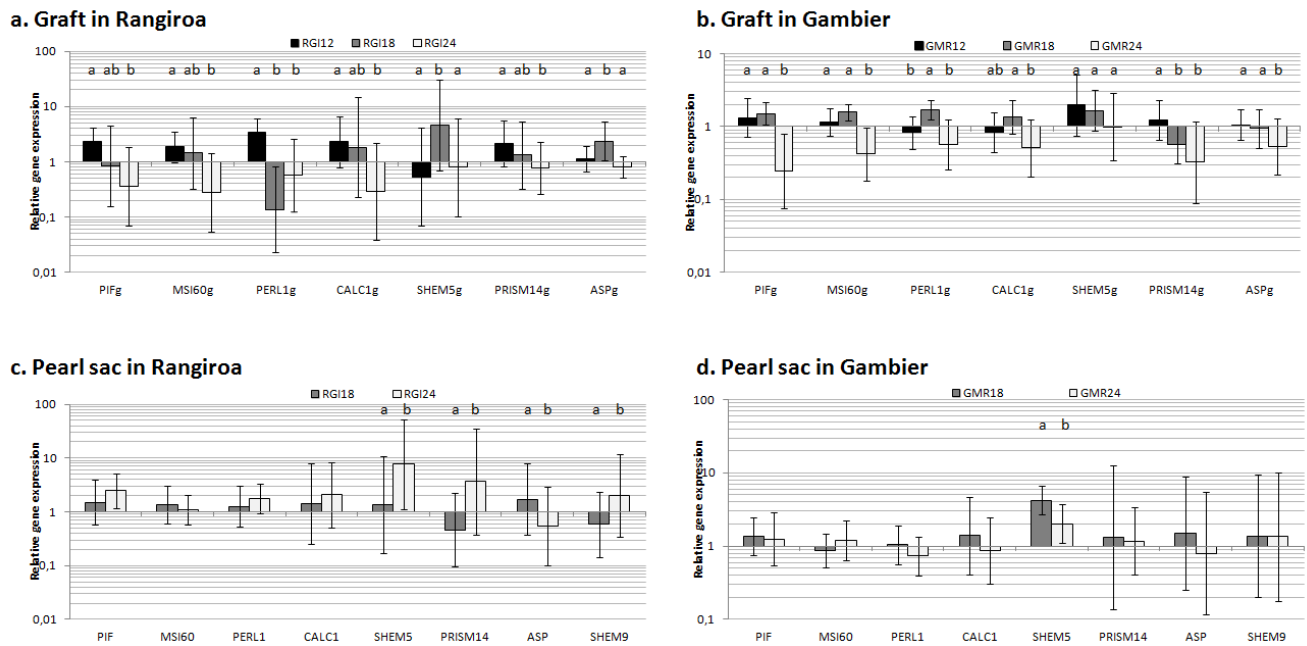


Figure 5.3 : Relative expression of biomineralisation genes in *P. margaritifera*. Relative expression of 7 genes in the mantle at Rangiroa (a) and Gambier (b) for donors aged 12, 18 and 24 months (dark to light grey). Relative expression of 8 biomineralization genes in the pearl sac in Rangiroa(c) and Gambier (d) for donors aged 18 and 24 months (dark and light grey respectively). Y axes are in logarithmic scale. Error bars correspond to standard deviations. Statistical differences between the age groups are indicated by letters ($p < 0.05$).

In Rangiroa, four candidate genes were not significantly different in the pearl sac between the two donor age categories PIF, MSI60, PERL1 and CALC1.

Among the eight candidate genes studied in the pearl sac at Gambier, only the expression of SHEM5 was significantly different in the pearl sac between the two donor age categories ($p = 0.0002$).

4.4. AGE EFFECT ON SHELL GROWTH

At the time of grafting, the donor oyster width, height and thickness were recorded. Results for shell growth with respect to donor age and culture site are described in Figure 5.4. No difference between the two sites at fixed age was significant for the donor that were younger than 24 months: *i.e.* only 24 months old donor oysters at (RGI24 and GMR24) were significantly different between Rangiroa and Gambier sites for shell height (with average measurements of 81.0 ± 9.2 mm and 71.9 ± 6.7 mm respectively) and shell thickness (with average measurements of 18.4 ± 3.4 mm and 21.4 ± 2.6 mm respectively). The growth curves show the maximum slope for donors less than 18 months old for Rangiroa and Gambier biometric parameter.

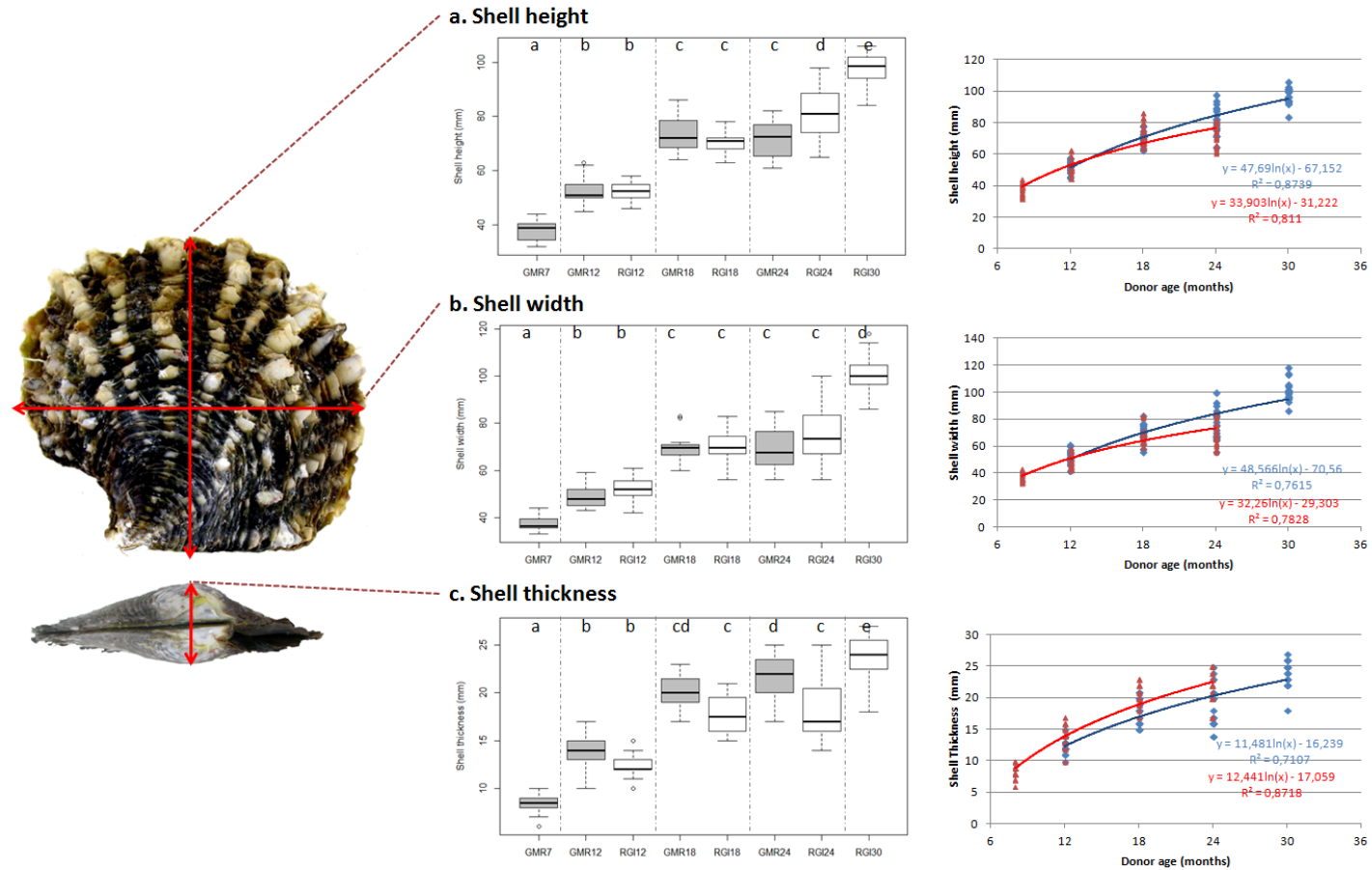


Figure 5.4 : *Pinctada margaritifera* donor oyster shell biometric parameters. a. Shell height, b. width and c. thickness were measured at each graft time (GMR7 corresponds to 7 months old at Gambier location, GMR12 and RGI12 to 12 months old, GMR18 and RGI18 to 18 months old and GMR24 and RGI24 to 24 months and RGI30 to 30 months old at Rangiroa location). Each box-plot has the following elements: 1) median (solid bar in the box-plot); 2) 25th to 75th percentile (rectangular box); 3) 1.5*interquartile range (non-outlier range of the box whiskers); 4) minimum and maximum values (extreme dots); and 5) outlier values (outside box whiskers). Statistical differences between the age groups are indicated by letters ($p < 0.05$). The growth curve on the right contains two curves: Shell growth in Rangiroa in blue and in Gambier in red with their corresponding equations.

5. DISCUSSION

This study is the first to evaluate the impact of pearl oyster age on pearl size (including nacre weight and thickness) and on the expression levels for a panel of eight genes involved in shell biomineralisation in *P. margaritifera*. This study is also the first to examine the impact of pearl oysters aged less than 2 years on pearl quality traits. We found that donor age impacts the size of the pearl, pearl grade and surface quality. Donor age also impacts the gene expression level of aragonite-related genes (*Pif-177*, *MSI60*, *Perline*), calcite-related genes (*Aspein*, *Shematin5*, *Prismalin*, *Shematin9* (only for pearl sac tissues)) as well as the gene implicated in both layers (*Nacrein*) that we monitor.

The major result of this study is that donor age has an impact on pearl size. In fact we found that pearls from the oldest donors, 30 months in Rangiroa and 24 months in Gambier, yielded the lightest and the thinnest pearls. The ageing of the donor's mantle cells clearly alter the quantity of nacreous deposit. Grafts originating from young donors produced bigger pearls than grafts produced by older donors (more than 24 months). This result may be related to the donor shell's growth curve where maximum growth occurs before 18 months. As a general rule, growth rates are directly related to bivalve age. *P. margaritifera* undergoes rapid shell growth until it reaches 18 months and then the rate slowly begins to decrease as it begins to invest more energy into reproduction (Pouvreau *et al.* 2000). Pouvreau *et al.* (2000) also confirmed that growth differences between atolls became highly significant for 2-year-old pearl oysters and similarly, we observed that 24-month-old donors were significantly different in shell height and thickness between Rangiroa and Gambier (in Rangiroa, oysters had greater heights and in Gambier, oysters were thicker). The pearl itself is structurally identical to the nacreous layer of the shell consisting of calcium carbonate aragonite (Taylor 1969). This physiological background could explain the ability of grafts originating from younger individuals to produce bigger pearls in association with cellular growth activity and this idea was further supported by the graft biomineralisation analysis which showed a higher level of expression for the aragonite candidate gene (PIF, MSI60 and PERL) in the younger donor oyster.

In the present study, we found a donor impact that confirms family and donor effect on pearl size as previously described in Ky *et al.* (2013) and Tayale *et al.* (2012). In a previous study, we demonstrated that donor shell biometry at fixed age was not correlated with pearl size while the recipient shell biometry impacted pearl size (Blay *et al.* 2017). Pearl size results from a complex interplay between the donor and the recipient oyster. When the donor is in a “growth period” (i.e. young stage), the cell’s graft will deposit more nacre on the pearl and if paired with a recipient oyster also undergoing a period of elevated growth, we would expect pearl size to be maximized. A previous study also led to a similar hypothesis where they showed a relationship between shell growth performances of families selected and used as graft donors and the final weight of the cultured pearls produced (Ky and Le Moullac 2017). In Pouvreau *et al.* (2000), increments in nacreous deposition equalled $7.2 \mu\text{m}\cdot\text{d}^{-1}$ during the second year of the life cycle and decreased with the age of the pearl oyster, reaching a mean value of $3.1 \mu\text{m}\cdot\text{d}^{-1}$ during the fourth year of the life cycle and confirming that growth rate in shell or tissue is directly related to oyster age.

Age for donor and recipient oysters is a parameter that is important to consider. A donor aged between 12 and 18 months seems to be the ideal candidate for a maximum nacreous deposition. These results were confirmed with the graft biomineralisation analysis where in GMR30, the oldest donor oysters showed the lowest level of expression for the aragonite candidate gene (PIF, MSI60 and PERL). PIF was previously found to be positively correlated with shell deposition rates in *P. margaritifera* (Joubert *et al.* 2014) and thus with pearl size (nacre weight and thickness) (Blay *et al.* 2016). A parallel can be drawn with plants, where a study demonstrated that increases in height is clearly associated with age, where scions from younger donors grew faster during the initial period (first year) than scions from older donors in the radiate pine (Mencuccini *et al.* 2006).

Among the various phenotypes of pearl quality traits surveyed in this work, those concerning grade and surface defects deserve special consideration. Our results clearly demonstrate that grade and surface defects were of poor quality (more D-R pearls and > 5 defects) for donor oysters older than 24 months as well as for donors less than 12 months. For pearls produced by donors younger than 12 months, we are faced with a technical problem versus a physiological one. This technical problem occurs because

the use of very young oysters (i.e. small sized individuals, 3.78 ± 0.36 cm mean height) for the graft limits the precision in choosing the perfect tissues for the graft and leads to poor pearl quality. For pearls produced from donors older than 24 months, the aging of the pearl sac cells is likely to explain the reduction in pearl quality. This result is supported by an analysis of the expression level for the panel of 8 genes involved in the biomineralization of graft tissue. Results showed a minimum expression for genes involved in aragonite production in 24-month-old donor oysters from both Gambier and Rangiroa. Older cells are characterized by several detrimental changes that are likely to alter gene expression. We demonstrated in a recent study that the recipient oyster regulates the metabolism of the pearl sac by supplying nutrition throughout the pearl formation period (Le Pabic *et al.* 2016; Blay *et al.* 2017; Ky *et al.* 2017). Recipient oysters might regulate the expression of biomineralization genes in the pearl sac and this could explain the weak difference between donor age categories for pearl sac expression levels. We confirmed results reported by Ky *et al.* (2017), where 2-year-old graft cells improved pearl grade, predominantly through a higher proportion of 0 surface defects.

In the present study we can conclude that using donors that are too young (<12 months) or too old (>24 months) decreases the quality of the pearls produced. These results could be compared to a human graft model where, for example, the impact of donor age on liver transplantation has been analyzed in several retrospective studies with contradictory conclusions. Some studies reported similar graft survival in older and younger donors (Emre *et al.* 1996; González *et al.* 2002), but in others, a higher incidence of poor initial function (Busquets *et al.* 2001), arterial complications (Vivarelli *et al.* 2004) and decrease graft survival (Serrano 2009) was reported. No doubt, *in fine*, that donor age has a role on the quality of the graft result. Similarly, our study demonstrates that donor age influences the pearl phenotype and that there is potential to improve pearl size and quality in *P. margaritifera* if donor age is optimized. Donor age (between 12 and 18 months) with high potential for nacre deposition and high biomineralization potential will increase cultured pearl size and quality.

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AUTHORS CONTRIBUTIONS

Conceived and designed the experiments: CB CLK. Performed the experiments: CB CLK. Analysed the data: CB. Contributed reagents/ analysis tools: CLK SP. Draft the paper: CB, CLK, SP. Revise the manuscript: CB CLK SP.

Chapitre 6

Crossing phenotype heritability and candidate gene expression in grafted black-lipped pearl oyster, an animal chimera

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**Crossing phenotype heritability and candidate gene expression in grafted black-lipped pearl oyster,
an animal chimera**

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1. ABSTRACT

Grafting mantle tissue of a donor pearl oyster into the gonad of a recipient oyster results in the formation of a chimera, the pearl sac. The phenotypic variations of this chimera are hypothesized to be the result of interactions between the donor and recipient genomes. In this study, the heritability of phenotypic variation and its association with gene expression were investigated for the first time in the chimera formed during *P. margaritifera* pearl production. Genetic variance was evaluated at three different levels, 1) before the graft operation (expression in graft tissue), 2) after grafting (pearl sac tissue expression in chimera) and 3) on the product of the graft (pearl phenotype traits) based on controlled bi-parental crosses and the F1 generation. Donor related genetic parameter estimates clearly demonstrate heritability for nacre weight and thickness, darkness and colour, surface defects and grade, which signifies a genetic basis in the donor oyster. In graft relative gene expression, the value of heritability was superior to 0.20 in for almost all genes (only *Pif-177* $h^2 = 0.11$), while in pearl sac, heritability estimates were low ($h^2 < 0.10$) (except for *CALCI* and *Aspein* ($h^2 > 0.20$)). Pearl sac expression seems to be more influenced by residual variance than the graft, which can be explained by environmental effects that influence pearls sac gene expression and act as a recipient additive genetic component. The interactions between donor and recipient are very complex and further research is required to understand the role of the recipient oysters on pearl phenotypic and gene expression variances. Donor oyster-derived genetic parameters are an initial step towards developing a selective breeding program to improve pearl traits.

2. INTRODUCTION

Quantitative genetics is a powerful framework to explore the complex genetic architecture of phenotypic traits (Kruuk & Hadfield 2007). The fraction of the phenotypic variability that is of transmittable genetic origin is called heritability (Falconer & Mackay 1996; Roff 1997; Lynch & Walsh 1998). Quantitative genetic approaches have been designed to determine to what degree this phenotypic variation is genetically rather than environmentally determined (Falconer 1989). Broad-sense heritability (H^2) estimates the proportion of phenotypic variation due to all genetic effects, whereas narrow-sense heritability (h^2) estimates the proportion of phenotypic variation due to heritable genetic variation alone (Visscher *et al.* 2008). Recent reports of substantial heritability for gene expression and new estimation methods using marker data highlight the relevance of investigating heritability in the genomics era. At the transcriptome level, gene expression profiling has become a popular technique used to quantify regulatory changes in messenger (m)RNA expression. Indeed, gene expression acts as an intermediate phenotype between genotypes and complex traits (Nica and Dermitzakis 2008; Li *et al.* 2012; Goldinger *et al.* 2013). To investigate heritability, the expression profile of a gene in a segregating population can be treated as a quantitative trait and its additive genetic variance estimated (Visscher *et al.* 2008; Powell *et al.* 2013; Wright *et al.* 2014; McCairns *et al.* 2016). Genetic variation underlying gene expression levels has been well established and reported in the literature, with the transcript levels for the majority of genes being heritable to some degree (Price *et al.* 2011; Grundberg *et al.* 2012; Powell *et al.* 2012), but inconsistency in heritability principles raises questions about the transmission process.

Heritability is of great relevance for breeding strategy as it measures the potential response to selection (Lynch and Walsh 1998; Falconer 1989; Mousseau 1998). In cultivated populations, the selection procedure chosen needs to be the best adapted to the breeding plan, allowing assessment of genetic parameters in few generations based on a small effective population. In the context of cultured pearl production by the *Pinctada* genus, the complexity of the graft leading to a chimera type complex makes it more complicated to understand the heritability of any phenotypes or candidate gene expression.

In the plant kingdom, the heritability of grafted-induced phenotypic changes suggests that regulatory processes underlying the scion-rootstock communication also involve a genetic component (Tsaballa *et al.* 2013). Some studies have demonstrated the exchange of genetic material between cells in grafted plants (Stegemann & Bock 2009). Recently, increasing effort has been made to determine how macromolecules are transferred between scions and rootstocks in grafted plants to reveal the mechanism that controls graft-induced changes in plant traits (Paultres *et al.* 2016). Grafting is characterized by tight connections between cells with different genomes, providing the possibility of interactions or cell communication between genetically divergent cells, resulting in a profound perturbation of the cellular environment (Cao *et al.* 2016). Chimeras provide one of the most interesting environments in which to investigate the transmission of genetic material and the resulting phenotypic variation. Thus, the phenotypic variations of the chimera are hypothesized to be the result of interactions between the different genomes.

In the case of pearl bivalve aquaculture based on a grafting operation, previous genetic studies have primarily focused on determining genetic parameters for shell growth, aiming to detect any significant genetic variation for shell growth in the pearl mussel *H. cumingi* (Jin *et al.* 2012) and in the pearl oysters *Pinctada fucata martensii* (He *et al.* 2008; Wang *et al.* 2010) and *P. maxima* (Kvingedal *et al.* 2010). For *P. margaritifera*, genetic analyses based on heritability estimations are still lacking for both quantitative pearl traits and expression levels of some biomineralization genes. A study was made on *P. maxima*, with the estimation of the genetic parameters (heritability and genetic correlations) of commercially important pearl traits (Jerry *et al.* 2012). The production of cultured pearls is both unique and biologically complex in comparison to any other aquaculture industry. *P. margaritifera* produces valuable pearls as a result of the biomineralization process of a mantle graft originating from a donor oyster, inserted together with a nucleus, into the gonad of a recipient oyster (Southgate 2011). The grafting process therefore associates two distinct genotypes, each of which maintains its own genetic identity throughout the life of the grafted organism (the recipient), but which survive together as a genetic chimera due to a unique symbiotic relationship (Mudge *et al.* 2009). Exploring the heritability of candidate gene expression in the graft tissue

(donor) and pearl sac (chimera), and the heritability of pearl phenotypic traits (product of the chimera) is vital to understanding the phenotypic variations induced by the grafting process and the recipient environment.

This original study aimed to evaluate *P. margaritifera* genetic variance for both pearl traits and biomineralization gene expression levels, based on a multi-cross design that made it possible to consider parental and segregating progeny contributions at three material levels: 1) the mantle graft tissue gene expression, 2) the pearl sac tissue (chimera) gene expression and 3) the final product at harvest, the pearl phenotypes. Most previous studies have estimated the genetic contribution to phenotypic traits and, more recently, examined relative gene expression, but they have rarely crossed the traits and the gene expression in the same analysis. In the present study, heritability will then be estimated from parents to progenies within different bi-parental crosses, making it possible to evaluate character transfer in a two-generation framework. The representative panel of genes encoding proteins involved in the biomineralization process that we screened in the graft and pearl sac were: 1) aragonite: *Pif-177*, *MSI60* and *Perline*; 2) calcite (*Aspein*, *Shematin* and *Prismalin*); and 3) for proteins implicated in both layers, *Nacrein* (Marie *et al.* 2012; Joubert *et al.* 2010; Xiang *et al.* 2013).

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

Nine bi-parental *P. margaritifera* families (named A1, B2, D2, F5, G6, H6, H7, I6 and I7) were produced in the Ifremer hatchery system facilities in Vairao, Tahiti, French Polynesia, using female and male broodstock from Mangareva Island (Gambier Archipelago, French Polynesia). Spawning was induced by thermal shock (Ky *et al.* 2015). Nine families were produced in two distinct periods (i.e. two separate controlled breedings, #1 and #2), 5 families (A1, B2, D2 and F5) using 4 females and 3 males (in March 2013), and 4 families (G6, H6, H7, I6 and I7) using 3 females and 2 males (in August 2013). Figure 6.1 illustrates the breeding design, showing that individuals 2, 6 and 7 (males) and H and I

(females) were used in multiple combinations. Artificial breeding, larval rearing and oyster culture procedures were conducted using the protocol developed by Ky *et al.* (2013).

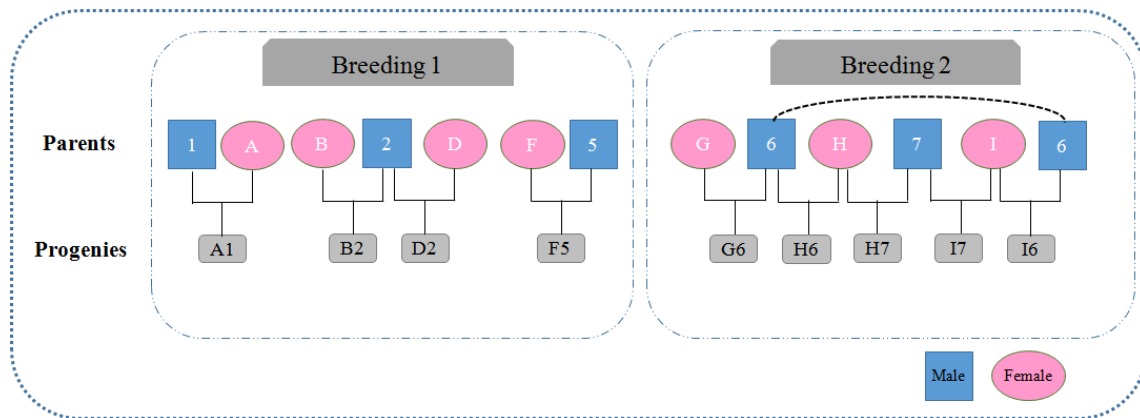


Figure 6.1 : *Pinctada margaritifera* crossbreeding design for the production of the nine half-sib families used as graft donors. Females and males were named with letters and numbers respectively.

Individuals of the nine families that would be used as donor oysters were randomly selected and transferred by air to Mangareva Island (Gambier Archipelago), allowing the oysters to be cultured in natural environmental conditions. Two months prior to nucleus implantation, oysters from the nine progenies were taken from the rearing station and stored ready for use in the grafting procedure.

3.2. GRAFTING PROCEDURE

As the grafting operation itself may influence cultured pearl quality, all grafts were performed under standard production conditions by a single expert at the Regahiga Pearl Farm using a single nucleus size of 1.8 BU (5.45 mm diameter; Imai Seikaku Co. Ltd., Japan). All recipient pearl oysters were obtained by natural spat collection from the wild in the Mangareva lagoon. They were selected based on visible health status (colour of the visceral mass and gills), shell size appearance, and muscle resistance when prising the shells slightly open.

A total of four different experimental grafts were performed; two using the parents of the two breeding designs (one per breeding) and two others using the progenies (one per breeding). For breeding #1, all the five parents were used as donors, covering a total of 229 grafts, with for A ($n = 28$ grafts), B ($n = 36$), D (n

= 37), F ($n = 29$), 1 ($n = 23$), 2 ($n = 36$), 5 ($n = 40$). For breeding #2, 200 grafts were produced with a standard 40 grafts per parent. Concerning the progenies, 20 donors per families were used, with donors providing 1260 grafts for breeding #1 and 2000 grafts for the breeding #2. At 45 days post-grafting, recipient oysters were checked to estimate nucleus retention and oyster mortality rates as described in Ky *et al.* (2014). After this check, recipient oysters that had retained their nuclei were drilled and fixed to chaplets for long term culture and each chaplet was labelled according to the corresponding donor oyster for traceability.

3.3. PEARL QUALITY VARIABLES

After 18 months of culture in Regahiga lagoon, the cultured pearls were harvested and placed separately in compartmented boxes that allowed traceability between the pearls and corresponding donor oyster family. Once harvested, cultured pearls were cleaned and five variables were measured to characterize their quality:

- The size of the cultured pearls was assessed by measuring nacre thickness and weight.
- Cultured pearl shape was characterized in two ways: the presence / absence of circle(s) and the shape category (“b” for baroque and semi baroque, “o” for oval and drop, “r” for round and semi-round pearls).
- The colour of the pearls was evaluated on the basis of the darkness of their colour and their visually perceived colour category, which is conferred by pigments (bodycolor: grey, white and yellow), and secondary colours (overtone: green, aubergine and peacock).
- The cultured pearl grade was determined for each sample according to the official A–D Tahitian classification (Journal Officiel 2001 n° 30, 26 July 2001) from the most to least valuable quality: A, B, C, D and Rejects (*rebuts*).
- The surface defects and lustre (components of cultured pearl grade) were determined separately so that they could be analysed independently.

Quality traits were evaluated as described in Ky *et al.* (2013). To ensure homogeneity in parameter assessment, all evaluations were made visually (without a jeweller's loupe) by 2 operators working together and cross checking.

3.4. GENE EXPRESSION VARIABLES

The formation of the molluscan shell nacre is regulated to a large extent by a matrix of extracellular macromolecules that are secreted by the shell-forming tissue and the mantle (Ellis and Haws 1999). Recently, the number of genes identified as coding for molluscan shell matrix components has increased (Susuki *et al.* 2009; Miyamoto *et al.* 2005, 2013; Marie *et al.* 2012; Joubert *et al.* 2010; Montagnani *et al.* 2011; Huang *et al.* 2012; Suzuki and Nagasawa 2013; Shi *et al.* 2013). In order to identify variability in gene expression in the graft process, we sampled 3 to 5 grafts per donor during the graft operation and pearl sacs during harvest (preserved in RNAlater® and stored at -80°C for subsequent RNA extraction). We then evaluated relative gene expression by screening aragonite-related genes (*Pif-177*, *MSI60* and *Perline*), calcite-related genes (*Aspein*, *Shematin* and *Prismalin*) and one gene implicated in both layers (*Nacrein*) (Blay *et al.* 2017). Total cellular RNA was extracted from the initial graft tissues and harvested pearl sacs (final graft stage) using TRIZOL reagent (Life Technologies) according to the manufacturer's recommendations. RNA was quantified using a nanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). For each sample, 3 µg of total RNA were treated with DNase using a DNA-free Kit (Ambion) to degrade any potential DNA contaminants. For each sample, 0.5 µg of total RNA were reverse-transcribed using a Transcriptor First Strand cDNA Kit (Roche) and amplified by real-time PCR on a Roche Light Cycler® 480 using a set of forward and reverse primers (Blay *et al.* 2017). Two other genes were used as "housekeeping genes": *REF1* (Joubert *et al.* 2014) and *GAPDH* (Lemer *et al.* 2015). The amplification reaction contained 5 µL LC 480 SYBR Green I Mast (Roche), 4 µL cDNA template, and 1 µL of primer (1µM), in a final volume of 10 µL. Each run included a positive cDNA and a blank control for each primer pair. The PCR reactions consisted of a first step of 10 min at 95°C followed by 40 cycles consisting of: 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. At the end, an additional

cycle was performed from 55 to 95°C, increasing by 0.1°C every second, to generate the dissociation curves and to verify the specificity of the PCR products. All measurements were performed on duplicate samples and all analyses were based on the Ct values of the PCR products. Relative gene expression was calculated using two reference genes *GAPDH* and *REF1*, by the $2^{-\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001), as follows: Relative expression $_{(\text{target gene, sample } x)} = 2^{-(\Delta\text{Ct}_{\text{sample, sample } x} - \Delta\text{Ct}_{\text{calibrator, sample } x})} = 2^{-\Delta\Delta\text{Ct}}$. Here, the ΔCt calibrator is the mean of the ΔCt values obtained for the tested gene. The delta threshold cycle (ΔCt) is calculated by the difference in Ct for the target and reference genes. The relative stability of the *GAPDH* and *REF1* combination was confirmed using NormFinder (Stability value for best combination) (Anderson *et al.* 2004). PCR efficiency (E) was computed for each primer pair by determining the slopes of standard curves obtained from serial dilution analysis of a cDNA to ensure that E ranged from 90 to 110%.

3.5. STATISTICS

The normality of data distribution and homogeneity of variances were tested for pearl size and graft and pearl sac relative gene expression data using the Shapiro–Wilk test and Bartlett’s test. When necessary, transformations were used to adjust data to this distribution (logarithm or square root).

We first evaluated the “family effect” on culture pearl trait and gene expression among the progenies of the controlled breeding (Table 1). An ANOVA was performed to test for “family effect” on cultured pearl weight, thickness and gene expression in the graft among the progenies. To test for “family effect” on pearl sac gene expression, ANOVA was performed on progenies of breeding #1 only (pearl sacs from breeding #2 were not harvested). If the overall test was significant, a Dunn procedure, including Bonferroni correction for multiple tests, was performed among all pairs of families. Qualitative classes based on cultured pearl surface defects, lustre, grade, darkness and circles were re-encoded to give quantitative scores that would enable the mean value of progenies to be obtained for each criterion, thus allowing them to be ranked. For each criterion, Kruskal–Wallis tests were then applied to compare the

progenies. For the cultured pearl colour and shape categories, differences and family effects were evaluated using χ^2 tests (Ky *et al.* 2013).

Quantitative genetic parameters and variance components were estimated using an animal model (Kruuk 2004) based on the donor oyster family relationships. The analyses were implemented in R© software using the Markov Chain Monte Carlo for generalized linear mixed models (MCMCglmm) package (Hadfield 2010). We considered the phenotype y_i of the individual i as a variation around the average population phenotype μ as a function of the pedigree of the individual and other factors. The model was:

$$y_i = \mu + a_i + e_i$$

In this equation, μ stands for the average population phenotype, a_i is called the breeding value and accounts for the influence of the additive effect on the phenotype, and e_i is a residual accounting for the variation not captured by the phenotype. Host genetic variation was considered as a common environmental effect. “Animal” was included as a random effect. The heritability (h^2) was estimated as the ratio of the additive genetic variance to total phenotypic variance.

$$h^2 = \sigma_a / \sigma_p = \sigma_a / (\sigma_a + \sigma_f + \sigma_r)$$

Where σ_a is an estimate of the additive variance, σ_f is an estimate of random variance and σ_r is an estimate of the residual variance. When summed, these three components add up to the total phenotypic variance σ_p .

In all cases, the differences were considered statistically significant when p values were lower than 0.05. Statistical analyses were performed using R© software (version 3.2.1).

4. RESULTS

Cultured pearl quality traits were recorded and analysed on a total of 2241 samples, resulting from grafts made using tissue from the parental and progeny generations. Variations in pearl quality traits and both graft and pearl sac relative gene expressions are given in tables S1, S2, S3 and S4. Results are presented in three sections below corresponding to: 1) family and cohort effects on pearl quality and gene expression, 2) heritability of cultured pearls traits, and 3) heritability of gene expression levels.

4.1. FAMILY EFFECT ON PEARL QUALITY TRAITS AND GENE EXPRESSION

The average nacre thickness among the 2241 harvested pearls was 0.11 cm, with minimum and maximum values of 0.01 and 0.37 cm, respectively. The average nacre weight was 0.62 ± 0.29 g, with minimum and maximum values of 0.05 and 3.35 g, respectively. The nacre weight and thickness for each family and parent are given in table S1. Very highly significant “family effects” were demonstrated for the nacre thickness and weight ($p < 0.0001$) (Table 6.1). The cultured pearl quality traits are described in table S2. Overall, analyses comparing differences in pearl quality indicators among the nine families showed a significant “family effect” for all traits except lustre (Table 6.1).

Analyses comparing differences in relative gene expression of the graft from the nine families showed a highly significant “family effect”, with the main differences between the nine families (Table 6.1). This effect was the least significant for relative expression of the *Pif-177* gene ($p = 0.01$). Relative gene expressions in the graft among the eight genes are given in table S3. All differences between the nine families for each gene are shown in Fig S1.

The comparison of the relative expression of the eight genes in the pearl sac showed a significant “family effect” for four genes *Aspein* ($p = 0.01$), *MSI60* ($p = 0.01$), *Shematrin9* ($p = 0.01$) and *Nacrein* ($p = 0.04$) (Table 6.1). Pearl sac relative gene expressions are given in table S4.

Tableau 6.1 : Significance of the fixed family effect on pearl quality traits and gene expression in *P. margaritifera* progenies; 1918 harvested pearls were examined for nine pearl quality traits, and eight genes coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177*, *MSI60* and *Perline*), the prismatic layer (*Aspein*, *Shematin9*, *Prismalin* and *Shematin5*), and both the prismatic and the nacreous layers (*Nacrein*) were studied in 164 graft tissue pieces and 73 pearl sacs.

Pearl Traits	nacre weight	nacre thickness	circle	shape	surface defect(s)	lustre	grade	darkness	colour
(n = 1918)	***	***	**	*	***	ns	***	*	**

Graft	<i>Pif-177</i>	<i>MSI60</i>	<i>Perline</i>	<i>Nacrein</i>	<i>Aspein</i>	<i>Shematin9</i>	<i>Prismalin</i>	<i>Shematin5</i>
(n = 164)	**	***	***	***	***	***	***	***

Pearl sac	<i>Pif-177</i>	<i>MSI60</i>	<i>Perline</i>	<i>Nacrein</i>	<i>Aspein</i>	<i>Shematin9</i>	<i>Prismalin</i>	<i>Shematin5</i>
(n = 73)	ns	*	ns	*	*	*	ns	ns

p<0.0001 =***, p<0.01 = **, p<0.05 = *, ns = not significant

4.2. HERITABILITY OF CULTURED PEARL TRAITS

Heritability estimates for donor-derived pearl quality traits are given in Table 6.2. A moderate to low heritability was found for darkness ($h^2 = 0.37$; 95% CI [0.30, 0.44]), nacre weight ($h^2 = 0.34$; 95% CI [0.27, 0.41]), nacre thickness ($h^2 = 0.29$; 95% CI [0.22, 0.36]), surface defects ($h^2 = 0.21$; 95% CI [0.15, 0.28]), grade ($h^2 = 0.19$; 95% CI [0.11, 0.25]), colour ($h^2 = 0.16$; 95% CI [0.11, 0.23]) and lustre ($h^2 = 0.12$; 95% CI [0.06, 0.18]). For these heritable expression traits, the genetic variance component explained between 12 and 37% of the total variance. However pearl shape and presence / absence of circle(s) showed low heritability values ($h^2 = 0.02$; 95% CI [0.00, 0.06] and $h^2 = 0.05$; 95% CI [0.01, 0.10], respectively) attributable to the donor.

4.3. HERITABILITY OF RELATIVE GENE EXPRESSION LEVELS

Heritability estimates for donor-derived relative gene expression in the graft are given in Table 6.2. With the exception of *Pif-177* transcript levels, which showed only a low heritability, *MSI60* ($h^2 = 0.42$; 95% CI [0.24, 0.63]), *Perline* ($h^2 = 0.47$; 95% CI [0.22, 0.72]), *Nacrein* ($h^2 = 0.37$; 95% CI [0.22, 0.54]), *Aspein* ($h^2 = 0.37$; 95% CI [0.22, 0.54]), *Shematin9* ($h^2 = 0.37$; 95% CI [0.22, 0.54]), *Prismalin* ($h^2 = 0.37$; 95% CI [0.22, 0.54]), *Shematin5* ($h^2 = 0.37$; 95% CI [0.22, 0.54]), *Pif-177* ($h^2 = 0.02$; 95% CI [0.00, 0.06]) and *Aspein* ($h^2 = 0.05$; 95% CI [0.01, 0.10]) showed low heritability values attributable to the donor.

= 0.33; 95% CI [0.14, 0.51]), *Prismalin* ($h^2 = 0.44$; 95% CI [0.27, 0.6]), *Shematin5* ($h^2 = 0.35$; 95% CI [0.21, 0.52]) and *Shematin9* ($h^2 = 0.25$; 95% CI [0.11, 0.41]) showed moderate to high heritability.

Regarding relative gene expression in the pearl sac, heritabilities are given in Table 6.2. Except *Aspein*, which showed a high heritability ($h^2 = 0.41$; 95% CI [5E-5, 0.77]), and *Nacrein*, which showed a moderate heritability ($h^2 = 0.25$; 95% CI [5E-5, 0.67]), expression levels of all other genes had low heritabilities ($h^2 < 0.10$).

Tableau 6.2: Heritability (and CI 95%) for: a. pearl quality traits in *P. margaritifera* (N = 2241); b. mantle graft tissue relative gene expression levels (N = 176); and c. pearl sac tissue relative gene expression level (N = 80). Eight genes were studied coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177*, *MSI60* and *Perline*), prismatic layer (*Aspein*, *Shematin9*, *Prismalin* and *Shematin5*), or both (*Nacrein*).

a. Pearl quality traits		nacre weight	nacre thickness	circle	shape	surface defect(s)	lustre	grade	colour	darkness
h ²		0.34	0.29	0.05	0.06	0.21	0.12	0.19	0.16	0.37
CI 95%		(0.27, 0.41)	(0.22, 0.36)	(0.01, 0.10)	(1E-3, 0.06)	(0.15, 0.28)	(0.06, 0.18)	(0.11, 0.25)	(0.11, 0.23)	(0.30, 0.44)

b. Graft tissue relative gene expression		<i>Pif-177</i>	<i>MSI60</i>	<i>Perline</i>	<i>Nacrein</i>	<i>Aspein</i>	<i>Shematin9</i>	<i>Prismalin</i>	<i>Shematin5</i>
h ²		0.11	0.42	0.47	0.37	0.33	0.25	0.44	0.35
CI 95%		(4E-4, 0.29)	(0.24, 0.63)	(0.22, 0.72)	(0.22, 0.54)	(0.14, 0.51)	(0.11, 0.41)	(0.27, 0.60)	(0.21, 0.52)

c. Pearl sac relative gene expression		<i>Pif-177</i>	<i>MSI60</i>	<i>Perline</i>	<i>Nacrein</i>	<i>Aspein</i>	<i>Shematin9</i>	<i>Prismalin</i>	<i>Shematin5</i>
h ²		0.03	0.09	0.07	0.25	0.41	0.06	0.03	0.05
CI 95%		(2E-4, 0.11)	(2E-4, 0.27)	(3E-4, 0.23)	(5E-5, 0.67)	(5E-5, 0.77)	(4E-5, 0.21)	(4E-5, 0.15)	(4E-5, 0.23)

5. DISCUSSION

The present approach in a complex animal graft model evaluates the genetic parameters of relative gene expression of the graft tissue (at grafting time), the pearl sac tissue (at harvest), together with the pearl quality trait phenotypes (i.e. the product of the grafting procedure). We report for the first time in *P. margaritifera* the variation together in the phenotype and in the transcription response (i.e., gene expression). In this framework, discussion will focus on: 1) the heritability for gene expression of biomineralization candidates in the graft and the pearl sac, 2) the link between the pearl phenotype and gene expression, and 3) the final pearl phenotype and how it can be integrated into selection programs.

5.1. GENE EXPRESSION HERITABILITY

It is well known that traits under genetic control are likely to demonstrate higher heritability values than those whose variability is highly influenced by the environment (Fisher 1930; Falconer and Mackay 1996; Lynch and Walsh 1997). The data of the present study indicate that the parental effects on gene expression level are much stronger in the mantle graft, than in the pearl sac. From our study, the value of heritability was moderate to high ($h^2 > 0.20$) in graft relative gene expression for almost all genes (only *Pif-177* showed a low heritability; $h^2 = 0.11$), whereas heritability values were low for expression in the pearl sac ($h^2 < 0.10$) (except for *Nacrein* ($h^2 = 0.25$) and *Aspein* ($h^2 = 0.41$)). The pearl sac therefore seems to be more influenced by residual variance than the mantle graft provided by the donor oyster. The residual variance can mainly be explained by environmental effects that influence pearl sac gene expression and act as a recipient additive genetic component. Unfortunately, we were unable to estimate recipient oyster-derived genetic parameters for relative gene expression because recipient oysters were obtained from natural spat collection in which we could not control the natural variance. Previous studies observed a significant correlation between recipient shell size and harvested pearl size in *P. fucata martensii* (Wada and Komaru 1996), and in *P. margaritifera* (Ky *et al.* 2016; Blay *et al.* 2017). So pearl size is likely to have a recipient additive genetic component. The low heritability levels of gene expression

in pearl sac corroborate a recipient additive genetic component and suggest that the deposition of nacre on the pearl may be dependent on the capacity of the recipient oyster to gather energy and allocate it to cellular growth and nacre deposition processes (Wada and Jerry 2008; Le Pabic *et al.* 2016). Moreover, this low heritability could be induced by grafting, particularly by the recipient cellular environment. A recent histological examination and chronological description of pearl sac development in *P. margaritifera* (Kishore and Southgate 2016) showed that complete attachment of the mantle graft tissue to the host tissues had taken place by 14 days after grafting. The newly developed pearl sac could barely be distinguished as foreign tissue present in host oysters at this stage. In fact, the pearl sac had attained the visible characteristics of the host tissue and could not be differentiated as foreign tissue by 18 days after grafting. In the plant kingdom, grafting is characterized by a tight connection between cells, providing the possibility of interactions or cell communication between different cell lineages and resulting in a profound perturbation of the cellular environment (Cao *et al.* 2016). Grafting involves contact between heterologous cells at the stock and scion junction. It has been previously shown that that cells of the scion and stock individuals become linked to each other, and that genetic material (macromolecules including DNAs, RNAs and protein) can be transported between them (Jackson *et al.* 2001; Li *et al.* 2013; Wang *et al.* 2016; Cao *et al.* 2016). Recently, several studies have reported that endogenous small RNAs can be transmitted in chimeras during grafting and induce epigenetic modifications such as DNA methylation and RNA silencing, without changing the DNA sequence (Haque *et al.* 2007; Molnar *et al.* 2010; Wu *et al.* 2010; Li *et al.* 2013; Wang *et al.* 2016). Interactions between scions and rootstocks are complex but they mutually affect the graft zone and research has increasingly attempted to uncover the processes involved in these interactions (Wang *et al.* 2016). In the present animal model, detailed mechanisms enabling intercellular molecular transport need further research in order to confirm or refute their likeness with plant kingdom chimera and propose mechanisms that could help us to understand how this environment could moderate heritability in pearl sac gene expression.

Moreover the present heritability values based on pedigree assignment explained only a minority of the expected heritable fraction. Although the majority of transcripts appear to have genetic variation for expression in the graft, less than 50% of the total phenotypic variation is typically explained by additive effect. Other non-additive genetic effects contribute significantly to transcriptional variation. This variance is known as “missing heritability” and its underlying factors and mechanisms are not precisely established (Trerotola *et al.* 2015). Gene expression heterogeneity can be influenced by cell cycle position, stochastic expression or epigenetic effects (Parts *et al.* 2014). In recent decades, some studies clarify that non-genetic sources of heritable phenotypes play a role in phenotypic variations (Jablonka & Lamb 2008; Danchin *et al.* 2011; Laland *et al.* 2014). In particular, epigenetic modifications (defined as heritable changes in chromatin structure and DNA methylation) impact gene expression (Migicovsky & Kovalchuk 2011) without affecting the underlying genomic sequences (Gibney *et al.* 2010; Trerotola *et al.* 2015). Epistatic components need to be integrated by estimating the contribution of non-genetic factors (Koch 2014). In the present study, further work needs to be done on epistatic variance. Furthermore, genetic regulation does not only happen at the transcription level, further investigation on the expression of matrix protein in the pearl sac at the protein level should be made.

5.2. RELATIONSHIP BETWEEN PEARL PHENOTYPE AND GENE EXPRESSION

Gene expression levels constitute an intermediate step toward final phenotype expression (Hubner *et al.* 2005; Emilsson *et al.* 2008; Chakravarti *et al.* 2013; Parts *et al.* 2014). Some studies have combined genetic data and genome-wide gene expression analysis to try to understand the genetic basis of gene expression (Brem *et al.* 2002; Schadt *et al.* 2003; Cheung *et al.* 2003). In such studies, mRNA levels are the phenotypes of interest and are subject to experimental, environmental and genetic sources of variation that can be estimated. It was first demonstrated that, within populations, statistically significant estimates of heritability were found for gene expression in a much larger proportion of genes than would be expected by chance (Schadt *et al.* 2003; Cheung *et al.* 2003). Such evidence of heritability for gene expression is important because statistical power to detect gene variants that affect gene expression

depends on heritability (Visscher *et al.* 2008). In the present study, it was not possible to combine genetic data and genome-wide gene expression analysis, but it was possible to combine data on gene expression in the pearl sac with phenotype traits to show the relationship between the final pearl phenotype and gene expression level.

Nacrein and *Aspein* were the only transcripts in the pearl sac for which the heritability estimates are rather high or moderate. Shell matrix proteins play a key role in the shell bio-mineralization process. Some genes encoding the proteins of the calcified matrix have been identified and are known to be specifically involved in the formation of the nacreous layer and/or prismatic layer, playing a role in crystal nucleation, orientation, polymorph and morphology during deposition of the two shell layers (Joubert *et al.* 2010; Montagnani *et al.* 2011; Marie *et al.* 2012). *Aspein* is thought to play a key role in calcite precipitation (Takeuchi *et al.* 2008; Isowa *et al.* 2012). In contrast, *Nacrein*, which is found in both the nacreous and prismatic layers of the shell, exhibits carbonic anhydrase activity and probably functions as a supplier of bicarbonate ions and a regulator of calcium carbonate crystal formation (Miyamoto *et al.* 2013; Liu *et al.* 2012; Suzuki & Nagasawa 2013). In previous studies, we found a significant correlation between relative gene expression of *Aspein* in the pearl sac with the component of quality traits (surface defects, lustre and grade), and with colour. Furthermore a significant correlation was found between *Nacrein* relative gene expression with colour of the pearl (and no significant correlation in graft tissue) (Blay *et al.* 2017). These two candidates seem to be involved in pearl quality and colour phenotypes. However, further work is still required to refine our understanding of the exact role of *Aspein* and *Nacrein* in the pearl phenotype, because our studies revealed high levels of additive expression in pearl sac, thus providing evidence for a genetic basis for this variation, which could be used in breeding programs.

5.3. FINAL PHENOTYPE

Heritability allows a comparison of the relative importance of genetics and environment in the variation of traits within and across populations, and is a proxy parameter for predicting the response to selection (Visscher *et al.* 2008). Whatever the mechanism implied in pearl formation, the most important is the final pearl phenotype and its heritability. Our results clearly demonstrate heritability for nacre weight and thickness, darkness and colour, surface defects and grade, signifying an important donor oyster effects with a genetic basis, while shape and presence / absence of circle(s) with low heritability were not strongly heritable or attributable to the donor. In fact, pearl shape is known to be mostly influenced by environmental factors (Ky *et al.* 2015). This study confirms the significant genetic role that the implanted mantle graft plays in the biomineralization process of cultured pearls (Arnaud-Haond 2007; McGinty *et al.* 2010; Blay *et al.* 2017). When heritability is high, the corresponding trait could be improved by selecting donor oysters with high genetic merit. Sufficient additive genetic variance in a selected trait is a prerequisite for selective breeding and good breeding efficiency is possible when levels are high (Gjedrem and Baranski 2010). From an applied point of view, this has major implications for any genetic selection strategies and for the black pearl industry in French Polynesia. Variation in additive and non additive genetic factors and environmental variance are population specific, meaning that heritability depends on the population. Therefore, the heritability in one population cannot be used to predict the heritability in another population for the same trait, although in practice heritabilities of similar traits are often similar across populations in the same or different species (Visscher *et al.* 2008). Therefore, selection programs aimed at improving traits such as pearl size, colour, darkness, surface defects and grade should be achievable through targeted donor oyster selection, whilst further work is required to understand the role of the recipient oysters on pearl phenotypic variance and gene expression variance.

6. CONCLUSIONS

The current study showed, for the first time, an additive genetic component attributable to donor oysters for gene expression in graft tissue and, to a lesser extent, in the pearl sac and for harvested pearl phenotype (excluding pearl shape and circle). The interaction between donor and recipient are very complex and research has increasingly attempted to uncover the processes involved in these interactions and the resulting graft-induced phenotypic changes, for example by studying molecular mechanisms and endogenous factors. Moreover, establishing a direct link between pearl phenotype and candidate gene expression remains an important next step if we are to understand its role in *P. margaritifera* selection potential in a breeding program. This study provided a good understanding of heritability estimates for pearl phenotypes and gene expression in this chimera model.

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AUTHOR CONTRIBUTIONS

CB followed the graft experiments at the pearl farm and carried out the molecular analysis in the laboratory. CB analysed the data and drafted the manuscript. CLK designed the experiments from the breeding to the grafting in the "parent-progenies" procedure. CLK was the supervisor of CB's PhD,

together with SP, and both SP and CLK helped to revise and enrich the manuscript. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

7. SUPPLEMENTARY INFORMATION

Table S1: Nacre weight (in g) and nacre thickness (in cm) of cultured pearls harvested following grafting with *P. margaritifera* donors from 12 parents and the nine progenies. Female and male parents were named by letters and numbers, respectively. *n* corresponds to the number of pearls harvested per parent or progeny.

	Parents in breeding #1							Parents in breeding #2					Progenies of breeding #1				Progenies of breeding #2				
	1	2	5	A	B	D	F	6	7	G	H	I	A1	B2	D2	F5	G6	H6	H7	I6	I7
<i>n</i>	17	25	32	22	28	27	22	33	15	35	32	35	171	129	178	188	247	252	250	255	248
Nacre weight (g)																					
Means	0.49	0.42	0.38	0.29	0.48	0.32	0.51	0.50	0.54	0.41	0.40	0.67	0.52	0.71	0.59	0.58	0.65	0.55	0.59	0.83	0.79
SD	0.25	0.20	0.16	0.22	0.19	0.13	0.17	0.21	0.19	0.15	0.11	0.22	0.23	0.30	0.29	0.23	0.29	0.21	0.25	0.35	0.30
Min	0.18	0.18	0.13	0.05	0.16	0.11	0.26	0.23	0.35	0.15	0.23	0.28	0.16	0.25	0.15	0.16	0.27	0.14	0.20	0.23	0.24
Max	1.02	1.16	0.84	1.13	0.86	0.65	0.88	1.23	0.89	0.73	0.63	1.34	1.54	2.30	1.91	1.26	2.13	1.54	1.65	3.35	1.99
Nacre thickness (cm)																					
Means	0.11	0.09	0.08	0.06	0.10	0.08	0.11	0.10	0.11	0.08	0.09	0.13	0.05	0.08	0.07	0.07	0.12	0.11	0.12	0.15	0.14
SD	0.05	0.03	0.03	0.04	0.03	0.03	0.02	0.03	0.03	0.03	0.02	0.03	0.04	0.04	0.05	0.04	0.04	0.03	0.04	0.04	0.04
Min	0.03	0.04	0.02	0.01	0.04	0.02	0.06	0.04	0.07	0.03	0.04	0.07	0.02	0.01	0.02	0.03	0.06	0.03	0.05	0.05	0.06
Max	0.21	0.17	0.16	0.19	0.16	0.14	0.15	0.18	0.19	0.15	0.13	0.22	0.21	0.26	0.24	0.18	0.27	0.23	0.23	0.37	0.26

Table S2: Cultured pearl quality traits from the *P. margaritifera* experimental graft. Number of cultured pearls (and percentage in brackets) for group of parent breeding and each progenies: 1) shape categories (“**b**” for baroque and semi baroque, “**o**” for oval and drop, “**r**” for round and semi-round pearls); 2) pearl circles (“**0**”= absence and “**1**”= presence); 3) surface defect classes (“**0**” = 0 defects, “**1**” = 1–5 defects, “**2**” = 6–10 and “**3**” = >10 defects); 4) lustre levels (“**0**” = absence of lustre, “**1**” = moderate lustre and “**2**” = high lustre); 5) classification grade (“**A**” to “**D**” and **Rejects**); 6) darkness level (**low**, **moderate** and **high** darkness) and visual color categories (“peacock”, “green”, “grey” and “other”: corresponding to white, aubergine and yellow pearls).

	Shape			Circle		Surface defect				Lustre			Grade					Darkness			Colour			
	b	o	r	0	1	0	1	2	3	0	1	2	A	B	C	D	R	low	moderate	high	peacock	green	grey	other
Parents breeding #1 (n = 173)	57 (32.9)	47 (27.2)	69 (39.9)	144 (83.2)	29 (16.8)	24 (13.9)	83 (48.0)	42 (24.2)	24 (13.9)	17 (9.8)	110 (63.6)	46 (26.6)	27 (15.6)	58 (33.5)	43 (24.8)	23 (13.3)	22 (12.7)	17 (9.8)	151 (87.3)	5 (2.9)	27 (15.6)	86 (49.7)	41 (23.7)	19 (11.0)
Parents breeding #2 (n = 150)	58 (38.7)	60 (40.0)	32 (21.3)	90 (60.0)	60 (40.0)	2 (1.3)	44 (29.3)	30 (20.0)	74 (49.4)	5 (3.3)	124 (82.7)	21 (14.0)	6 (4.0)	53 (35.3)	44 (29.3)	44 (29.4)	3 (2.0)	20 (13.3)	106 (70.7)	24 (16.0)	49 (32.7)	27 (18.0)	61 (40.7)	13 (8.6)
A1 (n = 171)	58 (33.9)	46 (26.9)	67 (39.2)	137 (80.1)	34 (19.9)	15 (8.8)	95 (55.6)	38 (22.2)	23 (13.4)	10 (5.8)	116 (67.8)	45 (26.3)	43 (25.1)	53 (31)	44 (25.7)	23 (13.5)	8 (4.7)	35 (20.5)	110 (64.3)	26 (15.2)	31 (18.1)	48 (28.1)	76 (44.4)	16 (9.4)
B2 (n = 129)	50 (38.8)	39 (30.2)	40 (31.0)	93 (72.1)	36 (27.9)	7 (5.4)	44 (34.1)	42 (32.6)	36 (27.9)	10 (7.7)	97 (75.2)	22 (17.1)	14 (10.8)	32 (24.8)	42 (32.6)	32 (24.8)	9 (7)	16 (12.4)	78 (60.5)	35 (27.1)	9 (7.0)	40 (31.0)	77 (59.7)	3 (2.3)
D2 (n = 178)	77 (43.3)	45 (25.3)	56 (31.4)	142 (79.8)	36 (20.2)	8 (4.5)	71 (39.9)	39 (21.9)	60 (33.7)	21 (11.8)	127 (71.3)	30 (16.9)	27 (15.2)	50 (28.1)	44 (24.7)	33 (18.5)	24 (13.5)	44 (24.7)	99 (55.6)	35 (19.7)	19 (10.7)	50 (28.1)	104 (58.4)	5 (2.8)
F5 (n = 188)	73 (38.8)	56 (29.8)	59 (31.4)	143 (76.1)	45 (23.9)	7 (3.7)	74 (39.4)	70 (37.2)	37 (19.7)	8 (4.3)	139 (73.9)	41 (21.8)	37 (19.7)	59 (31.4)	57 (30.3)	31 (16.5)	4 (2.1)	12 (6.4)	106 (56.4)	70 (37.2)	57 (30.3)	73 (38.8)	55 (29.3)	3 (1.6)
G6 (n = 247)	134 (54.2)	48 (19.4)	65 (26.3)	161 (65.2)	86 (34.8)	4 (1.6)	56 (22.7)	94 (38.1)	93 (37.6)	18 (7.3)	178 (72.1)	51 (20.6)	13 (5.3)	37 (15.0)	89 (36.0)	98 (39.7)	10 (4.0)	48 (19.4)	148 (59.9)	51 (20.7)	35 (14.2)	84 (34.0)	104 (42.1)	24 (9.7)
H6 (n = 252)	111 (44.0)	59 (23.4)	82 (32.6)	177 (70.2)	75 (29.8)	1 (0.4)	98 (38.9)	85 (33.7)	68 (27.0)	22 (8.7)	174 (69.1)	56 (22.2)	22 (8.7)	71 (28.2)	73 (29.0)	82 (32.5)	4 (1.6)	47 (18.7)	153 (60.7)	52 (20.6)	70 (27.8)	100 (39.7)	71 (28.2)	11 (4.4)
H7 (n = 250)	99 (39.6)	54 (21.6)	97 (38.8)	193 (77.2)	57 (22.8)	3 (1.2)	143 (57.2)	65 (26.0)	39 (15.6)	28 (11.2)	166 (66.4)	56 (22.4)	16 (6.4)	104 (41.6)	73 (29.2)	47 (18.2)	10 (4.0)	53 (21.2)	157 (62.8)	40 (16.0)	37 (14.8)	91 (36.4)	103 (41.2)	19 (7.6)
I6 (n = 255)	116 (45.5)	70 (27.5)	69 (27.0)	207 (81.2)	48 (18.8)	5 (1.9)	118 (46.3)	66 (25.9)	66 (25.9)	26 (10.2)	154 (60.4)	75 (29.4)	29 (11.4)	76 (29.8)	69 (27.0)	65 (25.5)	16 (6.3)	43 (16.9)	154 (22.7)	58 (21.1)	38 (14.9)	107 (42.0)	99 (38.8)	11 (4.3)
I7 (n = 248)	100 (40.3)	61 (24.6)	87 (35.1)	191 (77.0)	57 (23.0)	2 (0.8)	108 (43.6)	72 (29.0)	66 (26.4)	16 (6.5)	162 (65.3)	70 (28.2)	28 (11.3)	92 (37.1)	55 (22.2)	57 (23.0)	16 (6.4)	48 (19.3)	147 (59.3)	53 (21.4)	23 (9.3)	93 (37.5)	102 (41.1)	30 (12.1)

Table S3. Relative gene expression in graft tissue of genes coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177*, *MSI60*, *Perline*), the prismatic layer (*Aspein*, *Shematrín9*, *Prismalin* and *Shematrín5*), and both prismatic and nacreous layers (*Nacrein*) for parents and progenies of breedings #1 and #2.

		<i>Pif-177</i>	<i>MSI60</i>	<i>Perline</i>	<i>Nacrein</i>	<i>Aspein</i>	<i>Shematrín9</i>	<i>Prismalin</i>	<i>Sheamtrín5</i>
Parents in breeding #1 (n = 7)	A	0.56	0.33	0.36	0.37	0.20	0.13	1.14	0.62
	B	0.64	0.59	0.77	1.69	0.76	1.93	4.97	9.60
	D	1.55	0.87	1.28	3.57	0.95	1.49	4.92	9.31
	F	2.81	2.33	2.00	3.52	1.40	1.94	2.32	4.36
	1	0.68	0.84	0.89	0.99	0.76	0.13	0.42	3.47
	2	0.75	0.32	0.54	3.53	1.12	1.57	2.88	9.87
	5	2.65	1.59	1.61	1.78	1.36	3.78	3.33	0.08
Parents in breeding #2 (n = 5)	G	1.38	0.68	1.09	0.30	1.09	1.86	1.49	1.49
	H	0.82	0.39	0.65	2.85	0.85	1.76	2.75	0.56
	I	1.00	0.69	1.18	6.88	1.29	1.75	2.66	0.89
	6	4.35	1.81	1.75	7.34	1.59	3.59	2.50	1.00
	7	2.13	0.67	1.50	1.13	0.57	0.79	2.47	0.31
Progenies of breeding #1 (n = 70)	A1	0.67	0.51	0.50	0.38	1.67	1.56	2.89	0.29
	B2	1.32	0.85	1.03	0.93	1.44	1.85	2.39	0.16
	D2	1.21	0.53	0.82	0.82	1.54	1.80	2.49	0.41
	F5	0.79	0.54	0.67	0.68	1.22	1.40	1.89	1.24
Progenies of breeding #2 (n = 94)	G6	1.02	1.32	1.36	3.40	0.97	0.75	0.32	2.56
	H7	0.89	1.34	1.22	1.72	0.71	0.69	0.63	4.85
	I7	1.04	1.58	1.20	0.26	0.65	0.50	0.27	1.04
	H6	1.03	1.57	1.18	2.13	0.79	0.75	0.62	2.48
	I6	1.11	1.84	1.52	1.04	0.86	0.74	0.61	0.80

Table S4. Relative gene expression in pearl sac of genes coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177*, *MSI60*, *Perline*), the prismatic layer (*Aspein*, *Shematin9*, *Prismalin* and *Shematin5*), and both prismatic and nacreous layers (*Nacrein*) for progenies and parents of breeding #1.

		<i>Pif-177</i>	<i>MSI60</i>	<i>Perline</i>	<i>Nacrein</i>	<i>Aspein</i>	<i>Shematin9</i>	<i>Prismalin</i>	<i>Shematin5</i>
Parents in breeding #1 (<i>n</i> = 7)	A	0.18	0.30	0.38	0.26	5.78	0.08	2.16	0.20
	B	0.48	0.61	0.69	2.00	7.64	2.70	16.96	0.65
	D	0.38	0.37	0.48	1.03	0.30	0.39	1.81	1.09
	F	0.64	0.66	0.53	0.50	0.33	0.04	0.83	0.07
	1	0.56	0.56	0.61	0.68	0.34	0.13	1.86	0.27
	2	0.76	0.49	0.74	2.72	2.37	0.36	1.83	0.69
	5	0.88	0.81	1.29	2.30	0.41	0.11	3.24	0.12
Progenies of breeding #1 (<i>n</i> = 73)	A1	1.09	0.90	0.95	0.67	0.76	0.85	0.59	0.68
	B2	1.25	1.11	1.34	1.92	3.40	1.50	0.67	1.16
	D2	1.15	1.09	1.29	0.91	0.63	1.96	1.20	1.52
	F5	1.17	1.59	0.94	0.90	0.42	1.10	0.90	1.22

Fig S1. Relative expression profiles of eight biomineralization gene markers in mantle grafts of *P. margaritifera* assessed by real-time PCR for the progenies: *Pif-177* (A), *MSI60* (B), *Perline* (C), *Nacrein* (D), *Aspein* (E), *Shematrix9* (F), *Prismalin* (G), and *Shematrix5* (H). Different letters indicate statistically significant differences determined by ANOVA.

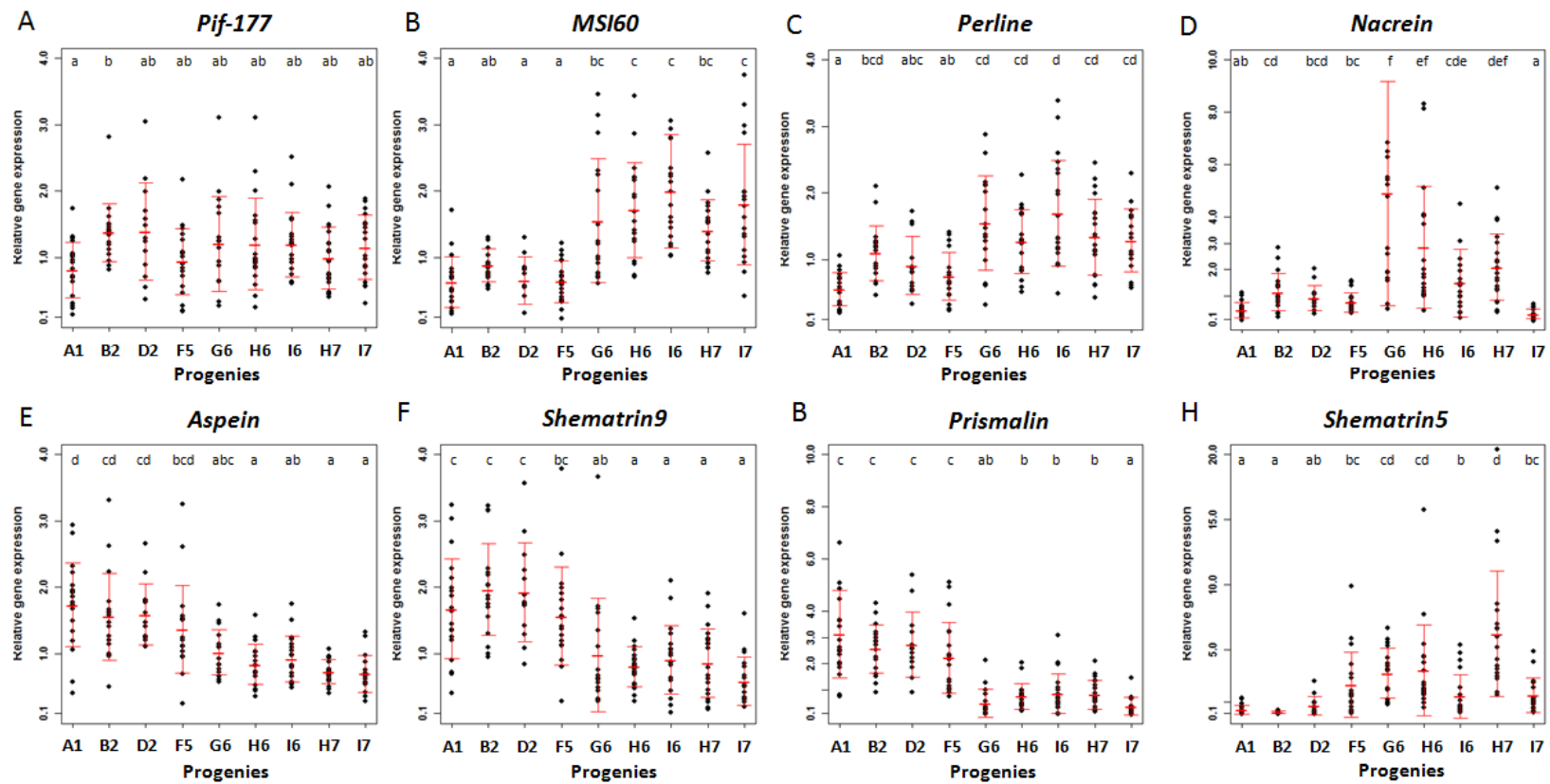
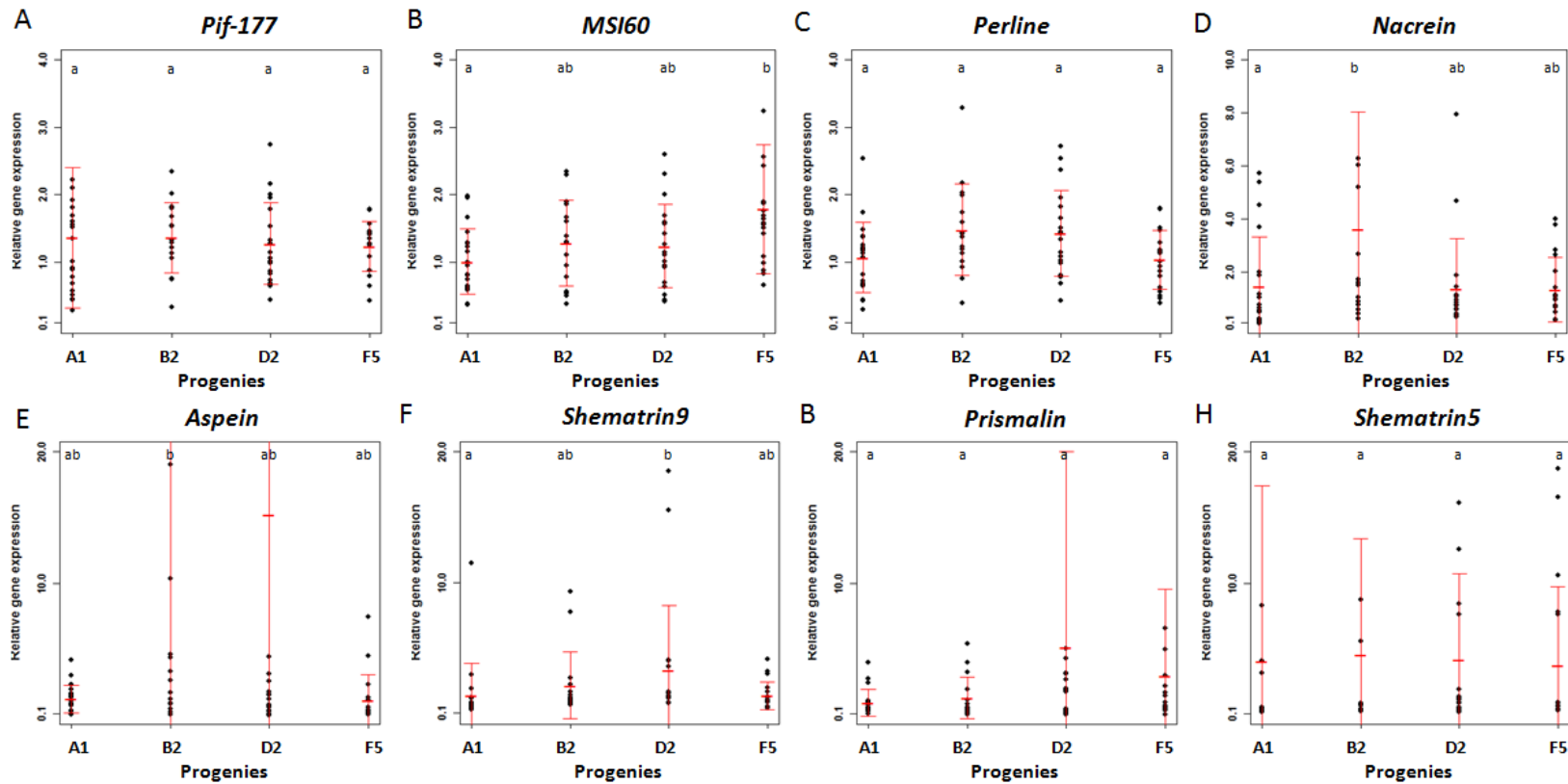


Fig S2. Pearl sac relative expression of the eight selected marker genes of the biomineralization of *P. margaritifera*. Relative expression profiles (real-time PCR) of *Pif-177* (A), *MSI60* (B), *Perline* (C), *Nacrein* (D), *Aspein* (E), *ShematrIn9* (F), *Prismalin* (G), *ShematrIn5* (H) in progenies. Different letters indicate statistically significant differences determined by ANOVA.



Chapitre 7

Discussion générale et perspectives

Cette thèse avait pour principal objectif d'évaluer le déterminisme génétique des caractères d'intérêt perlicole chez *P. margaritifera*. L'objectif est donc de définir les rôles respectifs de ces deux huîtres dans un contexte d'allogreffe, en examinant à la fois 1) leurs contributions phénotypiques sur les traits de qualité de la perle et 2) leurs contributions moléculaires sur l'expression des gènes de biominéralisation de la perle. Pour répondre aux différents objectifs, des familles d'huîtres ont été produites en éclosion, puis élevées et enfin étudiées via des greffes expérimentales dans les conditions de production. Des approches transcriptomiques, via l'analyse de l'expression des gènes, ont été mises en œuvre et ont permis d'approfondir nos connaissances sur d'éventuels marqueurs de qualité de la biominéralisation. Les huit marqueurs utilisés sont issus des travaux de thèse de Joubert (2011) et Marie *et al.* (2012).

1. SYNTHÈSES DES RESULTATS ET DISCUSSION GÉNÉRALE

1.1. CONTRIBUTION DE L'HUITRE DONNEUSE ET RECEVEUSE AUX PHÉNOTYPES DE LA PERLE

Les **chapitres 2, 4, 5 et 6** de cette thèse ont permis d'approfondir nos connaissances sur la contribution des deux huîtres impliquées dans le processus biologique de la greffe. En effet, afin de pouvoir améliorer la qualité de la perle, il est important de comprendre la part de la contribution des facteurs génétiques intervenant sur sa réalisation (Wada and Jerry 2008).

Les résultats du **Chapitre 2**, visant à estimer les variations phénotypiques dans le cadre d'un dispositif cultural bi-localisé (Rangiroa *vs.* Tahaa), ont révélé que le poids et l'épaisseur du dépôt perlier (la taille de la perle) ne sont pas affectés par l'environnement (*i.e.* site de culture) (Figure 7.1). Cependant une autre étude sur 5 sites a démontré un effet du site de culture sur la taille de la perle, même si l'atoll de Rangiroa et l'île de Tahaa donnent les mêmes résultats pour ce critère phénotypique de part leur position intermédiaire dans l'aire géographique de la Polynésie française (Le Pabic *et al.* 2016). Notre étude porte sur des donneuses issues de familles d'éclosion étudiées en parallèle sur les deux sites alors que la

seconde porte sur des donneuses issues de collectage sans répliqua sur les différents sites. Nos résultats montrent clairement un effet famille et donneuse sur la taille de la perle. Il sera donc intéressant d'étudier des familles issues d'écloserie en répliqua sur un nombre plus important de site de culture, afin de pouvoir conclure quand à l'effet site de culture à l'échelle de la Polynésie française.

D'après de précédentes études, la taille de la perle semble donc être dépendante de l'huître donneuse de greffon (Tayale *et al.* 2012 ; Ky *et al.* 2013 ; Blay *et al.* 2016 **chapitre 2** de cette thèse). Afin d'affiner ces connaissances, nous avons exploré l'impact de la taille de l'huître receveuse et de l'huître donneuse de greffon à âge fixe sur la taille de perle (**Chapitre 4**). Nos résultats ont montré une corrélation positive entre la taille de l'huître receveuse et celle de la perle. Mais étonnamment aucun effet de la taille de l'huître donneuse de greffon sur celle de la perle n'a été observé. On a également confirmé un effet huître donneuse de greffon sur certains paramètres de qualité de la perle (excepté la forme et le cerclage) (Figure 7.1). Chez *P. maxima*, McGinty *et ses collaborateurs* (2010, 2011) se sont intéressés à définir le rôle à la fois de l'huître donneuse et receveuse sur le phénotype de la perle et particulièrement sur la taille et la couleur. Ces études ont établi que l'amélioration de la valeur marchande des perles dépend de la sélection génétique des huîtres donneuses de greffon. Au travers de xénogreffes entre *P. maxima* et *P. margaritifera* il a été montré: 1) l'expression de gènes impliqués dans le processus de biominéralisation de l'huître donneuse dans l'huître receveuse (McGinty *et al.* 2010) et 2) l'influence espèce-spécifique de l'huître donneuse sur les caractères d'intérêts perlicoles (McGinty *et al.* 2011). L'importance de l'huître donneuse chez *P. margaritifera*, vis-à-vis de la couleur de la perle produite, est aussi illustrée par la relation directe avec la coloration de la nacre, présente au niveau de la face interne de la coquille de l'huître donneuse. Les premiers résultats sur la sélection génétique familiale pour ce caractère montrent l'existence d'une propension marquée au sein des familles à donner à l'issu de la greffe une tendance de couleur plutôt qu'une autre au niveau des perles produites (Tayale *et al.* 2012; Ky *et al.* 2013, 2016, 2017). Ce chapitre 4 met donc en évidence le rôle de l'huître receveuse sur le phénotype taille de la perle. La physiologie de la receveuse au cours de la culture est à considérer. Quelques travaux ont été réalisés notamment sur

l'influence du mode de culture sur la production de perle ou de mabé. D'après Kishore and Southgate (2016), le mode de culture (chapelet ou panier) n'influence pas la taille mais la forme de la perle chez *P. margaritifera*. La seconde étude concernant la production de mabé chez *Pteria sterna* ne montre pas d'influence de la technique de culture ni sur la croissance des huîtres receveuses ni sur la taille de la perle (Ruiz-Rubio *et al.* 2006).

L'huître donneuse de greffon a donc un rôle sur le phénotype couleur mais également sur le phénotype taille. Cependant d'après les résultats de notre chapitre 4, la taille de la donneuse n'impacterait pas celle de la perle, mais un effet donneuse et un effet famille existent sur ce phénotype (Tayale *et al.* 2012 ; Ky *et al.* 2013, Blay *et al.* 2017). Nous avons donc étudié le phénotype taille sous un autre angle : l'âge (**chapitre 5**). En effet, le vieillissement des cellules affecte les organismes vivants que ce soit un simple individu unicellulaire ou multicellulaires. Le vieillissement est défini comme une perte progressive des fonctions tissulaires au cours du temps (Medwar 1955 ; Flatt 2012). Dans les systèmes de greffe, de nombreuses études mettent en avant un effet de l'âge du greffon sur les résultats de la greffe notamment chez l'humain. Une étude, par exemple, se portant sur la greffe du foie, a montré que la jeunesse du donneur impacterait significativement la survie du receveur (Kubota *et al.* 2017). Au cours de cette thèse, nous avons démontré pour la toute première fois chez les huîtres perlières, un effet de l'âge de l'huître donneuse de greffon sur les phénotypes de la perle (chapitre 5). Dans ce chapitre, l'effet de l'âge de la donneuse de greffon a été démontré chez des individus de 7 à 30 mois sur les phénotypes « taille » et « qualité » de la perle (grade et défauts). En effet, les donneuses de moins de 24 mois donnent des perles plus lourdes et plus épaisses que celles issues des donneuses de plus de 24 mois, et ce, sur les deux familles dans les deux environnements de culture différents (*i.e.* Rangiroa et Mangareva). De plus, notre étude démontre une nouvelle fois que la qualité des perles est diminuée chez les donneuses de plus de 24 mois, mais également pour les perles issues des jeunes donneuses (7 mois). De précédents travaux avaient étudié l'effet de l'âge de la donneuse et de la receveuse, mais seulement sur les phénotypes couleur et qualité de la perle (grade, défauts et lustre) montrant ainsi un impact positif de la jeunesse des donneuses

sur la qualité des perles (Ky *et al.* 2016). Nos résultats prouvent qu'il existe bel et bien un âge optimal jouant sur la taille et la qualité, situé entre 12 et 18 mois (Figure 7.1).

Les approches de génétiques quantitatives utilisées dans les travaux de cette thèse (**chapitre 6**) ont permis d'identifier les paramètres génétiques des populations de donneuses de greffon issues d'écloserie (parent/descendant). Cette étude nous a permis de dissocier la part de variance de génétique additive (attribuée à la donneuse) de la variance environnementale (comprenant la receveuse) sur l'expression de gènes de biominéralisation et des caractères d'intérêt perlicole. Ces analyses révèlent une héritabilité de l'huître donneuse de greffon sur les paramètres de qualité de la perle, avec un maximum pour le caractère foncitude ($h^2 = 0.37$) et un minimum pour les caractères forme ($h^2 = 0.06$) et cerclage ($h^2 = 0.05$) de la perle (**chapitre 6**) (Figure 7.1). Les connaissances actuelles sur les bases génétiques chez l'huître perlière sont réduites le plus souvent aux estimations d'héritabilité au niveau des traits de croissance de la coquille et de leurs corrélations phénotypiques avec la taille de la perle (Velayudhan *et al.* 1996 ; Wada 1984, 1986). Seuls Jerry et ses collaborateurs (2012) ont étudié les paramètres génétiques des traits phénotypiques de la perle chez *P. maxima*. Ces auteurs ont obtenu des valeurs d'héritabilité de la donneuse proches de nos estimations avec une héritabilité également minimale pour la forme ($h^2 = 0.06$).

1.2. CONTRIBUTION MOLECULAIRE DE LA DONNEUSE ET RECEVEUSE A LA FORMATION DE LA PERLE

Après avoir analysé la contribution des huîtres donneuses et receveuses sur les traits phénotypiques de la perle, il est également important de comprendre la formation de la perle d'un point de vue moléculaire au travers de l'expression de gènes de biominéralisation issus des travaux de thèse de Joubert C. (2010) et Marie *et al.* (2012). Chez les mollusques, l'épithélium du manteau secrète une matrice organique composée de polymorphes de carbonate de calcium inorganique et de polymères organiques (structure protéique) qui régule la calcification de la coquille (Fougrouse *et al.* 2008). Plusieurs études ont suggéré que les gènes du manteau responsables de la formation de la coquille sont aussi responsables de la

formation de la perle. Bien que de nombreuses protéines aient été isolées grâce à des approches biochimiques et moléculaires afin de comprendre la formation de la coquille, la fonction de ces protéines demeure ambiguë (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 ; Joubert *et al.* 2010 ; Montagnani *et al.* 2011 ; Marie *et al.* 2012 ; Li *et al.* 2017). Depuis quelques années l'expression de la matrice protéique a été examinée dans le sac perlier (Inoue *et al.* 2010, 2011 ; Wang *et al.* 2009 ; McGinty *et al.* 2012 ; Zhao *et al.* 2012 ; Blay *et al.* 2016). Afin de mieux comprendre le rôle de chacune des deux huîtres et de comprendre la formation de la perle d'un point de vue moléculaire, nous avons étudié l'expression de gènes de la matrice protéique de biominéralisation aussi bien au niveau du greffon (au moment de la greffe) que du sac perlier (au moment de la récolte), et ce dans différents contextes. Les influences de l'environnement ont été évaluées via l'étude du site de culture ou de la cinétique de la formation de la perle. Les influences de la génétique ont été analysées via les études sur la taille de la donneuse à âge fixe et variable et les estimations d'héritabilité (**Chapitre 2 à 6**).

Dans cette thèse, nous avons étudié le niveau d'expression de PIF-177 au sein du sac perlier sur 5 familles dans le cadre d'une greffe bi-localisée (Rangiroa vs. Tahaa) (**chapitre 2**). Aucun impact du site de culture n'a été observé sur le niveau d'expression de PIF-177 au sein du sac perlier, alors que son niveau d'expression est corrélé au poids/épaisseur de la perle. Ces résultats sont confirmés par les travaux réalisés sur les 5 sites où l'environnement n'influence pas l'expression des gènes de biominéralisation étudiés (Le Pabic *et al.* 2016). Au sein du **chapitre 3**, l'expression d'un panel de 8 gènes codant des protéines de biominéralisation au cours du temps de culture de la perle (durant les 9 premiers mois) a été mesurée. Ces résultats ont permis de démontrer l'influence de l'expression de certains gènes sur la qualité de la perle. En effet, un fort taux d'expression des gènes Prismalin 14, Aspein ou encore Shematrin9 engendre des perles avec plus de défauts de surface, un grade inférieur ou encore une présence importante de dépôt de surface (Figure 7.1). Ceci est également confirmé par une autre étude de cette thèse (**chapitre 4**), où l'on

observe une corrélation entre le niveau d'expression du panel de gènes dans le sac perlier et les traits de qualité de la perle, mais également sur les paramètres biométriques de la coquille de l'huître receveuse. Ceci suggère que l'huître receveuse peut jouer un rôle déterminant dans la régulation de l'expression des gènes de biominéralisation au sein du sac perlier. Ces résultats sont en adéquation avec les paramètres génétiques évalués dans cette thèse (**chapitre 6**), où l'on montre des valeurs d'héritabilité plutôt élevées pour les greffons alors qu'elles sont très faibles au niveau du sac perlier. Ce résultat démontre une part de variance environnementale non négligeable et pouvant être assimilée par l'huître receveuse. De précédentes études ont également observé une corrélation significative entre la taille de l'huître receveuse et la taille de la perle chez *P. Fucata martensii* (Wada and Komaru 1996) et chez *P. margaritifera* (Ky et al. 2016 ; Blay et al. 2017) corroborant qu'une part de la variance environnementale peut être attribuable à la receveuse. Dans le **chapitre 5**, l'expression des gènes de biominéralisation a été étudiée pour la toute première fois en fonction de l'âge de l'huître donneuse. Le niveau d'expression est influencé par l'âge au niveau du greffon pour les gènes codant des protéines intervenant dans la formation de l'aragonite (PIF 177, MSI60, Pearlin) et dans les deux couches (Nacrein). Toutefois, il n'y a pas de différence entre les différentes catégories d'âge pour ces mêmes gènes au niveau du sac perlier. Les donneuses les plus jeunes ont un potentiel de biominéralisation plus élevé en ce qui concerne les gènes aragonitiques. Les rares études reliant l'expression de gène à l'âge chez des huîtres perlières se concentrent sur la formation de la coquille durant l'ontogénèse comme chez *P. fucata* (Miyazaki et al. 2010 ; Fang et al. 2011).

1.3. BILAN

Les différents résultats de ces chapitres améliorent considérablement nos connaissances sur la réalisation des traits phénotypiques et moléculaires de la perle en définissant la contribution de l'huître donneuse et receveuse (Figure 7.1).

D'un point de vue phénotypique, cette thèse a mis en évidence un rôle non négligeable de l'huître receveuse sur le phénotype taille de la perle. La physiologie de la receveuse au cours de la culture est donc

à considérer. D'autres études sont à envisager comme par exemple l'influence de l'âge de l'huître receveuse sur la taille de la perle, même si des premières réponses ont été fournies par l'étude de Ky et ses collaborateurs (2016). L'utilisation de jeune receveuse pourrait être une piste. Bien que son rôle soit déterminant quant au phénotype taille de la perle, l'huître receveuse a longtemps été ignorée. Nos résultats ont également démontré la complexité du phénotype taille de la perle. En effet plusieurs facteurs interagissent sur ce critère phénotypique, soit l'huître receveuse, soit l'huître donneuse au travers des effets donneuses, de l'âge et des estimations d'héritabilité (Figure 7.1). Il existe une part de variance génétique entre l'huître donneuse et les phénotypes qualité de la perle (couleur et foncitude, qualité (grade, lustre et défauts de surface)), alors que cet effet est moindre pour la forme et le cerclage. (Figure 7.1). Ces travaux de thèse ont montré une analogie au niveau du processus de greffe entre le monde végétal et animal. En effet, le porte greffe (receveur) chez les végétaux est souvent choisi par rapport à sa compatibilité, son adaptation au sol, sa vigueur, sa forme, sa résistance aux maladies, mais aussi pour sa faculté à diriger la croissance végétative. Le greffon (donneur) quant à lui est choisi sur une base qualitative comme par exemple pour le fruit ou la fleur au niveau de sa couleur ou encore de son goût. Actuellement, les études au niveau du monde végétal se penchent essentiellement sur les interactions greffon/ porte greffe et les différents échanges entre les deux individus. Il a été montré une communication entre les cellules mais également des échanges incluant du matériel génétique entre le greffon et le porte greffe comme par exemple entre *Brassica Juncea* et *B. oleracea* (Jackson 2001 ; Li *et al.* 2013 ; Wang *et al.* 2016 ; Cao *et al.* 2016). Dans notre modèle, des recherches sur l'existence et les mécanismes de transport moléculaire intercellulaire entre donneur et receveur seraient très intéressantes et permettraient d'approfondir nos connaissances sur le rôle de chacune des deux huîtres et leurs interactions.

Au niveau moléculaire, ces travaux de thèse sont les premiers à corréler l'expression de gènes avec les paramètres de qualité de la perle au niveau du sac perlier chez *P. margaritifera* (Figure 7.1). Ces travaux ont donc permis d'étudier la cinétique d'expression de gènes corrélés à 1) la synthèse de la calcite et/ou de l'aragonite, 2) la croissance ou la qualité de perle, depuis la greffe jusqu'à la récolte. L'étude de

l'influence des protéines de biominéralisation sur le processus de la perle en formation est à son commencement, seules quelques études ont observé l'expression de gènes au niveau du sac perlier (Takeuchi and Endo 2006 ; Wang *et al.* 2009 ; Inoue *et al.* 2010, 2011 ; Gu *et al.* 2016). Gu *et al.* (2016) mettent en avant un effet de l'huître receveuse sur l'expression des gènes de biominéralisation dans le sac perlier. En comparant les niveaux d'expression entre le manteau et le sac perlier, ils obtiennent des niveaux d'expression bien inférieurs pour le sac perlier, résultats en adéquation avec ceux obtenus par d'Inoue *et al.* (2011). Les niveaux d'expression de MSI60 et Aspein sont inférieurs dans le sac perlier par rapport au manteau. Nos travaux ont également montré une différence au cours de la formation de la perle du niveau d'expression des gènes de biominéralisation corroborant les résultats d'Inoue *et al.* (2011), où certains gènes ont un faible niveau d'expression durant les premières phases et d'autres plus tard dans le processus de la biogénèse de la perle. D'après nos résultats, le niveau d'expression des gènes ne semble pas être seulement régulé par les gènes de l'huître donneuse de greffon, mais d'autres facteurs comme par exemple l'âge, la receveuse ou les conditions environnementales peuvent réguler l'expression des gènes de biominéralisation (Figure 7.1). Nous avons également mis en évidence trois gènes fortement impliqués dans les phénotypes de la perle au travers de différents chapitres à savoir PIF 177, Aspein et Shematin9 (Figure 7.1).

Estimer l'expression des gènes biomarqueurs de l'activité minéralisatrice chez *P. margaritifera* en fonction de diverses conditions environnementales permettrait d'approfondir le rôle de l'huître receveuse dans l'expression de gènes au niveau du sac perlier. Une étude chez *P. margaritifera* a montré que l'expression de la plupart des gènes de biominéralisation étudiés serait régulée par la concentration en microalgue et la température au niveau du manteau (Joubert *et al.* 2014). Une thèse actuellement en cours sur ce sujet étudie le niveau d'expression des gènes codant des protéines impliquées dans les processus de biominéralisation au sein des tissus biominéralisateurs avec cette fois-ci le sac perlier et le greffon en réponse à des questions d'ordre écophysiologiques. Les résultats de cette étude permettront d'identifier des gènes biomarqueurs potentiels de la croissance de la coquille via l'analyse du niveau d'expression des

gènes au sein du manteau et du sac perlier en réponse à la combinaison des différents paramètres environnementaux (niveaux trophiques et températures) (*i.e.* RNA-seq) (Thèse en cours Latchere O.).

Au cours de cette thèse, nous avons explicitement montré que l’huître donneuse de greffon ne fait pas que former le sac perlier suite au processus de greffe mais contribue à certains traits de qualité de la perle et à l’expression des gènes de biominéralisation. Mais nous avons également clairement montré que l’huître receveuse jusque là souvent ignorée, a elle aussi une influence majeure dans la formation de la perle notamment sur l’expression de sa taille mais également sur la régulation de l’expression des gènes de biominéralisation (Figure 7.1).

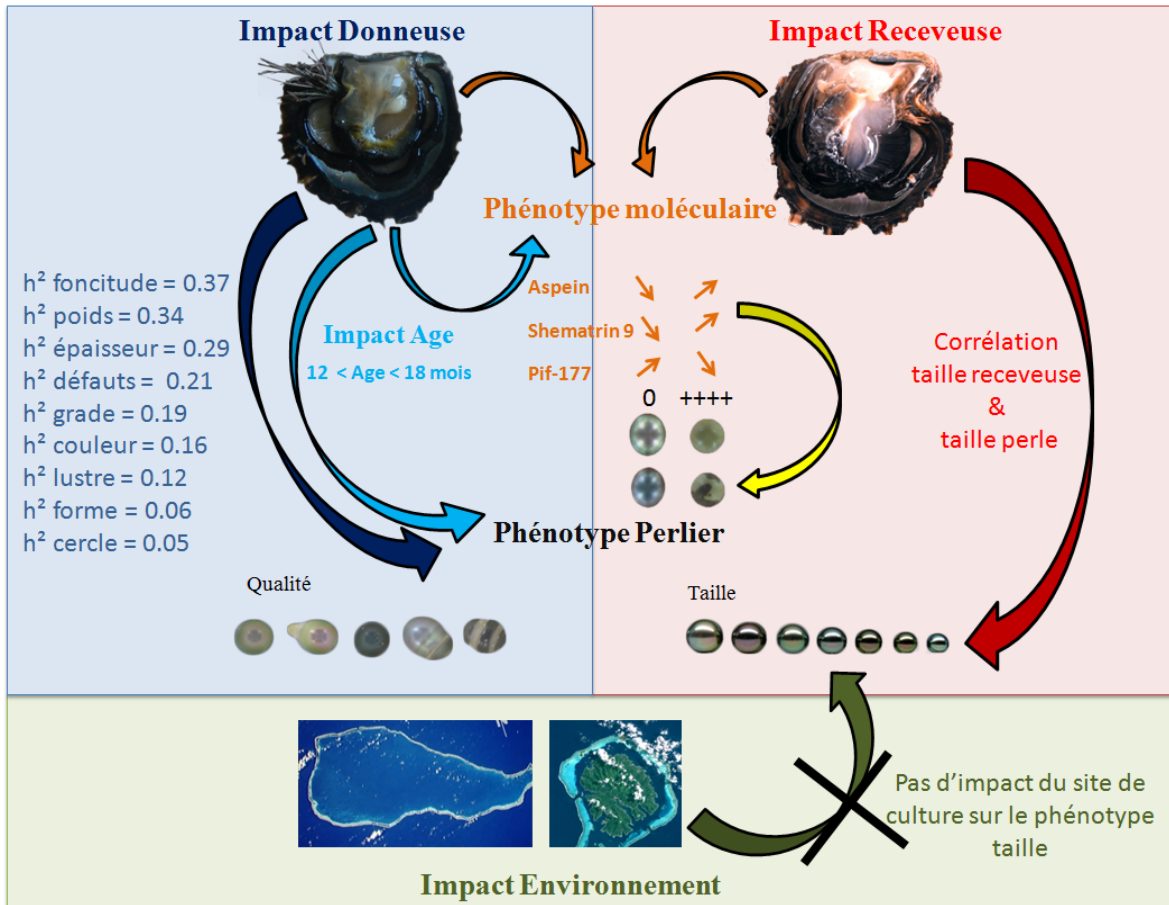


Figure 7.1 : Synthèse des principaux résultats concernant les contributions de l’huître donneuse et receveuse sur les phénotypes de la perle et l’expression des gènes de biominéralisation. h correspond à l’héritabilité au sens étroit.

2. PERSPECTIVES

2.1. D'UN POINT DE VUE APPLIQUE

Les travaux réalisés au cours de cette thèse ont un objectif appliqué, celui d'améliorer la productivité de l'activité perlicole polynésienne et de contribuer à un redressement du secteur économique dans son ensemble. En effet, cette thèse a permis d'apporter des connaissances fondamentales sur le processus de formation de la perle et de biominéralisation dans une optique concrète d'amélioration de la qualité de la perle de *P. margaritifera*. La production de perle de culture peut, en effet, être optimisée via la sélection d'huîtres perlières de hautes propriétés biominéralisatrices permettant d'obtenir des récoltes plus homogènes et de meilleures qualités (au vue des estimations d'héritabilité et des effets familles de première génération produite en éclosérie). A l'heure actuelle, le schéma de production de perle de culture chez *P. margaritifera* en Polynésie Française utilise des huîtres perlières issues de collectage de naissains en milieu naturel, source unique d'approvisionnement en huîtres perlières donneuses et receveuses. Les huîtres perlières sont collectées directement dans la nature, contrairement aux espèces concurrentes produites en éclosérie. Jusqu'à présent, l'abondance en huître perlière et la facilité du collectage dans la nature ont été des atouts de taille pour le développement de l'industrie perlicole en Polynésie. Mais les pays concurrents n'ayant pas eu cette ressource ont développé depuis 25 ans des lignées d'huîtres dont la taille et la couleur des perles sont optimisées. C'est le cas par exemple aux Philippines (Jewelmer) et en Birmanie (Tasaki) où la sélection de *P. maxima* permet la production de perles dorées de grandes tailles. Au travers de la sélection génétique, les phénotypes taille et couleur de la perle pourront être optimisés. A l'heure actuelle en Polynésie française, trois écloséries existent : deux expérimentales et une privée. La sélection génétique familiale d'huîtres perlières en est donc à son commencement. De façon concrète, la production en éclosérie d'huître perlière sélectionnée permettra de disposer d'huîtres perlières de haute propriété biominéralisatrice. Une thèse en collaboration entre un perliculteur privé, l'Ifremer et le CRIOBE devrait commencer en 2017 avec pour sujet : « Sélection génétique de l'huître perlière *Pinctada*

margaritifera et génomique fonctionnelle du déterminisme de la qualité de la perle ». Les objectifs de la thèse seront 1) de gérer la diversité génétique en éclosion via l'étude de la variabilité génétique au sein des descendants produits en éclosion en les comparant à des témoins sauvages issus de collectage naturel, 2) d'estimer le progrès génétique réalisé avec des animaux issus des premiers cycles de sélection sur la couleur et la croissance et 3) d'identifier et d'étudier l'expression des gènes clefs impliqués dans le potentiel de croissance des huîtres perlières.

En parallèle, afin de contrôler l'impact de l'exploitation de *P. margaritifera* sur la biodiversité, des études de conservation génétiques devront être entreprises. Des connaissances sur la structure génétique sont indispensables afin de définir les limites et maintenir une aquaculture durable. Du fait de l'exploitation des huîtres issues du collectage naturel et de transferts depuis les lagons qui recrutent au sein des fermes (principalement dans l'archipel des Tuamutu et Gambier) (Le Pennec 2010), des flux de gènes sont créés au travers des archipels (Arnaud-Haond *et al.* 2007 ; Lemer and Planes 2012). Ces études de variabilité génétique entre des individus sauvages et issus d'éclosion pourront être faites à l'aide d'un jeu de marqueurs type SNP (pour « single nucleotide polymorphism »). Elles permettront d'étudier le polymorphisme en identifiant des génotypes à travers l'ensemble du génome avec une résolution supérieure aux marqueurs de types microsatellites utilisés dans les précédentes études (Arnaud-Haond *et al.* 2007 ; Lemer and Planes 2012). Ces études évalueront l'impact des huîtres issues d'éclosion ou encore issues de collectage (utilisé en perliculture) sur la population sauvage. Une abondante littérature existe sur les salmonidés tels que la truite ou le saumon, où les interactions entre individus d'élevages et sauvages peuvent être intentionnelles ou accidentelles. L'impact de l'élevage sur les populations naturelles est un sujet d'actualité, avec notamment l'incidence génétique des mélanges et leurs conséquences phénotypiques voire adaptatives.

2.2. DE L'EXPRESSION AU ROLE DES PROTEINES

L'utilisation des techniques de biologie moléculaire et de génomique de plus en plus performantes permet de disposer aujourd'hui de diaporamas quasi-complets de gènes exprimés au sein des tissus biominéralisateurs pour une espèce donnée (Jackson *et al.* 2006; Clark *et al.* 2010; Joubert *et al.* 2010; Berland *et al.* 2011 ; Huang *et al.* 2013). Plusieurs transcriptomes concernant les tissus minéralisateurs sont disponibles chez les huîtres perlières du genre *Pinctada*, soit pour le manteau chez *P. margaritifera* (Joubert *et al.* 2010), *P. martensii* (Shi *et al.* 2013), *P. maxima* (Jones *et al.* 2013 ; Deng *et al.* 2014), *P. fucata* (Kinoshita *et al.* 2011), soit pour le sac perlier chez *P. martensii* (Zhao *et al.* 2012), *P. maxima* (McGinty *et al.* 2012). Ces listes exhaustives de gènes et de transcrits sont une source considérable d'informations qui ont permis d'améliorer les connaissances sur les protéines impliquées dans le processus de biominéralisation. Cependant, ces méthodes ne renseignent pas sur leurs rôles exacts dans la formation et la régulation des processus de biominéralisation. En effet, il serait intéressant de décrire la structure primaire des séquences protéiques et de prédire la formation de structures secondaires ou tertiaires, ou encore de révéler la présence de motifs fonctionnels. Identifier les modifications post traductionnelles et la caractérisation structurale des protéines identifiées dans la littérature permettraient de déterminer l'activité biologique et d'aller vers une caractérisation fonctionnelle plus approfondie. Quelques travaux ont étudié le coté fonctionnel des protéines (Addadi *et al.* 1987; Marin *et al.* 2000, 2007; Marxen *et al.* 2003; Marie *et al.* 2008). Au cours de cette thèse, le niveau d'expression de gènes au sein du greffon et sac perlier a été évalué en réponse à diverses questions comme la cinétique d'expression (**chapitre 3 à 5**), l'analyse de la variabilité individuelle/familiale (**chapitre 3 & 6**), ou encore le site de culture (**chapitre 2**) et ont permis ainsi d'affiner le rôle de certains transcrits sur la qualité de la perle.

D'autres techniques permettraient d'augmenter nos connaissances sur la fonctionnalité de ces protéines comme par exemple la mise en place d'expériences de génétique inverse qui déterminerait la fonction d'un gène (et donc de la protéine qu'il code) par l'inhibition de la fonction et l'observation du phénotype qui en résulte. Quelques études d'ARN interférent (RNAi) ont été réalisées afin de caractériser la fonction

de plusieurs protéines impliquées dans la biominéralisation notamment chez *P. fucata* (Suzuki *et al.* 2009 ; Fang *et al.* 2011) ou encore *P. martensii* (Yan *et al.* 2014). Des études utilisant cette technique sont actuellement en cours chez *P. margaritifera* au sein du laboratoire de l'Ifremer Tahiti.

2.3. PLASTICITE PHENOTYPIQUE ET SELECTION SUR LA DIVERSITE PHENOTYPIQUE

Comprendre l'impact de l'action de la sélection (*i.e.* naturelle ou contrôlée) et de la plasticité phénotypique (*i.e.* pressions environnementales) sur la diversité des populations et prédire la réponse des espèces dans un contexte actuel de changement global est crucial. En effet, face aux variations environnementales, la sélection et la plasticité phénotypique peuvent être traduites par de la variance des populations et des modifications du phénotype moyen. La modification du phénotype par la sélection naturelle ou contrôlée est visible uniquement à l'échelle populationnelle, soit au sein d'une même génération par élimination de certains individus et/ou au cours des générations par modification des fréquences alléliques modifiant ainsi le phénotype moyen et la diversité phénotypique de la population. Tandis qu'une modification phénotypique par plasticité sur chaque individu plastique peut entraîner une modification à l'échelle de la population visible au sein d'une génération (Pigliucci 2001). Cette adaptabilité du phénotype à un environnement hétérogène provient de la plasticité du mode de régulation des gènes autrement dit la plasticité épigénétique. Récemment, il a été montré que cette plasticité épigénétique (par exemple les méthylations de l'ADN) peut être héritable et transférée sur plusieurs générations (Duncan *et al.* 2014). Dans un contexte de changement global de nombreuses études se penchent sur ce sujet (Zhang *et al.* 2013 ; McCairns *et al.* 2016 ; Trerotola *et al.* 2015 ; Roquis *et al.* 2016). La diversité génétique est un paramètre essentiel quant à la capacité d'adaptation des populations au cours du temps et face aux pressions environnementales. Prédire comment une population de *P. margaritifera* peut survivre et s'adapter face aux pressions environnementales est donc crucial aussi bien d'un point de vue évolutif que perlicole. Prenons l'exemple du déterminisme de la couleur, des travaux

antérieurs ont montré l'existence d'un lien phénotypique étroit entre la couleur de la coquille interne (Ky *et al.* 2017) ou encore la couleur de chair (travaux réalisés durant cette thèse mais non présentés) de l'huître donneuse de greffons et la couleur des perles récoltées, mais également un impact significatif de certains facteurs environnementaux (comme par exemple la profondeur de culture) sur l'expression de la coloration coquillière (plasticité phénotypique). Chez les mollusques, la coloration de la coquille est un caractère qui apparait soit par l'iridescence de la coquille en lien avec la structure cristalline (Snow *et al.* 2004), soit par l'intégration de pigments au sein de la coquille, soit par combinaison des deux. Les facteurs environnementaux pourraient induire un changement de la conformation de l'ADN par modifications épigénétiques. Malgré ces découvertes récentes, les mécanismes moléculaires sous-jacents à l'expression du phénotype « couleur », ainsi que l'origine de l'hétérogénéité de la distribution de ce trait dans les populations restent mal connus. Une thèse a commencé sur ce sujet avec pour objectif d'appréhender cette question à l'échelle globale du phénotype, du génome, et de l'épigénome, au sein de populations d'élevage et de populations naturelles via différents types de séquençage NGS. Les objectifs de cette thèse sont 1) de caractériser de façon quantifiable le phénotype couleur, 2) d'identifier le phénotype moléculaire (gène exprimé) en lien avec la pigmentation (*i.e.* RNA-seq), 2) d'identifier les variants génétiques associés (*i.e.* RAD-seq), et enfin 4) d'étudier l'implication des mécanismes épigénétiques dans l'expression de la plasticité phénotypique de la couleur (*i.e.* Chip-seq). Le séquençage du génome de *Pinctada margaritifera* réalisé fin 2016 (assemblage en cours) devrait aider en ce sens et apporter de nouvelles perspectives.

3. CONCLUSIONS

Globalement, le travail réalisé au cours de cette thèse a permis de mieux comprendre les facteurs affectant la formation de la perle et sa qualité. En utilisant à la fois des données phénotypiques et transcriptomiques, nos résultats ont permis de définir quelle est la contribution de la receveuse et de la donneuse à la formation de la perle, mais également de confirmer et d'affiner quels gènes influencent les traits phénotypiques de la perle chez *P. margaritifera*. Cette thèse a révélé le rôle non négligeable de l'huître receveuse aussi bien au niveau du phénotype de la perle (notamment de la taille de la perle) qu'au niveau moléculaire. Ces recherches ont également montré pour la première fois les paramètres d'héritabilité aussi bien d'un point de vue moléculaire que phénotypique. Les futurs programmes de sélection visant à l'amélioration de la qualité de la perle devront donc s'intéresser aussi bien à l'huître donneuse de greffon qu'à l'huître receveuse, suivant les critères de sélection envisagés. Cette étude aura également montré l'importance d'examiner la complexité des interactions donneur/receveur.

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ANNEXE 1 : INFLUENCE DU NIVEAU DE DEPOTS PERLIER SUR LE GRADE ET LA COULEUR

Influence of nacre deposition rate on cultured pearl grade and colour in the black lipped pearl oyster *Pinctada margaritifera* using farmed donor families

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ABSTRACT

Cultured pearl nacre deposition rate in *P. margaritifera* oysters was studied to examine its influence on Tahitian classification grade and colour and to further our understanding of using selected donor oysters produced in a hatchery system for the improvement of these traits. A large-scale grafting experiment using 150 farmed donor oysters was realised in Rangiroa atoll (French Polynesia). After 18 months of culture, 874 pearls were harvested and 7 pearls quality traits were recorded: 1) nacre deposition rate assessed by nacre thickness and weight, 2) classification grade, lustre and surface defects, and 3) colour assessed by pearl colour darkness level and visual colour categories. Nacre deposition rate significantly influence pearl grade, surface defects, darkness level and colour categories, but not the lustre. Grade A pearls had the thickest and heaviest nacre on average, compared with grade D pearls and rejects. In addition, pearls without any surface defect had thicker and heavier nacre. Pearls with low darkness were the thinnest and the lightest. Pearls of aubergine-peacock secondary colours were the thickest and the heaviest. These results highlight the importance of rapid nacre deposition rate for increasing the production of grade A pearls, with less surface defects and dominance of a black tone colour. Fast nacre deposition may, however, have a negative impact on other pearl quality traits, such as pearl lustre. This study demonstrates the importance of examining relationships between pearl quality traits in a way that provides solid information that can be directly used for breeding and genetic selection programs.

Keywords: Cultured pearl / Farmed donor families / French Polynesia / Nacre deposition rate / Pearl colour / Pearl grade / Pearl quality / *Pinctada margaritifera*

1. INTRODUCTION

The black-lip "pearl oyster", *Pinctada margaritifera* (Linnaeus 1758) var. *cumingi* (Reeve), which occurs throughout the Indo-Pacific region, is an abundant marine mollusc in the lagoons of French Polynesia. In this area, cultured pearls are the main focus of aquaculture and the business of this organic gem production is well established. In fact, cultured pearls are produced by grafting a round nucleus made of shell with a piece of mantle from a donor mollusc into the gonad of a host mollusc. The mantle tissue grows around the nucleus and becomes a pearl sac, which secretes successive nacre layers on the nucleus. This process results in the formation of a cultured pearl in the space of approximately 20 months (Taylor and Strack 2008). In this tropical ecosystem, the production of cultured pearl represents the most valuable export industry and is the second most important source of income for French Polynesia after tourism. This industry is currently undergoing an unprecedented crisis, with profits showing a continuing decline, due to a combination of factors in which overproduction plays an important role: quantity has been favoured to the detriment of quality, in a context of world economic crisis (Le Pennec and Buestel 2010). Furthermore, it is estimated that only 5 percent of harvested pearls can be classed as grade A quality according to the local regulatory control standards (Ellis and Haws 1999). In this context, an increase in the production of perfect pearls would therefore be a considerable advantage for the industry: favouring quality over quantity.

Tahitian cultured black pearl quality and hence, their value and beauty, is determined following a wide range of criteria, including pearl size, nacre thickness and weight, pearl classification grade, surface quality and lustre, colour (bodycolour and overtone), darkness level are important factors. Pearl size (ranging from 8.0 mm to 20 mm) determines value, with larger pearls generally commanding higher prices. More specifically, according to Tahitian government regulations, all exported pearls must be screened by the "*Maison de la Perle*" using X-ray technology to ensure that their nacre thickness is at least 0.8 mm. Cultured pearls with thin nacre are rejected. Tahitian classification grading is an evaluation made with the naked eye, which grades pearls into 5 classes: A, B, C, D and rejects, the last of which are

below class D quality and cannot be exported from Tahiti (see description in Materials and Methods section). This classification grading is determined by two components: pearl surface quality and pearl lustre. A remarkable specificity of the *P. margaritifera* "pearl oyster" is its ability to produce a wide range of pearl colours, where the predominant bodycolours are white, yellow and grey and secondary colours are green, aubergine and peacock (Tayale *et al.* 2012). These secondary colours (overtones) may be present in a variety of combinations and are considered a plus factor. Generally, the darker a Tahitian pearl, the more valuable it is, especially for the Asian market.

For pearl-producing mollusc, relationships between phenotypic criteria and their influence on pearl quality traits and/ or relationships between pearl quality traits are insufficiently documented. Some studies have shown phenotypic correlations between pearl weight and shell traits, e.g., for *P. fucata* (Wada 1984; Velayudan *et al.* 1996; He *et al.* 2008). Wada and Komaru (1996) studied the phenotypic correlation between size of pearls and shell valve weight in *P. fucata martensii* oyster and found a small positive relationship whereby oysters with heavier valves produced larger pearls. Another study, on *H. cumingii*, showed that improving mussel body length or weight in culture indirectly led to improved pearl size and weight (Jin *et al.* 2012). For *P. margaritifera*, McGinty *et al.* (2010) studied nacre deposition rate through a xenografting experiment between *P. maxima* and *P. margaritifera*, demonstrating that such xenografting influenced pearl quality and that the donor oysters played a significant role. Tayale *et al.* (2012) showed the evidence of wild donor effect on cultured pearl quality from a duplicated grafting experiment on *P. margaritifera*. This study also revealed the existence of correlations between cultured nacre thickness and: 1) grade and 2) colour components (darkness level and "visual categories"). More recently, Ky *et al.* (2013) showed the existence of family effect (i.e. oysters produced by hatchery system) on pearl quality and highlights the possibility of setting up a sib genetic selection plan for *P. margaritifera*.

To fill this gap in our knowledge about the black-lipped pearl oyster, the present study set out to examine and confirm, what has been observed in Tayale *et al.* (2012) using wild donors, on the influence of cultured pearl nacre thickness and weight, i.e. nacre deposition rate, on cultured pearl grade and colour,

by: 1) using here farmed donor oysters produced by hatchery system and 2) studying more precisely the relationships between seven quality traits; nacre thickness, nacre weight, classification grade, pearl surface defect, lustre, darkness level and visual colour categories. For this, a sample of cultured pearls providing of a large grafting experiment was used. This experimental graft was done using 100 farmed donors from 10 fullsib families, with the grafting operations all performed by a single professional grafter and receivers (provided from a unique spat collection) reared in a monoculture site in the lagoon of Rangiroa (Tuamotu archipelago - French Polynesia) in order to minimise “environmental factors” (Ky *et al.* 2013). From a genetic point of view, this work constitutes the first description of the potential of selecting donor oysters through sib selection for fast nacre deposition and its effect on pearl quality traits. From an aquaculture point of view, this study open the way in French Polynesia for the implantation of oyster hatchery, where the application of modern genetic breeding methods will increase the proportion of high quality pearls by producing selected lines of donor oysters.

2.MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Ten bi-parental families were produced in the Ifremer hatchery facilities in Vairao (Tahiti, French Polynesia) between ten female and 10 male broodstock oysters. Artificial spawning, larval rearing, and oysters culture were made as described in Ky *et al.* 2013. Grafting experiment has been made in Gauguin's Pearl Farm on Rangiroa atoll (Tuamotu archipelago). The grafting operations were all performed by a single professional grafter in order to minimise "environmental factors" and receivers were provided from a unique spat collection. Experimental graft and pearl harvest are also described in Ky *et al.* 2013.

MEASUREMENT OF CULTURED PEARL QUALITY TRAITS

Cultured pearls were cleaned by ultrasonication in soapy water (hand washing) with a LEO 801 laboratory cleaner (2 L capacity, 80 W, 46 kHz), then they were rinsed in distilled water.

Pearl nacre deposition rate were assessed by nacre thickness and nacre weight. These two last component were measured as described in Ky *et al.* 2013.

Pearl grade (Table 1) was determined by a single professional expert according to the official A-D Tahitian classification (*Journal Officiel* 2001 n° 30, 26 July 2001) (Ky *et al.* 2013).

Tableau 1 : Summary of the official A-D Tahitian cultured pearl classification by grade (A, B, C, D and rejects) following surface defects and lustre properties (Journal Officiel 2001 n° 30, 26 July 2001).

Grade	Properties/ definitions
A	Highest quality. Free of defects on at least 90% of its surface, only minor imperfections on the other 10%. Very good lustre.
B	Good quality. Free of defects on at least 70% of its surface, a tolerance of 2 visible imperfections. Good lustre at minimum.
C	Medium quality. Free of defects on at least 40% of its surface, 10% of visible defects are tolerated. Medium lustre at minimum
D	Sufficient quality. With minor defects on 60% of its surface, 20% of visible defects tolerated or white holes. Low lustre at minimum
Rejects	Not saleable. With calcite and/ or organic deposit on at least 20% of its surface or a devitalized zone over 20% of its surface or nacre deposition inferior to 0.8mm. Rejects are destroyed.

For the following pearl quality traits: colour, darkness level, surface defects and lustre, data were visually classified by the naked eye (no magnification device like jeweler's loop as made by Tahitian pearl technicians of Maison de la Perle) into categories by two operators working in cooperation. Colour evaluation was made on the cultured pearls according to Ky *et al.* (2013) by the darkness of colour and the visually-perceived colour category, which is due to pigments (bodycolour: grey, white and yellow), and secondary colour (overtone: green, aubergine, and peacock).

STATISTICAL ANALYSIS

Before data analysis, pearl grade, surface defects and darkness of colour were re-encoded to give a quantitative score so that they could be ranked (Ky *et al.* 2013). Kruskal-Wallis tests followed by Dunn's

multiple comparison tests with a Bonferonni correction were performed to determine whether there were significant differences in nacre weight (g) or nacre thickness (mm) according to grade, surface defect class, darkness level or colour category (Winer *et al.* 1991). Kruskal-Wallis tests followed by Dunn's multiple comparison tests with a Bonferonni correction were performed to determine whether there were significant differences in lustre between pearl surface defect classes, and in pearl darkness class between the visual colour categories.

We considered a difference significant when the p -value was lower than 0.05 (Dagnelie 2007). Statistical analyses were performed using the R software program (version 2.14.1) and XLSTAT (version 2009.4.02).

3.RESULTS

RELATION OF NACRE THICKNESS-WEIGHT WITH PEARL CLASSIFICATION GRADE

The average nacre thickness among the 874 harvested pearls was 1.12 mm, with minimum and maximum values of 0.23 mm and 2.98 mm, respectively. The distribution of the 874 harvested pearls among the five pearl classification grades was as follows: 6.2% (N = 54) grade A, 20.7% (N = 181) grade B, 33.9% (N = 296) grade C, 26.1% (N = 228) grade D and 13.1% (N = 115) rejects.

Data analysis showed a very highly significant difference in nacre thickness between pearl grades ($p < 0.0001$). Figure 1a shows how grade A pearls have the thickest nacre (1.28 mm on average). Mean nacre thicknesses in grades B, C, D and reject were 1.16 mm, 1.08 mm, 1.10 mm and 1.16 mm, respectively. Grade A pearls therefore had nacre close to 15% thicker on average than grades C, D and 10% thicker on average than grade B and reject. Incidence of cultured pearl thickness among the five classification grade is illustrated in table 2.

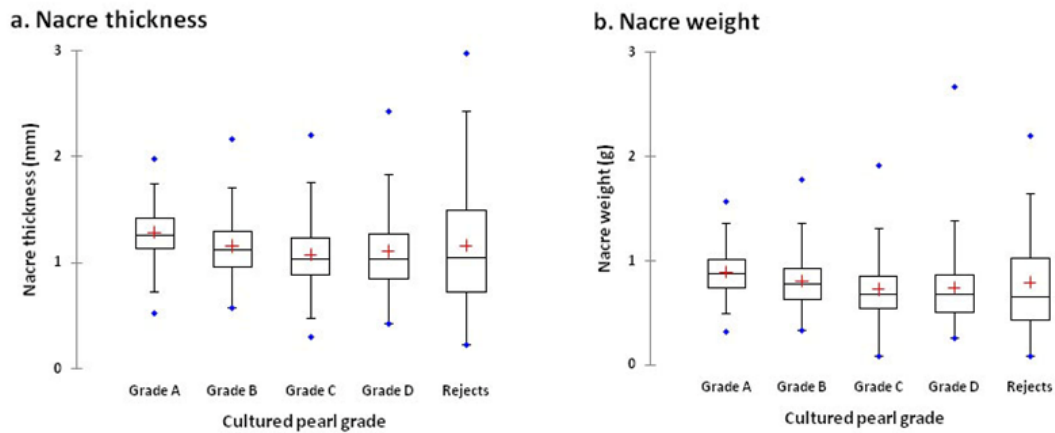








Figure 1: Nacre thickness (a, in mm) and nacre weight (b, in g) of cultured pearls according to the five classification grade: A, B, C, D and rejects. Each box-plot (McGill *et al.* 1978) has the following 6 elements: 1) mean ("+" cross in the box-plot); 2) median (solid bar in the box-plot); 3) 25th to 75th percentile (rectangular box); 4) 1.5*interquartile range (non-outlier range of the box whiskers); 5) minimum and maximum values (extreme dots) and 6) outlier values (outside box whiskers).

The average nacre weight among the 874 harvested pearls was 0.76 g, with minimum and maximum values of 0.08 g and 2.68 g, respectively. In addition, a very highly significant difference in cultured pearl nacre weight was found between pearl grades ($p < 0.0001$). Figure 1b shows how grade A pearls are the heaviest (0.90 g on average). Mean pearl weight of grades B, C, D and reject were 0.80 g, 0.73 g, 0.74 g and 0.80 g, respectively. Grade A pearls were therefore nearly 20% heavier on average than grade C and D cultured pearls. Table 2 illustrated influence of cultured pearls weight on classification grade.

Tableau 2 : Cultured pearl nacre deposition rate (thickness in mm and weight in g) and its influence on Tahitian classification grade (A, B, C, D and rejects) and visual colour categories (overtone: aubergine, peacock, green, and bodycolour: yellow, grey and white). The cultured pearl samples are ranked from the thickest and heaviest to the thinnest and lightest in weight.

Thickness (mm)	Weight (g)	Grade	Colour	Photo*
1.24	0.87	A	Aubergine	
1.21	0.84	B	Peacock	
1.10	0.75	B	Green	
0.99	0.62	C	Yellow	
0.85	0.48	D	Grey	
0.65	0.46	R	White	

*Images not scaled to size

RELATION OF NACRE THICKNESS-WEIGHT WITH PEARL SURFACE DEFECTS AND LUSTRE

The 874 harvested pearls were distributed among the four surface defect classes as follows: 7.1% (N = 62) with no defects, 48.6% (N = 425) with 1 to 5 defect(s), 32.5% (N = 284) with 6 to 10 defects and 11.8% (N = 103) with more than 10 defects.

Cultured pearl nacre thickness was highly significant between the surface defect classes ($p = 0.001$). Figure 2a shows that pearls without any defects had the thickest nacre (1.21 mm on average). Pearls with 1 to 5 defect(s), with 6 to 10 defects and more than 10 defects showed 1.11 mm, 1.10 mm and

1.20 mm mean nacre thickness, respectively. Pearls without any defects had 10% thicker nacre than pearls with 6 to 10 defects.

In addition, a significant effect of surface defect class was recorded for cultured pearl nacre weight ($p = 0.028$). Figure 2b illustrates the mean nacre weight of the four surface defect classes, and shows that pearls without any defects were the heaviest (0.82 g on average). Mean weight of pearls with 1 to 5 defect(s), 6 to 10 defects and more than 10 defects weighed 0.76 g, 0.74 g and 0.82 g, respectively. Pearls without any defects were 11% heavier on average than pearls with 6 to 10 defects.

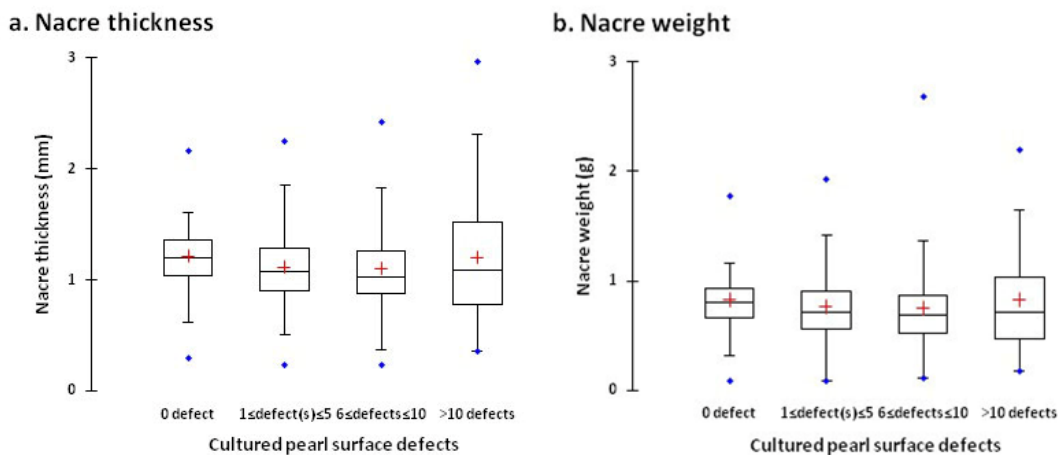


Figure 2 : Nacre thickness (a, in mm) and nacre weight (b, in g) of cultured pearls according to the four surface defect classes: 0 defect; $1 \leq \text{defect}(s) \leq 5$; $6 \leq \text{defects} \leq 10$; and > 10 defects. Each box-plot (McGill *et al.* 1978) has the following 6 elements: 1) mean ("+" cross in the box-plot); 2) median (solid bar in the box-plot); 3) 25th to 75th percentile (rectangular box); 4) 1.5*interquartile range (non-outlier range of the box whiskers); 5) minimum and maximum values (extreme dots) and 6) outlier values (outside box whiskers).

The 874 harvested pearls included 86.2% (N = 753) that had lustre and 13.8% (N = 121) without. No difference was recorded for nacre thickness ($p = 0.650$) or weight ($p = 0.505$), according to the presence or absence of lustre.

RELATION BETWEEN PEARL LUSTRE AND SURFACE DEFECTS

The proportion of cultured pearls with lustre varied among the four surface defect classes, as shown in Figure 3. The cultured pearls with no defects showed 93.5% of pearls with lustre, whereas those with 1 to 5 defect(s), 6 to 10 defects and more than 10 defects had lustre in 95.8%, 86.3% and 41.7% of

cases, respectively. A very highly significant effect was recorded for cultured pearl lustre ($p < 0.0001$) between these classes. Cultured pearls in the class with < 5 defects included more than twice as many with lustre than in the class with up to 10 defects.

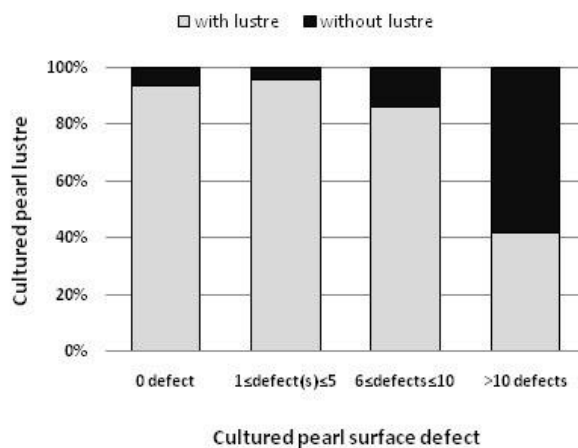


Figure 3 : Cultured pearl lustre distribution (% of pearls for each of the two lustre classes: with and without lustre) from each of the four surface defect classes: 0 defect, $1 \leq \text{defect}(s) \leq 5$, $6 \leq \text{defects} \leq 10$ and > 10 defects.

RELATION OF NACRE THICKNESS-WEIGHT WITH PEARL DARKNESS LEVEL

The 874 harvested pearls were distributed among the three darkness levels as follows: 23.3% (N = 204) with low, 49.7% (N = 434) with moderate and 27.0% (N = 236) with the high darkness level. Among these groups, there was a highly significant difference in nacre thickness between darkness levels ($p = 0.015$). Figure 4a illustrates the average nacre thickness distribution of the three darkness levels. The lightest coloured pearls (low level) had the thinnest nacre, with an average nacre thickness of 1.07 mm. The cultured pearls with the moderate darkness level had the thickest nacre, with an average nacre thickness of 1.15 mm. making them nearly 10% thicker than the lightest group. The darkest group of pearls had an intermediate mean nacre thickness of 1.12 mm.

Nacre weight was also significantly different between the three darkness levels ($p = 0.014$). Figure 4b shows the average nacre weight distribution of the three categories of darkness. The palest coloured pearls (low darkness level) were the lightest in weight, with an average nacre weight of 0.73 g. The

cultured pearls showing moderate darkness level were the heaviest, with an average nacre weight of 0.79 g. The moderate darkness level group was nearly 10% heavier than the lightest group. The darkest group of pearls had an intermediate nacre weight of 0.75 g.

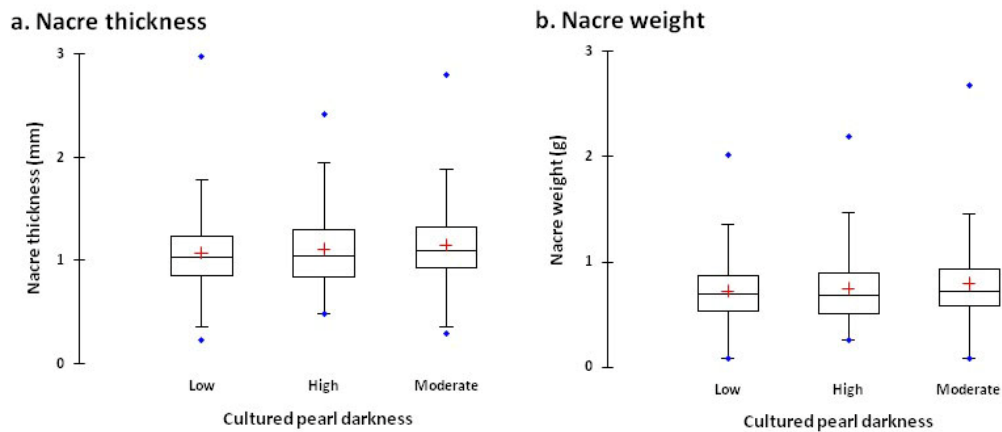


Figure 4: Nacre thickness (a, in mm.) and nacre weight (b, in g) of the cultured pearls among three darkness level: low, moderate and high darkness. Each box-plot (McGill *et al.* 1978) has the following 6 elements: 1) mean ("+" cross in the box-plot); 2) median (solid bar in the box-plot); 3) 25th to 75th percentile (rectangular box); 4) 1.5*interquartile range (non-outlier range of the box whiskers); 5) minimum and maximum values (extreme dots) and 6) outlier values (outside box whiskers).

RELATION OF NACRE THICKNESS-WEIGHT WITH VISUAL COLOUR CATEGORIES

Six colour categories were visually detected among the 874 harvested pearls. The three bodycolour were: 1) grey ($N = 249$; 28%), 2) yellow ($N = 37$; 4%) and 3) white ($N = 26$; 3%). The three secondary colour were: 1) green ($N = 488$; 56%), 2) peacock ($N = 59$; 7%) and 3) aubergine ($N = 15$; 2%).

A highly significant colour categories effect was recorded for nacre thickness ($p = 0.004$). Figure 5a illustrates the average nacre thickness of the six colour categories, ranked from the thickest to the thinnest, as follows: aubergine (1.29 mm), peacock (1.24 mm), green (1.13 mm), yellow (1.11 mm), grey (1.10 mm), and white (1.09 mm). Aubergine and peacock secondary colour pearls therefore had 18% and 13% thicker nacre respectively than white bodycolour pearls on average. Table 2 illustrated influence of cultured pearls thickness on colour categories.

A significant colour effect was recorded for nacre weight ($p = 0.0009$). Figure 5b shows the average nacre weight distribution of the six colour categories, ranked from the heaviest to the lightest, as follows: aubergine (0.91 g), peacock (0.86 g), green (0.77 g), yellow (0.77 g), white (0.75 g) and grey (0.74 g). Aubergine cultured pearls were therefore 23% heavier than white and grey bodycolours pearls on average. Incidence of cultured pearl weight among the six colour categories is illustrated in table 2.

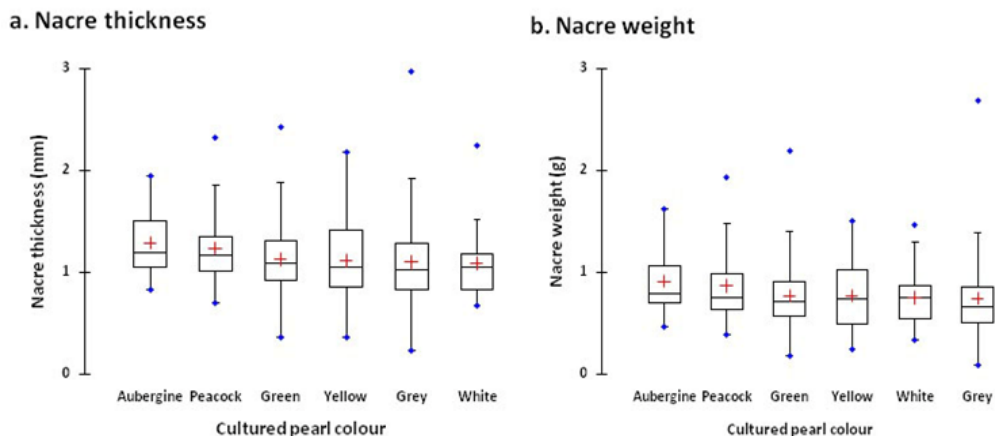


Figure 5 : Nacre thickness (a, in mm) and weight of nacre (b, in g) of cultured pearls from each colour categories. Cultured pearls are classed in the six colour categories: aubergine, peacock, green as secondary colours and, yellow, grey and white as bodycolour. Each box-plot (McGill *et al.* 1978) has the following 6 elements: 1) mean ("+" cross in the box-plot); 2) median (solid bar in the box-plot); 3) 25th to 75th percentile (rectangular box); 4) 1.5*interquartile range (non-outlier range of the box whiskers); 5) minimum and maximum values (extreme dots) and 6) outlier values (outside box whiskers).

RELATION BETWEEN PEARL DARKNESS LEVEL AND COLOUR CATEGORIES

Figure 6 shows the different darkness levels among each of the visual colour categories. A very highly significantly cultured pearl visual colour category effect was detected for darkness level ($p < 0.0001$). In figure 6, the six visual colours were ordered from the lightest coloured pearls (those exhibiting maximum number of pearls of the low darkness level), to the darkest (those exhibiting minimum number of pearls of the low darkness level), as follows: white (100%), yellow (54%), aubergine (27%), grey (24%), green (18%) and Peacock (7%).

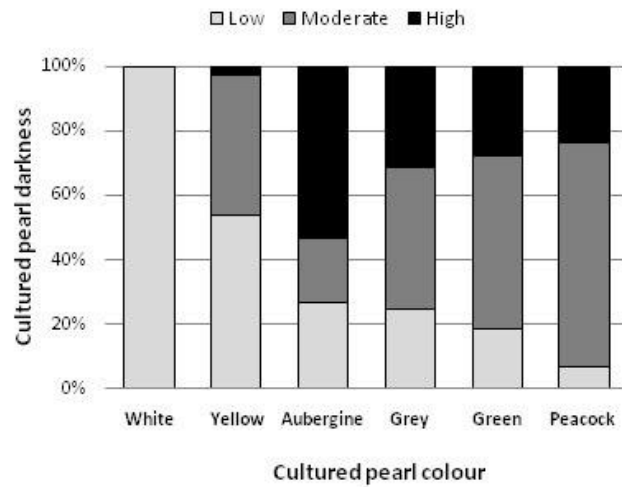


Figure 6 : Colour darkness distribution (% of each of the following categories: low, moderate and high darkness) in cultured pearls from each of the six visual colour categories (bodycolour and overtone). Colour categories are ranked from the lightest to the darkest as follows: white, yellow, aubergine, grey, green and peacock.

4. DISCUSSION

Nacre thickness and weight in cultured pearls produced from *P. margaritifera* are of major interest in the Tahitian pearl industry, because 1) cultured pearls with a nacre thickness less than 0.8 mm are rejected and 2) price also depends on pearl weight. In this study, measurement of nacre thickness and weight also offered a way to assess nacre deposition level, which clearly influenced two other main pearl quality traits: 1) classification grade and 2) pearl colour. This influence was also seen to affect some components of these quality traits, i.e., for classification grade, the number of surface defects was affected (although not directly lustre); and for the pearl colour, the darkness level and the visual colour categories observed.

INFLUENCE OF NACRE DEPOSITION RATE ON GRADE CLASSIFICATION AND ITS COMPONENTS

Grade classification is an important commercial criterion for cultured pearl pricing and sale at Tahitian cooperative auctions, with grade A the best valued. Our results revealed high nacre deposition rates for grade A pearls, which had the thickest and heaviest nacre on average, compared with grade D

pearls and rejects. This result is consistent with those obtained partially by Tayale *et al.* (2012) where, using grafts from wild donors, the authors demonstrated that correlations exist between cultured pearl nacre thickness and grade. This relationship has been found only in one of the two duplicate grafting experiment and has not been revealed for nacre weight.

Nacre thickness and weight are directly correlated with the nacre biomineralisation process. In fact, the biomineralisation phenomenon in molluscs is due to the property of the epithelial cells from the outer surface of the mantle tissue (which lines the inner surface of the shell) to synthesise different calcium carbonate polymorphs (Wilbur 1964; Wataba 1988). The nacre is formed by a hundred to thousand of aragonite crystal and protein matrix, which overlap alternately on the nucleus, as observed by electronic microscopy (Zhang and Xu 2013). The genome of the donor oyster, and its influence on the pearl biomineralisation process, is still present in the pearl sac of the recipient at the end of pearl formation (Arnaud-Haond *et al.* 2007). McGinty *et al.* (2010) showed that pearl quality traits were strongly influenced by the donor and not the recipient oyster as shown by a xenograft experiment using *P. maxima* and *P. margaritifera*. McGinty *et al.* (2011) demonstrated that the donor oyster is an important contributor to the mineralisation process in pearl culture. High biomineralisation capabilities may have contributed to a greater nacre deposition, as already observed in *P. maxima* (Kono *et al.* 2000; Müller 1997; Strack 2006).

More recently, Tayale *et al.* (2012) showed that *P. margaritifera* wild donor oysters can affect pearl quality traits and that genetic selection could be made of the "best" donors, making breeding programs possible. This is confirmed in the study of Ky *et al.* (2013) where family effect on pearl quality has been found, making possible sib selection. Consequently, there may be advantages for the pearl industry in using "pearl oysters" that exhibit rapid nacre deposition. Given the high level of nacre deposition observed here, molecular tools are currently developed in our laboratories as an approach to select for fast nacre deposition rate as a way to increase the overall proportion of grade A pearls produced by *P. margaritifera*, a trait that cannot be phenotypically selected from wild molluscs. However, care must

be taken with regard to possible effects that fast nacre deposition could have on other cultured pearl quality traits.

Cultured pearl surface defects and cultured pearl lustre are components of classification grade. For cultured pearl surface defects, our results show that samples without any defects had both the thickest and heaviest nacre. Fast nacre deposition rate could be one of the criteria to erase or to reduce the number of surface defects. Of course, other phenomena could explain this result, such as the impact of environmental factors during the culture period. As our experiment was conducted using a single location and period of culture (18 months) with the same single grafter making all the grafting operations in which nuclei of the same size were implanted, differences between nacre thickness and weight among the 874 pearls could be mainly explained by the genetic components of the donors (in this case issued from hatchery-bred families reared in culture). This important genetic implication in nacre deposition rate was confirmed by the xenograft studies realised by McGinty *et al.* (2010). In addition, Cuif *et al.* (2011) showed that the first nacreous materials deposited on the nucleus surface appear to be the main cause of irregularities that could be found in the future harvested pearls. If this initial matrix is not well established, we can hypothesize that, for the same rate of nacre deposition, the harvested pearls will present a much greater number of surface defects. Generally, implantation with *P. maxima* mantle tissue produced pearls with smoother surfaces (i.e., higher grades) than those from *P. margaritifera* donor tissue (McGinty *et al.* 2010). *P. maxima* is known to have a higher biomineralisation capacity than *P. margaritifera*. This suggests that fast nacre deposition rate (assessed here by nacre thickness and weight increase) decreases the number of surface defects on cultured pearls produced by *P. margaritifera*, and thereby also underlines the interest of selecting the "best" donors.

For cultured pearl lustre, our results show that pearls were not significantly thicker or heavier on average according to whether they had lustre or not. So there is no direct relation between nacre thickness / weight with the lustre. Lustre of pearls is known to be influenced by nacre deposition rate a few weeks prior to harvest time. Pearl farmers restrict pearl harvesting to winter as much as possible because nacre

deposition is at its slowest in this period; especially in Gambier archipelago, where the season is marked. This practice is believed to produce pearls with maximum lustre. Conversely, pearl harvest during summer season, where nacre deposition rate is at its highest in this period, is believed to produce pearls with lack of lustre. This is often observed in North Tuamotu Archipelago, where inter-season lagoon water temperature variation is low. A strong understanding of genetic correlations would be necessary to avoid inadvertent selection against non-target traits, such as the negative correlation observed between nacre deposition and lustre that has also been noted in the scientific literature (Alagarswami 1987; Snow *et al.* 2004). Snow *et al.* (2004) hypothesized that pearls with a brilliant lustre are produced by consistent and regular crystal formation. Nacre-based crystal formation is a complex biomineralisation process involving numerous genes (Joubert 2011). Pearl lustre is also known to be influenced by other environmental factors (Snow *et al.* 2004), in which indirect food availability, and thus cultural practice, e.g., frequency of oyster cleaning is very important (farmer pers. comm.).

INFLUENCE OF NACRE DEPOSITION RATE ON CULTURED PEARL COLOUR (DARKNESS AND "VISUAL COLOUR CATEGORIES")

Cultured pearl colour is defined according to two main characteristics: 1) the bodycolour, which is the dominant, overall colour of a pearl determined by a combination of several pigments; 2) the secondary colour, where some pearls display one or more additional overtones of colour in the reflection, but also diffusion and/ or diffraction from the top layers of the nacre surface. Combinations of bodycolours and overtones give *P. margaritifera* a wide range of colour appearances (Karampelas *et al.* 2011). Tayale *et al.* (2012) showed that nacre thickness and cultured pearl weight in pearls produced using wild donors were correlated with colour components, i.e., darkness levels and visual colour categories, with the palest pearl (i.e., white pearls) being the smallest (lowest thickness and weight). Here, this tendency is confirmed in pearls produced using donors from farmed families. Indeed, our results show that the palest pearls (low darkness level) had the thinnest layer of nacre and weighed the least.

The relationship between the six colour categories and darkness level, shown in figure 6, indicates an increase of darkness level (moderate + high levels) following the gradient: white < yellow < aubergine < grey < green < peacock. This gradient could represent a gradient of deposition of the dark tone, in which both melanin and metalloprophyrins have been implicated (Landman *et al.* 2001; Elen 2001). Peacock colour pearls are therefore particularly dark in tone, with only 14% of this colour group being classed as having low darkness level. This dark tone is a specificity of cultured pearls from *P. margaritifera* and corresponds to the so called "black pearls". *P. margaritifera* naturally secretes these pigments, which give predominantly "black pearls", with a base colour ranging from black to grey, depending on their quantity.

The present study highlights the fact that aubergine and peacock (mix of aubergine and green secondary colour) pearls, have the thickest and heaviest nacre deposition. In fact, aubergine and peacock pearls were on average 15% thicker and heavier than white pearls. Peacock pearls show a typical chromatic pattern, changing concentrically from greenish in the center to reddish at the peripheries. These chromatic patterns are known to be determined by the thickness, but also by the regularity of the nacre (Toyota and Nakauchi 2013). An interesting way of potentially increasing nacre thickness could be to select donor oysters that would produce mainly aubergine-peacock pearls, which can be done by identifying the corresponding inner shell coloration phenotype. This link between identification of donor inner shell coloration phenotype and corresponding pearl colour therefore needs to be studied. Nevertheless, for the *P. margaritifera* pearl industry, there is an evident interest in producing pearls of many different colours, especially for the European market. In the future, if the French Polynesian pearl industry comes to depend on a "hatchery system" (to provide a regular supply of donor oysters), donor lines could be developed in colours of interest.

The factors that contribute to the colour of a pearl include the oyster species, the thickness and number of layers of nacre, and the environmental quality of the culture zone where the receiver oyster is reared during pearl development (Snow *et al.* 2004). This is one reason why pearl farmers are becoming increasingly focused on raising environmental awareness. Although, research on colour determination in

cultured pearls is limited, the quality and nutritional content of the water is known to directly affect an oyster's health, growth rate and the quality of the nacre it produces (Linard *et al.* 2011). *P. margaritifera* is a bivalve with significantly higher algal clearance rates than *P. maxima* individuals of the same size (Pouvreau *et al.* 1999). Thus, if nutrition intake is compromised in these oysters, the nacre structure and quality of the pearl are likely to be negatively impacted, with a consequent effect on the colour.

5. CONCLUSION

In comparison to the correlations showing in Tayale *et al.* (2012) on *P. margaritifera*, this study: 1) was addressed to farmed donor mollusc (and not wild introduction), 2) concerned nacre weight (and not cultured pearl weight), with the notion of nacre deposition rate, 3) confirmed and specified the relationships between nacre thickness and weight, with pearl grade, pearl surface defects, pearl lustre, pearl darkness, pearl colour (bodycolour and overtone) and 4) revealed additional relationships between cultured pearl surface defect and lustre and pearl "colour categories" with darkness level.

Potential exists to improve the rapidity of pearl growth in *P. margaritifera*. The present study reveals that a high rate of nacre deposition (assessed by nacre thickness and weight), produces more grade A pearls, with less surface defects; this leads to maximum lustre, with a dominance of black tone, especially for aubergine and peacock cultured pearls. Conversely, low rate of nacre deposition produces more pearls of low grade, with more surface defects, minimum lustre and a dominance of pale toned colours, especially white bodycolour.

Selection of wild and farmed donor of *P. margaritifera* with high potential for nacre deposition could be used to increase cultured pearl quality. For such donor or family selection, molecular tools have already been selected through a high throughput gene expression study for the identification of 22 gene expression biomarkers for pearl quality and growth (Joubert 2011). Overall, the use of multi-trait selection approaches, which take into account quantitative genetic control, environmental effects and associated negative correlations may be the most effective strategy to improve pearl quality.

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ANNEXE 2 : INFLUENCE DE L'ENVIRONNEMENT SUR LA QUALITE DE LA PERLE



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Macro-geographical differences influenced by family-based expression on cultured pearl grade, shape and colour in the black-lip 'pearl oyster' *Pinctada margaritifera*: a preliminary bi-local case study in French Polynesia

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Abstract

In French Polynesia, the aquaculture of *Pinctada margaritifera* is carried out in numerous grow-out sites, located over three archipelagos (Gambier, Society and Tuamotu). To evaluate the impact of macro-geographical effects of these growing sites on pearl quality traits, five hatcheries produced families were used as homogeneous donor oysters in an experimental graft. The molluscs were then reared in two commercial locations: Tahaa Island (Society) and Rangiroa atoll (Tuamotu). At harvest, eight pearl quality traits were recorded and compared: surface defects, lustre, grade, circles, shape categories, darkness level, body and secondary colour and visual colour categories. Overall inter-site comparison revealed that: (1) all traits were affected by grow-out location except for lustre and round shape, and (2) a higher mean rate of valuable pearls was produced in Rangiroa. Indeed, for pearl grade, Rangiroa showed twice as many A-B and less reject samples than Tahaa. This was related to the number of surface defects (grade component): in Rangiroa, twice as many pearls had no defects and less pearls had up to 10 defects. Concerning pearl shape, more circled and baroque pearls were found in Tahaa (+10%). For colour variation, 10% more pearls have an attractive green overtone in Rangiroa than in Tahaa, where more grey body colour were harvested.

Lustre does not seem to be affected by these two culture site (except at a family scale). This is the first time *P. margaritifera* donor family have been shown to vary in the quality of pearls they produce depending on their grow-out location.

Keywords: *Pinctada margaritifera*, cultured pearl quality, grow-out location, environment

Introduction

Tahitian 'Black' South Sea cultured pearl production is based on aquaculture of *Pinctada margaritifera* a mollusc commonly known as the black-lip 'pearl oyster'. French Polynesia presently dominates black cultured pearl production, with 487 pearl farms, covering near 7800 ha of maritime surface area over 25 atolls or Islands distributed among three archipelagos: Gambier, Society and Tuamotu (DRMM 2013 statistics). Other countries of the Pacific region, such as the Cook Islands, Fiji islands or Micronesia, have also developed pearl farming industries based on *P. margaritifera* (Cartier, Krzemiński & Ito 2013). The production of a cultured pearl is a two-step process involving an initial 2 years oyster grow-out phase and then requiring a further 2 years after grafting to grow the actual pearl. The grafting process is essentially a surgical operation conducted by a trained technician, who inserts a small piece of tissue (*saibo*) cut from the

outer mantle edge of a sacrificed 'donor oyster', along with a small bead nucleus as for the most of saltwater pearls cultivation, into the gonad of a second 'recipient oyster'. Thus, the pearl culture process involves two oysters, each of which may genetically contribute directly or indirectly to one or more pearl quality traits, in interaction with the environment.

Tahitian cultured black pearl quality is determined according to a wide range of criteria, including pearl classification grade, surface quality and lustre, shape, colour (body colour and overtone), darkness level and size (Tayale, Gueguen, Treguier, Le Grand, Cochennec-Laureau, Montagnani & Ky 2012; Ky, Blay, Sham-Koua, Vanaa, Lo & Cabral 2013; Ky, Blay & Lo 2015). Tahitian classification grading (*Journal Officiel* 2001 no. 30, 26 July 2001) is an evaluation made with the naked eye (i.e. without any magnification device such as a jeweller's loupe), to grade pearls into five classes: A, B, C, D and R (rejects), the last of which are below class D quality and cannot be exported from Tahiti. This classification grading is determined by two components: pearl surface quality and lustre. Shape is also one of the principle determining factors of a cultured pearl's value; with the rounder the pearl, the more valuable it is (Ky, Blay, Sham-Koua, Lo & Cabral 2014). Circled pearls generally account for 25–30% of a Tahitian cultured pearl harvest. Another remarkable specificity of the *P. margaritifera* pearl oyster is its ability to produce a wide range of pearl colours (Ky *et al.* 2013). Cultured pearl colour is defined according to two main characteristics: (1) the body colour, which is the dominant, overall colour of a pearl determined by a combination of several pigments; (2) the secondary colour, which display one or more additional overtones of colour in the reflection, but also diffusion and/or diffraction from the top layers of the nacre surface (Karampelas, Fritsch, Gauthier & Hainschwang 2011). The predominant body colours are white, yellow, black and grey and the secondary colours are green, aubergine (red/purple) and peacock (a mix of aubergine and green) (Tayale *et al.* 2012; Ky *et al.* 2013; Ky, Blay, *et al.* 2014). Cultured pearl size (ranging from 8.0 to 20 mm) determines value, with larger pearls generally commanding higher prices (Blay, Sham-Koua, Vonau, Tetumu, Cabral & Ky 2014). The factors that contribute to cultured pearl quality include the oyster species and the environmental quality of the culture zone where the recipient oyster is reared during pearl develop-

ment (Gervis & Sims 1992; Southgate & Lucas 2008).

Understanding the influence of environment in the realisation of cultured pearl is particularly important, as numerous farms and grow-out sites are geographically distant and subject to disparate environmental regimes in French Polynesia. For example, in the context of a breeding program for pearl quality traits an understanding of the interactions between animals and environment is essential to ensure maximum genetic gains when multiple grow-out locations are used or targeted for the end product (Wada & Jerry 2008). To date, there have been few studies on family-specific interaction with environment in pearl oysters and all concern the silver-lip pearl oyster, *P. maxima*. Two of them have been restricted to survival and shell growth traits in (Kvingedal, Evans, Taylor, Knauer & Jerry 2008; Kvingedal, Evans, Lind, Taylor, Dupont-Nivet & Jerry 2010). Kvingedal *et al.* (2008) showed that families of 43-day-old *P. maxima* spat, when reared under different nursery culture conditions, exhibited environment-dependent growth patterns. Another study where potential environment impacts were examined in *P. maxima*, this time for pearl quality traits, was conducted in Indonesia by Jerry, Kvingedal, Lind, Evans, Taylor and Safari (2012) who reported significant interactions on cultured pearl size, colour, weight, shape and lustre due to differential ranking of families based on the relative performances at two commercial grow-out locations (Bali and Lombok). To date, no studies have treated the effects that environment could have on cultured pearl quality traits in *P. margaritifera*.

The aim of our study was therefore to evaluate the possible influence of macro-geographical grow-out sites on cultured pearl quality traits in *P. margaritifera*, namely: grade, surface defects, lustre, shape, circles, colour and its components (darkness level, body and secondary colours). This preliminary study will help for future breeding programs, that are likely to involve the rearing of oysters in geographically disparate locations. The present study was based on an experimental graft method where the grafting process was kept as uniform as possible by using same expert grafter, nucleus size, graft site and method (as for commercial grafting), donor oyster families (hatchery-produced) and recipient oyster source. To isolate environmental effects, grow-out was then conducted in two contrasting locations: Tahaa Island

lagoon (Society archipelago) and Rangiroa atoll lagoon (Tuamotu archipelago) (Fig. 1).

Materials and methods

Experimental animals

Five bi-parental families of pearl oysters, named F058, F612, F804, F805 and F806, were produced in a hatchery system at the Ifremer facilities in Vairao, Tahiti, French Polynesia. Spawning was induced by thermal shock following the procedures described in Ky *et al.* (2013). Breeding was conducted using five males and five females from the wild (unselected for growth performance or par-

ticular inner shell colour phenotype) broodstock pearl oysters from Takapoto atoll (Tuamotu Archipelago, French Polynesia). To avoid cross breeding contamination, the methods described in Ky *et al.* (2013) and Ky, Blay, *et al.* (2014) were used. Cleaning of female gametes, sperm motility checking and fertilization ratio were also described in the previously cited studies. After 24 h, the D-shaped larvae were collected on 45- μ m screens and each family was reared separately in a through flow culture system (Hui, Vonau, Moriceau, Tetumu, Vanaa, Demoy-Schneider, Suquet & Le Moullac 2011). Individuals of the five families, which were to be used as donor oysters, were grown at the field for 20 months and then transferred by plane to Ran-

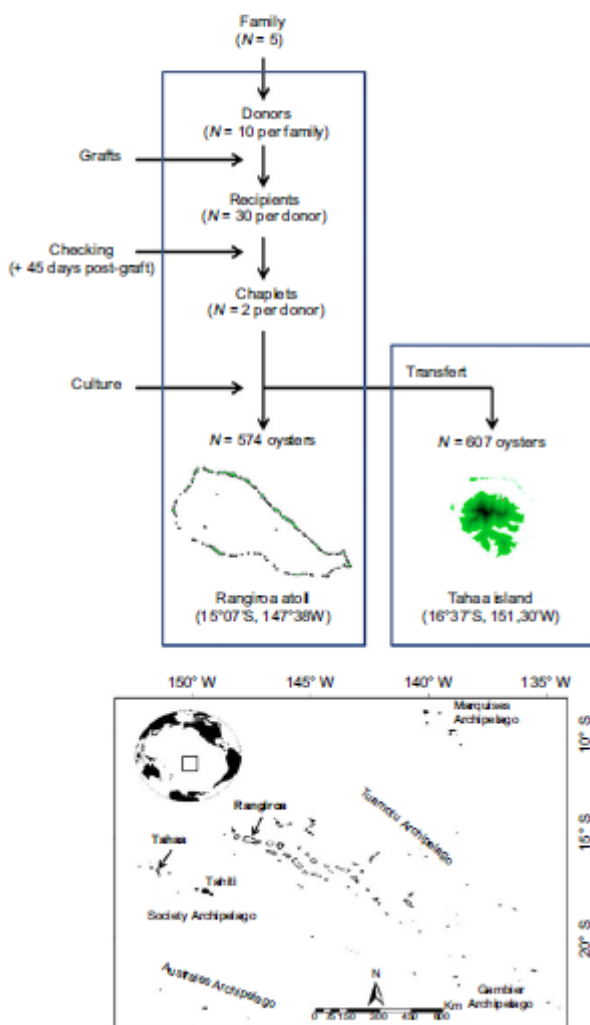


Figure 1 Experimental design of *Pinctada margaritifera* grafting, checking and culture operations. All the 1500 grafts operation was performed at Rangiroa. After checking, host oyster providing from each donor oyster were randomly split into two groups, one remain at Rangiroa and the second transferred to Tahaa. The harvests were realised in both sites.

giroa atoll (Tuamotu Archipelago), 2 months prior to nucleus implantation to allow the oysters to adapt to local environmental conditions.

Experimental grafting design

As the grafting operation may influence cultured pearl quality, all grafts were undertaken by a single professional technician. A total of 50 donors (10 per family) were used to perform 1500 grafts (30 grafts per donor) over a 3 day period in Gauguin's Pearl farm (Rangiroa atoll – Tuamotu archipelago) under the same conditions as for a commercial graft (Fig. 1). Each recipient pearl oyster came from natural spat collection from the wild. They were selected based on visible health status (colour of the visceral mass and gills), shell size appearance, and muscle resistance when the shells were pried open. Each recipient was grafted using a 2.4 BU nucleus (7.304 mm diameter; Nucleus Bio, Hyakusyo, Japan). At 45 days post graft operation recipient oysters were checked to estimate nucleus retention, nucleus rejection and oyster mortality rates were done as described in Ky, Molinari, Moe and Pommier (2014). After this check, recipient oysters that had retained their nuclei (no bead detected in the bags) were drilled and randomly fixed to chaplets (in the chaplets, oysters were attached in pairs to a rope with a monofilament fishing line) in two groups, after removing the net retention bags. As each chaplet was labelled according to the corresponding donor oyster (for traceability between donor and recipient), each donor oyster was represented by 2 chaplets. The 100 chaplets (each corresponding to one donor oyster) were split into two groups to be reared in two culture sites (50 chaplets per site), where each donor was equally represented per site. One of the two groups (607 oysters) was transferred by plane to Tahaa Island (16°37'S, 151°30'W), whereas the other (574 oysters) was maintained in Rangiroa atoll (15°07'S, 147°38'W) to evaluate site-specific environment influences on cultured pearl quality traits (Figs 1 and 2). The 'oysters' on chaplets were put in cooled iceboxes (without water) and the overall transfer operations took 4 h from water to water. Furthermore, pearl oysters were regularly cleaned in both sites, during the same periods and at the same frequency in order to remove biofouling (epibiota), which can hinder healthy oyster growth and pearl production.

Measurement of cultured pearl quality traits

After 16 months of culture in both Rangiroa and Tahaa sites, the cultured pearls were harvested and placed into a compartmented box that allowed traceability between sample pearls and corresponding donor oysters. Some *keshi* (small irregular shaped nacreous but non-nucleated pearls that form during the culture time after nuclei have been rejected) could also be harvested, but not graded. Cultured pearls were then cleaned by ultrasonication in soapy water (hand washing) with a LEO 801 laboratory cleaner (2 L capacity, 80 W, 46 kHz) according to Ky et al. (2013).

Cultured pearl quality traits: surface defects, lustre, grade, circle, shape, darkness level, and colour categories were evaluated visually (without loupe) by two operators working in cooperation, as described in Ky et al. (2013) and Ky, Blay, et al. (2014).

Environmental factors

For this inter-site comparison, made at the macrogeographic scale, the main environmental parameters (i.e. water temperature, nutrient levels), including seasonal variability, were deliberately not reported. Indeed, one goal of this study was to evaluate the impact that culture locations in French Polynesia had on cultured pearl quality traits realization over a wide geographic area. Both the environmental knowledge about the contrasting grow-out locations and the impact that food availability and temperature have on growth contribute to explaining the overall geographical variability observed in the present study. Indeed, these two main environmental parameters have been shown to influence growth and the biomineralization process in bivalves (Laing 2000; Pouvreau & Prasil 2000; Schöne, Houk, Freyre Castro, Fiebig, Oschmann, Kröncke, Dreyer & Gosseck 2005).

Statistical analysis

Differences in nucleus retention and rejection rate, oyster mortalities and harvested pearl and *keshi* rate in the five families in each grow-out location were evaluated using χ^2 tests.

Qualitative categories based on cultured pearl surface defects, lustre, grade, presence or absence of circles and darkness were re-encoded to give

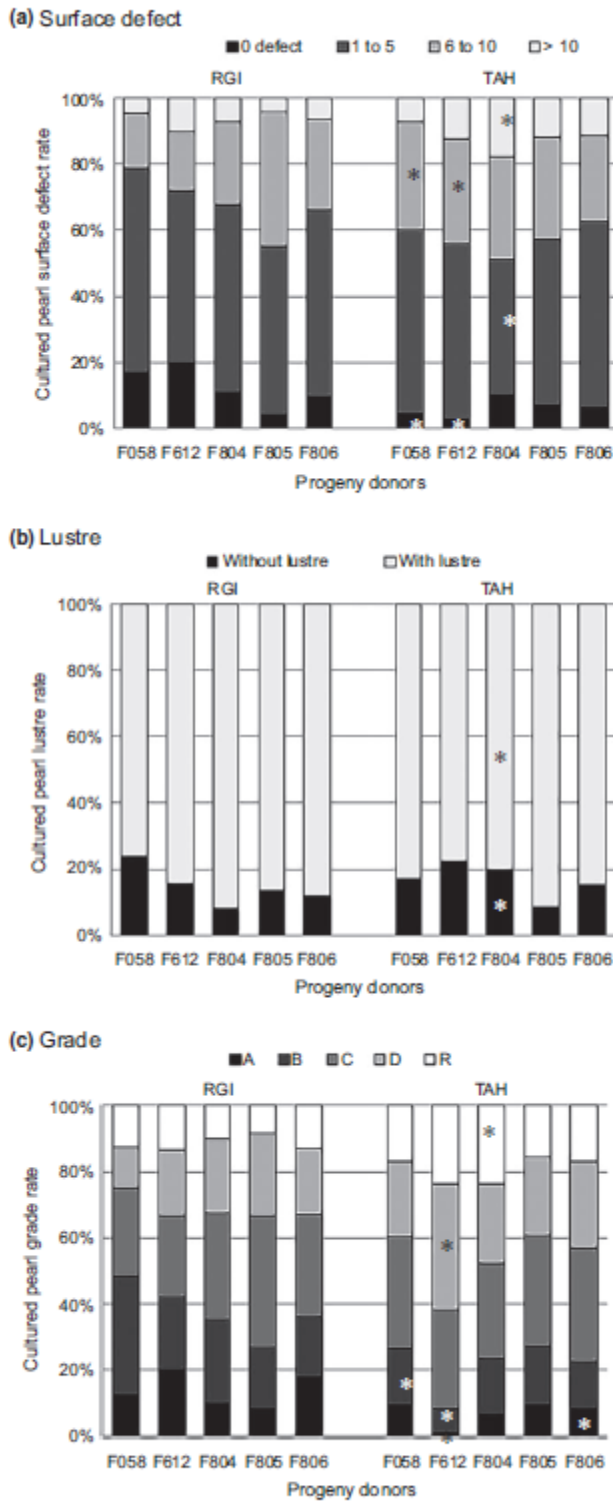


Figure 2 Proportions (%) of (a) each of the cultured pearl surface defect categories (four categories: no defect, 1–5 defects, 6–10 defects and more than 10 defects), (b) lustre (two categories: with or without lustre), (c) grade (five categories: A, B, C, D and R), for each of the five *Pinctada margaritifera* families reared at the Rangiroa (RGI) and Tahaa (TAH) grow-out sites. The data points significantly different ($P < 0.05$) for each family between the two grow-out sites are indicated with an asterisk (*) in the appropriate category on the TAH chart.

quantitative scores that would enable the mean value of families to be obtained for each trait, thus allowing them to be ranked. Scores from 0 to 4 were attributed to the different classes from the least to the most valuable (based on surface defects, lustre, grade circles and darkness) according to Ky *et al.* (2013) and Ky, Blay, *et al.* (2014). Due to non-normality of the variable distributions, non-parametric Kruskal–Wallis tests were used to test for differences between families in each grow out site.

For cultured pearl shape categories, presence/absence of secondary colour and visual colour categories, difference between families and effect of family in an intra-site level were evaluated using χ^2 tests.

Inter-culture site effect was evaluated for each quality trait category at two levels, by considering: (1) the cumulated value of the five families using χ^2 test, and (2) each family separately using Fisher's exact test (differences represented by asterisks in figure histograms).

In all tests, *P*-values lower than 0.05 were considered significant (Dagnelie 2007). All analyses were performed using XLSTAT (version 2009.4.02, Paris, France) and R software (version 2.14.1, The R Foundation for Statistical Computing, Auckland, New Zealand).

Results

Nucleus retention and cultured pearl harvest

The 1500 grafted pearl oysters showed an average rate of 78.6% nucleus retention ($N = 1181$), 15.2% ($N = 228$) nucleus rejection and 6.1% ($N = 91$) of mortality and/or predation (Table 1). No donor family effect was detected for nuclei retention ($P = 0.598$). In contrast, both nucleus rejection and mortality/predation showed significant donor family effects at 45 days post grafting: $P = 0.022$ (Table 1).

A total of 956 cultured pearls were harvested: 468 pearls in Rangiroa atoll and 488 pearls in Tahaa Island (Table 2). No significant donor family effect or site effect was detected for the rate of harvested pearls ($P = 0.414$ and $P = 0.363$ respectively). The rate of harvested keshi showed a highly significant site effect ($P = 0.004$). Significant donor family effect for harvested keshi was detected at both culture sites ($P = 0.044$ for Rangiroa and $P = 0.011$ for Tahaa). No family effect was detected for mortality/predation rate in

either culture site, although a highly significant site effect was detected ($P = 0.006$).

Cultured pearl surface defects and lustre as components of grade

For the cultured pearl surface defects, Table 3 showed the distribution in both site. Inter-culture site comparison revealed a very highly significant site effect ($P = 0.002$) for the two categories: (1) without any defects, and (2) with more than 10 defects (Table 3). Among the families, three showed a significant site effect: F058, F612 and F804. Family F058 showed a significantly ($P = 0.009$) higher proportion of pearls with no defects in Rangiroa, than in Tahaa, with nearly a fourfold difference (Fig. 2a). This family also produced a significantly greater percentage of pearls with 6–10 defects in Tahaa than in Rangiroa, with twofold difference ($P = 0.016$). Family F612 showed the same tendency as family F058, with: (1) 20.0% of pearls without any defects in Rangiroa, compared with 2.8% in Tahaa ($P = 0.0002$), and (2) nearly twice as many cultured pearls with 6–10 defects in Tahaa as in Rangiroa ($P = 0.021$) (Fig. 2a). Pearls from family F804 showed a significant site effect for: (1) 1–5 defects class ($P = 0.025$), with more in Rangiroa (+37.5%), and (2) >10 defects class ($P = 0.018$), with more in Tahaa (2.5 times more) (Fig. 2a). Concerning the donor family effect, a very highly significant effect was detected in Rangiroa ($P = 0.003$), whereas no significant effect was observed in Tahaa ($P = 0.339$).

For cultured pearl lustre, no significant site effect was detected at the inter-site comparison level ($P = 0.289$) (Table 3). Between the five families, F804 was the only one that showed a significant site effect for lustre ($P = 0.017$), with 10% more shiny pearls in Rangiroa than in Tahaa (Fig. 2b). Concerning the donor family effect, a significant effect was detected in Rangiroa ($P = 0.039$), whereas no significant effect was observed in Tahaa ($P = 0.107$).

For the cultured pearl grade, Table 3 showed the distribution in both site. Inter-culture site comparison revealed a very highly significant site effect on cultured pearl grade with $P < 0.0001$ for all categories except grade C, when average of all the families was considered (Table 3). Inter-culture site differences were shown in each of the families except for F805 (Fig. 2c). Pearls from family F058 showed a significant site effect for grade B

Table 1 Summary of grafted *Pinctada margaritifera* 'oysters' at 45 days post grafting: number and percentage (in brackets) of oysters by donor family that had retained their nuclei, rejected their nuclei, or died due to natural mortality and/or predation. After this checking operation, the oysters that had retained their nucleus were divided into two groups: one that was kept in Rangiroa and the other that was transferred to Tahaa

Donor families	t_0 Oysters grafted	t_{45} days post-graft				
		Oysters that retained their nuclei	Oysters that rejected their nuclei	Mortality and/or predation	Kept in Rangiroa	Transferred to Tahaa
F058	300	228 (76.0)	58 (19.3)	14 (4.7)	111	117
F612	300	243 (81.0)	44 (14.7)	13 (4.3)	117	126
F804	300	233 (77.7)	54 (18.0)	13 (4.3)	114	119
F805	300	240 (80.0)	32 (10.7)	28 (9.3)	117	100*
F806	300	237 (79.0)	40 (13.3)	23 (7.7)	115	122
Total	1500	1181 (78.7)	228 (15.2)	91 (6.1)	574	607

*23 oysters were lost during the culture period.

Table 2 Summary of the harvested *Pinctada margaritifera* cultured pearls in Rangiroa and Tahaa grow-out sites: number and percentage (in brackets) by donor family of cultured pearls, *keshi* and receiver oysters that had died due to natural mortality or predation after 16 months of culture

	Rangiroa			Tahaa		
	Harvested pearls	Keshi	Predation and/or mortality	Harvested pearls	Keshi	Predation and/or mortality
F058	89 (80.2)	5 (4.5)	17 (15.3)	83 (70.9)	14 (12.0)	20 (17.1)
F612	90 (76.9)	6 (5.1)	21 (18)	107 (85)	10 (7.9)	9 (7.1)
F804	99 (86.8)	0 (0.0)	15 (13.2)	107 (89.9)	4 (3.4)	8 (6.7)
F805	96 (82.0)	3 (2.6)	18 (15.4)	84 (84.0)	4 (4.0)	12 (12.0)
F806	94 (81.7)	1 (0.9)	20 (17.4)	107 (87.7)	3 (2.5)	12 (9.8)
Total	468 (81.5)	15 (2.6)	91 (15.9)	488 (83.6)	35 (6.0)	61 (10.4)

($P = 0.004$), with more in Rangiroa (+19%). F612 showed significant site effects for 3 grade categories: (1) grade A, with nearly 20-fold more in Rangiroa than in Tahaa ($P < 0.0001$); (2) grade B with nearly threefold more in Rangiroa than in Tahaa ($P = 0.004$); and (3) grade D with, inversely, nearly twofold more in Tahaa than in Rangiroa ($P = 0.004$) (Fig. 2c). Family F804 showed a significantly ($P = 0.009$) higher rate of grade R pearls in Tahaa than in Rangiroa (nearly twofold) (Fig. 2c). By contrast, family F806 had a significantly ($P = 0.04$) higher rate of grade A pearls in Rangiroa than in Tahaa (nearly twofold) (Fig. 2c). Data analysis showed a significant family effect on cultured pearl grade in Tahaa ($P = 0.05$), but none in Rangiroa ($P = 0.078$).

Circled cultured pearl and shape

Inter-culture site comparison revealed a very highly significant site effect on cultured pearl

circles, with $P = 0.003$. Rangiroa atoll showed a higher proportion (+10%) of cultured pearls without circles (68% vs. 59%) (Table 3). Inter-culture site comparison for incidence of circled pearl in each of the families showed a significant site effect in the three families F058 ($P = 0.0004$), F612 ($P = 0.0005$) and F804 ($P = 0.016$). All of these families presented a higher rate of pearls without circles in Rangiroa than in Tahaa; thus F058, F612 and F804 showed 75%, 83% and 70% without circles in Rangiroa, but in Tahaa they showed 49%, 61% and 53% respectively. The inter-site difference for this trait in these families therefore represented nearly 20%. A donor family effect was detected for the presence/absence in Rangiroa ($P < 0.0001$) and in Tahaa ($P = 0.037$).

Table 3 showed the distribution of the harvested pearl according to their shapes in both site. Inter-culture site comparison revealed no significant effect for the 'R' shape category

Table 3 Cultured pearl quality trait rates (%) and corresponding numbers (in brackets), recorded for the two grow-out sites: Rangiroa and Tahaa. Surface defects were encoded as follows: 0 (no defect), 1–5 defects; 6–10 defects and >10 (up to 10 defects). Lustre on the cultured pearl surface was encoded as follows: 'no' for absence and 'yes' for presence of lustre. Cultured pearl classification grade was encoded as follows: A, B, C, D and R (for Rejects). Circles on cultured pearls were encoded as follows: 'yes' for pearls with circles and 'no' for the samples without. For shape categories, 'B' corresponds to baroque or semi-baroque shapes; 'O' to oval, drop or button shape and 'R' to round or semi-round shapes. Cultured pearl colour was classified in three ways: darkness level (low, moderate and dark), body colour or secondary colour, and visual colour categories (grey, white, yellow, aubergine, green and peacock). The traits significantly different between the two grow-out locations at $P < 0.05$, $0.01 < P < 0.05$ and $P < 0.001$ are indicated with 1, 2 or 3 asterisk(s) (*), respectively, and NS for not significant

	Categories	Harvested cultured pearls			Inter-site significance
		Total	RGI	TAH	
Surface defect	0	9.2 (88)	12.2 (57)	6.4 (31)	**
	1–5	53.3 (509)	55.6 (260)	51.0 (249)	NS
	6–10	28.1 (269)	25.8 (121)	30.3 (148)	NS
	>10	9.4 (90)	6.4 (30)	12.3 (60)	**
Lustre	No	15.6 (149)	14.3 (67)	16.8 (82)	NS
	Yes	84.4 (807)	85.7 (401)	83.2 (406)	NS
Grade	A	10.1 (97)	13.7 (64)	6.8 (33)	***
	B	19.0 (182)	24.0 (112)	14.3 (70)	***
	C	31.5 (301)	31.0 (145)	32.0 (156)	NS
	D	23.9 (228)	20.0 (94)	27.4 (134)	**
	R	15.5 (148)	11.3 (53)	19.5 (95)	***
Circle	No	63.3 (605)	68.0 (318)	58.8 (287)	**
	Yes	36.7 (351)	32.0 (150)	41.2 (201)	**
Shape	B	52.8 (505)	47.2 (221)	58.2 (284)	***
	O	9.0 (86)	12.8 (60)	5.3 (26)	***
	R	38.2 (365)	40.0 (187)	36.5 (178)	NS
Darkness level	Low	33.1 (316)	29.9 (140)	36.1 (269)	*
	Moderate	57.5 (550)	57.5 (269)	57.6 (281)	NS
	Dark	9.4 (90)	12.6 (59)	6.3 (31)	***
Colour class	Body colour	46.8 (447)	40.6 (190)	52.7 (257)	***
	Secondary colour	53.2 (509)	59.4 (278)	47.3 (231)	***
Visual colour	Grey	41.2 (394)	34.8 (231)	47.3 (231)	***
	White	1.4 (13)	1.1 (7)	1.6 (8)	NS
	Yellow	4.2 (40)	4.7 (18)	3.7 (18)	NS
	Aubergine	4.8 (46)	8.3 (39)	1.4 (7)	***
	Green	40.5 (387)	45.7 (173)	35.5 (173)	***
	Peacock	7.9 (76)	5.4 (51)	10.5 (51)	**

($P = 0.268$). By contrast, a very highly significant site effect was recorded for 'O' shape ($P < 0.0001$), with a near twofold more in Rangiroa than in Tahaa (Table 3). In addition, for the 'BQ' shape, 10% more were found in Tahaa. Inter-site comparison between each of the families revealed that two of them showed a significant site effect: F612 ($P = 0.001$) and F804 ($P = 0.017$). Family F612 showed a higher proportion of 'BQ' cultured pearls in Tahaa (+15%) in comparison with Rangiroa ($P = 0.023$) and, inversely, a higher proportion of 'O' shape in Rangiroa (+9 times) than in Tahaa ($P < 0.001$). Family F804 has the same tendency as family F612 for 'BQ' shape, which was more frequent

($P = 0.05$) in Tahaa than in Rangiroa (+13%). A higher proportion of 'O' shape was also detected ($P = 0.013$) in family F804 in Rangiroa, with 8% of pearls in the 'O' category compared with 1% in Tahaa. In this last site, a significant family effect was detected ($P = 0.05$) and none in Rangiroa ($P = 0.208$).

Cultured pearl colour: darkness level and visual colour categories

Inter-culture site comparison revealed a very highly significant site effect on cultured pearl darkness level ($P = 0.002$). Twice as many dark pearls were produced in the Rangiroa site ($P = 0.001$) as

in Tahaa. By contrast, the Tahaa site exhibited 6% more of the palest cultured pearls than in Rangiroa ($P = 0.043$) (Table 3). Inter-culture site comparison in each of the five families, revealed significant family effect for three of them: F058 ($P = 0.002$), F612 ($P = 0.019$) and F806 ($P < 0.0001$). Family F058 showed: (1) nearly twice as many pale pearls in Tahaa than in Rangiroa ($P = 0.013$); (2) nearly 5 times more dark pearls in Tahaa than in Rangiroa ($P = 0.04$); and (3) more moderate darkness level pearl in Rangiroa (+25%) than in Tahaa ($P = 0.001$). Family F612 and F806 presented higher rates of dark cultured pearls in Rangiroa than in Tahaa with nearly 3 times and 10 times more respectively (Fig. 3a). Concerning the donor family effect, a very highly significant effect was detected in Rangiroa ($P < 0.001$), whereas no significant effect was observed in Tahaa ($P = 0.229$).

Inter-culture site comparison revealed a very highly significant site effect on cultured pearl body and secondary colour ($P = 0.0002$). Rangiroa atoll showed a higher proportion (+12%) of cultured pearls with secondary colours than Tahaa (Table 3). Inter-culture site comparison for each of the five families showed that three were significantly different between sites: F612 ($P = 0.023$), F804 ($P < 0.0001$) and F806 ($P = 0.028$). All these families showed a higher proportion of pearls with secondary colour in Rangiroa than in Tahaa, with: +16% (F612), +41% (F804) and +14% (F806) (Fig. 3b). In both site, a very highly significant donor family effect was detected for this site.

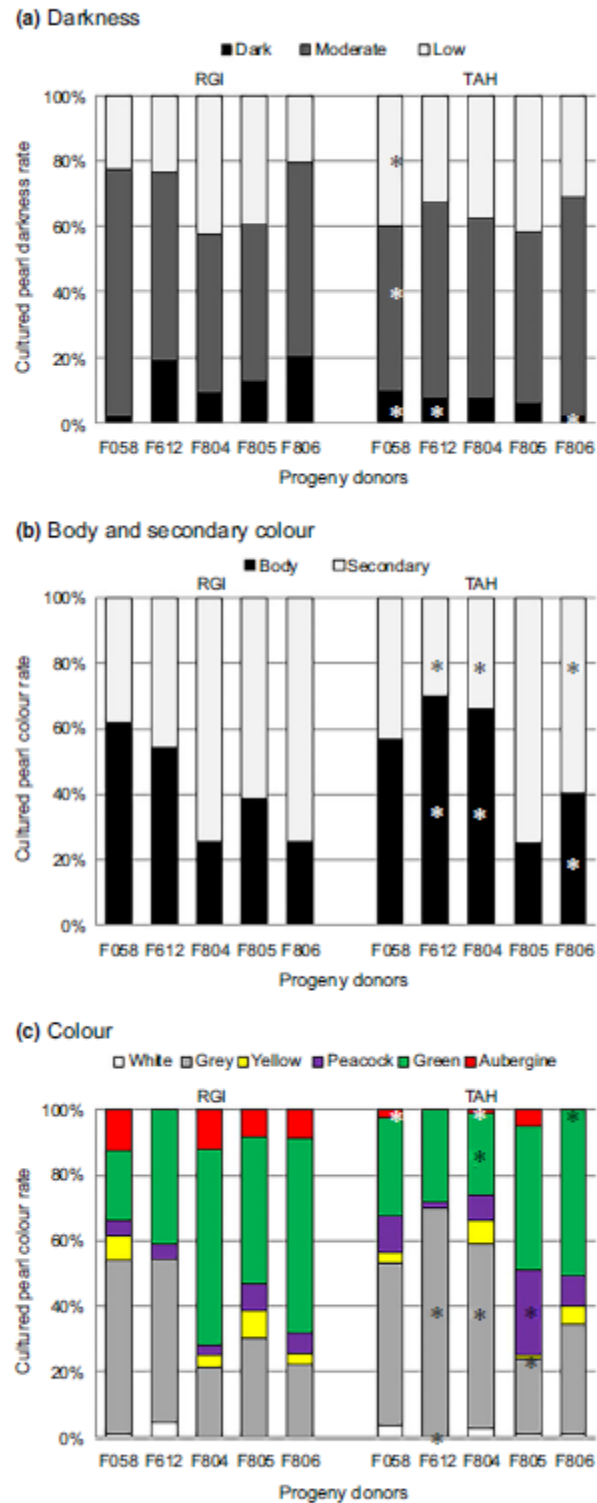
Table 3 showed the distribution of the harvested pearl according to their colour in both site. Inter-culture site comparison revealed a very highly significant site effect on cultured pearl colour ($P < 0.0001$). Rangiroa atoll showed a higher proportion of green and aubergine pearls ($P = 0.001$ and $P < 0.0001$ respectively) (Table 3). Tahaa site showed a higher proportion of grey and peacock samples ($P < 0.001$ and $P = 0.003$). Inter-culture site comparison between each of the families showed a significant site effect for all the families ($P < 0.001$) (Fig. 3c). Family F058 was only significantly different ($P = 0.01$) for the aubergine colour, which accounted for a far greater proportion in Rangiroa (5×) than in Tahaa. Family F612 showed three colour: grey, green and peacock in the Tahaa site. These three colours were also found in the Rangiroa harvest, but with the white colour in addition, which was absent from

Tahaa. For this family, the proportion of green pearls was significantly ($P = 0.05$) greater in Rangiroa than in Tahaa (+13%). In contrast, there were significantly more grey pearls in Tahaa than in Rangiroa ($P = 0.004$; +20%). Family F804 showed significantly ($P < 0.001$): (1) more aubergine (+11%) and green (+35%) pearls in Rangiroa than in Tahaa, and (2) more grey pearls (+35%) in Tahaa than in Rangiroa. Family F805 showed a significant site effect for yellow pearls, which were more frequent in Rangiroa (+8%) than in Tahaa. In contrast, this family had 8% peacock pearls in Rangiroa compared with 26% in Tahaa ($P = 0.001$) (Fig. 3c). Donor family F806 produced aubergine colour pearls in Rangiroa, but not in Tahaa. Conversely, no white pearls were harvested from this family in Rangiroa. In both site, a very highly significant donor family effect was detected for this site.

Discussion

This study was the first to examine whether macro-geographical differences influenced family-based phenotypic expression of cultured pearl quality traits in *P. margaritifera*. Accordingly, in this study we recorded eight cultured pearl quality traits in families of *P. margaritifera* when reared at two grow-out locations and showed that: (1) within each grow-out site, family effect was detected for grade, shape and visual colour categories, as already reported by Ky *et al.* (2013) working with hatchery produced families; (2) average grow-out site effect (comparison between mean value of the five families for each variable) existed for all variables except for lustre; and (3) family grow-out site effects (comparison between each of the family) also revealed variation in quality traits.

Family-specific pearl quality trait differences were found in our study, indicating the high levels of variability available for selection within populations. For cultured pearl grade (and its components: surface defects and lustre), the pattern of family grade was comparatively different between sites, whereby the most valuable families (from a pearl quality trait perspective) at one location tended not to be the most valuable at the other; for example, family F612 showed the highest rate of grade A pearls in comparison with the other families at the Rangiroa grow-out site, but had the lowest rate in Tahaa. This tendency was also



observed for the circle trait. Indeed, family F058 exhibited the highest proportion of cultured pearls without circles compared with the other families at the Rangiroa site, but was the one showing the lowest rate of circles in the Tahaa site. As the experimental graft was uniform (same: grafter, nuclei size and brand, donor oyster families and source of recipient), and animals reared during a same period, circle trait differences observed could not be attributed to grafter skill or seasonal variations (Ky, Nakasai, Molinari & Devaux 2015). In addition, these differences could also not be attributed to the 'oysters' transfer by plane as all the families were not affected in the same way. Therefore, we conclude that environment and interactions with family quality traits are of major consequence as results with the same donor families cultured at the Tahaa and Rangiroa sites differ for such quality traits, as classified by categories on a continuous scale and determined by expert eye of professional pearl graders. Although a previous study showed that inter-site differences in shell growth traits, quantitatively measured on a continuous scale in *Pinctada maxima* (Kvingedal et al. 2010), were of minor consequence between sites in Indonesia as the pattern of family growth performance was comparatively similar and ranking maintained, our present study has revealed environment effects with important ramifications. These effects should be considered in the design of pearl oyster breeding programs to improve quality traits. Nevertheless, our data suggest that the majority of family-determined quality traits, and thus the potential of oysters to produce high quality pearls, would be expressed regardless of which of the two sites the oysters were reared in.

The quality of a harvested cultured pearl is linked to the biomineralisation process, which is still poorly understood. Additionally, after the point of nucleus implantation into a host oyster, the farmer has limited control over how a pearl develops. The effect of site on pearl quality traits has never been previously evaluated in *P. margaritifera*. By contrast, the effect of site on shell growth had been evaluated, with previous studies suggesting that localised site effects significantly influence growth in black-lip pearl oysters. As the mantle, which is responsible for the biomineralisation process, is implicated in both shell growth and nacreous layers deposit on the nucleus, if site effects influence growth, they will probably also impact pearl quality, as confirmed by our results.

For example, both Sims (1994) and Pouvreau and Prasil (2000) demonstrated significant site-specific growth effects on *P. margaritifera* reared in the Cook Islands and French Polynesia, and Yukihiro, Lucas and Klumpp (2006) also found that growth rates differed greatly between *P. margaritifera* reared at two dissimilar sites within the Great Barrier Reef lagoon, Australia. Whilst the actual cause of these growth differences among grow-out sites is unknown, field studies show that water temperature, food availability, salinity and current flow rate all have an influence on growth (reviewed in Saucedo, Bervera-Leon, Monteforte, Southgate & Monsalvo-Spencer 2005). Differences in grow-out site between the lagoons of Tahaa Island and Rangiroa atoll were large and complex, involving variations in multiple parameters. One of the most important groups of factors is the variations in physical water parameters during the long period necessary for pearl culture. In fact, the biomineralisation process of a cultured pearl is known to be affected by both temperature and salinity (Kvingedal et al. 2008). Joubert, Linard, Le Moullac, Soyecz, Saulnier, Teaniniuraitemoana, Ky and Gueguen (2014) showed that shell growth is influenced by both microalgal concentration and temperature and also that these environmental factors regulate the expression of most of the gene matrix protein. Olson, Kozdon, Valley and Gilbert (2012) found a strong correlation between *P. margaritifera* shell nacre ultrastructure with environmental temperature and pressure. The Society archipelago, where Tahaa Island is located, is characterised by a significant rainy period (mostly during summer season) in comparison to Tuamotu archipelago, where Rangiroa atoll is located. Thus, seasonal variation in salinity may be more accentuated in Tahaa than in Rangiroa. These rainy periods in Tahaa may also drain some minerals and other nutrients or pollutants from the mountains, which did not occur in the atoll of Rangiroa as there are no mountains there. Such differences would also have an impact on food availability and the nutritional value of the specific and differing microalgae populations that grow in the two contrasting grow-out locations. Given that the sites experience different annual water temperature, different phytoplankton abundance and species, and are exposed to different current velocities, these environmental effects are likely to have contributed to the site-specific differences in cultured pearl quality detected.

Conclusions

In this experiment, there were significant differences in cultured pearl quality traits recorded on pearls produced with grafts from five full-sib families reared under the two contrasting grow-out sites. Even though the number of families we evaluated in this experiment was relatively low, the fact that we found differences in family response for cultured pearl quality traits indicated that environment and interaction with family effect on cultured pearl quality expression are important in *P. margaritifera*. In addition, awareness of their potential impact may need to be considered if oysters are going to be reared under disparate environments such in future genetic selection programs.

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ANNEXE 3 : INFLUENCE DE L'AGE SUR LE GRADE ET LA COULEUR



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Age-dependence of cultured pearl grade and colour in the black-lipped pearl oyster *Pinctada margaritifera*

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Abstract

Pinctada margaritifera is an economically important marine bivalve species for cultured pearl production in French Polynesian aquaculture. In order to evaluate the influence of donor oyster age on pearl quality traits, experiments were conducted over 6 years using both grafts and surgrefe operations. At harvest, six pearl quality traits were recorded and compared: surface defects, lustre, grade, darkness level and visual colour. Analysing the quality traits of pearls harvested in the initial graft process and those of pearls obtained from surgrefe experiments allowed a comparison of the influence of pearl sac cells originating from the initial mantle graft, which aged together with their recipient oysters. The results demonstrated a significant decrease between these successive grafts in lustre, grade (A-B-C), darkness level, and green colour – traits that are of major importance in the pearl market. The duplicated graft experiment allowed the comparison of donor oyster families at 2 and 5 years old, where a mantle graft was inserted into recipient oysters aged 2.5 years. The results showed the same tendencies to a lesser extent, with (i) an improved pearl grade, predominantly through a most important rate of 0 surface defect category, and (ii) a green/grey ratio in favour of the younger donor. A comparison between the graft-surgrefe and the duplicated graft experiments also highlighted: (i) the indirect role played by the younger recipient oysters, which must be optimized for optimal pearl quality realization, and (ii) the complex interplay between the donor and recipient oysters.

Keywords: pearl oyster age, *Pinctada margaritifera*, surgrefe, pearl grade, pearl colour

Introduction

Pinctada margaritifera (Linnaeus, 1758) (Mollusca, Bivalvia, Pteriomorpha) is a marine pearl-producing mollusc, principally cultivated in French Polynesia. *P. margaritifera* is found throughout the coral areas of the Indo-Pacific, but is particularly abundant in the atolls of French Polynesia. Cultured Tahitian pearl production is based on a surgical operation, which consists of introducing a round nacreous bead (these 'nuclei' are made from the shell of a freshwater mussel from the Mississippi River) into the gonad of a 'recipient' oyster, together with a small piece of mantle tissue (a graft of ~4 mm²) from a dissected 'donor' oyster (Kawakami 1952; Haws 2002). A recent report from the *Institut de la Statistique de la Polynésie Française* (Talvard & Challier 2015) summarized 2013 pearl production data. During this year, pearl production was conducted on 25 islands and atolls, compared with 28 in 2008. The number of maritime concessions was 517, which is 50 more than in 2008. The two most productive archipelagos were the Tuamotu and Gambier Archipelagos, which represented 398 (6300 ha) and 79 (1260 ha) concessions respectively. Cultured pearl exportation made 65 billion Euros for French Polynesia, which constituted the first increase (+10%) for this industry since 2007. The two main places importing Tahitian-cultured pearls were Japan (50% of the exportation volume) and Hong Kong (46%). Several auctions also took place in Tahiti in 2013 where the average pearl price reached up to 1000 FCP per unit; this is the first time such a figure has been achieved in the last 5 years.

Tahitian-cultured pearl quality is assessed according to an official A D classification (*Journal*

Officiel de la Polynésie française 2005). This grading system takes into account two physical parameters: (i) the perfection of the pearl surface and (ii) its lustre. Overall, there are five grades: (i) Top Gem: a perfect cultured pearl with an excellent lustre; (ii) Grade A: a surface that is 90% free from imperfections, with a very beautiful lustre; (iii) Grade B: a smooth surface to 70% of the pearl, with a good lustre at minimum; (iv) Grade C: a smooth surface to 40% of the pearl, with a medium lustre at minimum; and (v) Grade D: weak lustre, with small imperfections on more than 60% of the pearl. Under Tahitian Government regulation, cultured pearls of a quality below Grade D cannot be exported from Tahiti, although they can be sold locally. The surface quality is judged by looking at diverse imperfections such as dimples, bumps, stripes, curls, grooves, organic deposits, swellings, growths and milky, discoloured spots. The lustre (or shine) refers to the more-or-less perfect reflection of light from the surface of the pearl (Blay, Sham-Koua, Vonau, Tetumu, Cabral & Ky 2014). Colour is generally linked to pearl value for *P. margaritifera* in the Asian market: the darker it is, the more valuable the pearl is. The predominant body colours of *P. margaritifera* cultured pearls are grey, yellow and white. Overtones (secondary colours) may be present in a variety of combinations, including green, aubergine (reddish purple), and peacock, and are considered a plus factor. A completely black pearl with no overtones is considered less desirable and may be worth 50% less than one of a similar quality with green overtones (Ky, Blay, Sham-Koua, Lo & Cabral 2014).

In the context of a breeding program for pearl quality traits, an understanding of the influence of genetics and the environment, as well as the interactions between the two, is essential to ensure maximum genetic gains in relation to the aquaculture of this particular pearl oyster species, as multiple grow-out locations are used for the end product. To date, studies on the basis of pearl quality traits have mainly focused on the genetics of the donor oyster. Indeed, the influence of the donor on pearl quality traits has been definitively demonstrated using reciprocal xenografts between *P. maxima* and *P. margaritifera* oysters, in which donors were found to have a significant influence on both colour and surface complexion (McGinty, Evans, Taylor & Jerry 2010). The xenografts revealed that when a *P. margaritifera* donor is used, the resulting pearls exhibit colours with a

black base (consistent with those of *P. margaritifera*), regardless of the host oyster species. Tayale, Gueguen, Treguier, Le Grand, Cochennec-Laureau, Montagnani and Ky (2012) and Ky, Blay, Sham-Koua, Vanaa, Lo and Cabral (2013) demonstrated significant donor and family effects on pearl-colour darkness and visually perceived colour (body colour and overtone), pearl surface defects, lustre and grade in relation to *P. margaritifera*, using individual wild donors and hatchery-bred families. The influence of the environment in the realization of pearl quality traits has also been reported to be particularly important, as shown by the recent study on *P. margaritifera* conducted on Tahaa Island and Rangiroa atoll, where overall inter-site comparison revealed that: (i) all traits were affected by grow-out location, except for lustre, and (ii) a higher mean rate of valuable pearls was produced in Rangiroa (Ky, Blay, Aiho, Cabral, Le Moullac & Lo 2015). In relation to *P. maxima*, significant interactions between cultured pearl colour and lustre were observed by Jerry, Kvingedal, Lind, Evans, Taylor and Safari (2012) at two commercial Indonesian grow-out locations (Bali and Lombok).

Despite this existing knowledge about the complex interplay between donor, recipient and environment, no studies have examined the effects that the age of the oysters might have on cultured pearl quality traits in *P. margaritifera*. Presently, most pearl production is realized by using both donor and recipient oysters of approximately 2–3 years old. This age range could be exceeded if surgreffe operations were performed. In fact, recipient oysters that produce pearls fitting the criteria for good quality may be seeded with another nucleus to produce larger pearls during a subsequent culture period. Such surgreffe operations can be performed several times (3–4 times maximum), over the course of which the recipient oysters will naturally age. The aim of our study, therefore, is to evaluate the possible influence of oyster age on cultured pearl quality traits in *P. margaritifera* – namely, grade, surface defects, lustre and colour and its components (darkness level, body colour, and secondary colours). This study was based on experimental grafts and surgreffe methods, in which the grafting process was kept as uniform as possible by using the same expert grafter, nucleus size, graft site, and method (as used for commercial grafting), donor oyster families (hatchery-produced) and recipient oyster

source. This study will help with management and propagation of future oyster-line breeding programmes in hatchery systems.

Materials and methods

Surgreffe experiment: donor and recipient oyster ageing

Ten bi-parental families of *P. margaritifera*, produced in the Ifremer hatchery facilities in Vairao (Tahiti, French Polynesia), were used as donors in a previous graft experiment (Ky et al. 2013). First, a pool of corresponding recipient oysters aged 2.5 years were grafted as part of this previous experiment (Ky et al. 2013), then they were used in the present surgreffe trial at 4 years by inserting a second nucleus into the oysters (Fig. 1). In order to minimize environmental effects, the surgreffe experiment was undertaken (i) on a single grow-out site on Rangiroa atoll

(Tuamotu Archipelago, French Polynesia); (ii) on one pearl farm (Gauguin’s Pearl Farm), overseen by one professional grafter, so as to minimize differences (the same grafter who performed the initial grafts described in Ky et al. 2013) and (iii) with the same nucleus size and brand: 3.5 BU nucleus (10.48 mm diameter and 1.84 g weight – Nucleus Hyakusyo, Hyogo, Japan). Figure 1 shows that, of the pearl oysters initially grafted, 711 were not used for the surgreffe experiment. This was due to mortality, rejection during culture and shell breakage caused by forced opening during harvest of pearls from the initial grafting operation. Consequently, 783 recipient pearl oysters were used in the surgreffe experiment (Fig. 1). After the surgreffe operations had been undertaken, the recipient pearl oysters were put on to chaplets in groups of 10 and were covered with a plastic mesh to avoid predation. During the culture time, the pearl oysters were cleaned every 6 months using high-pressure seawater (Kärcher®, Winnenden, Deutschland).

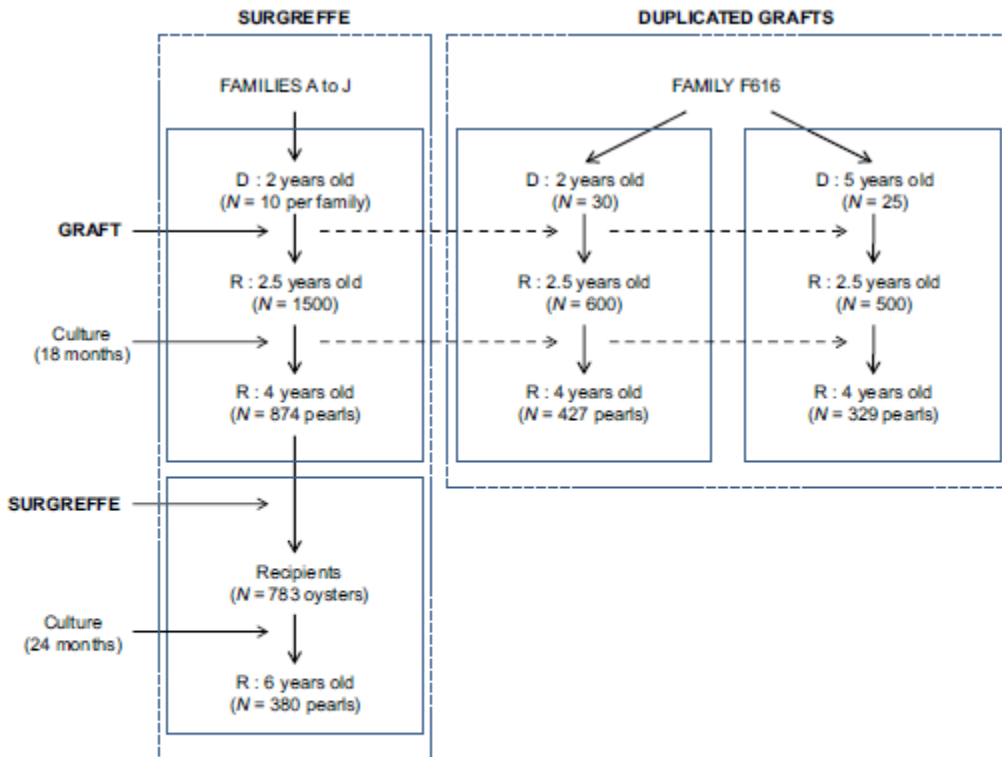


Figure 1 Experimental designs for the surgreffe method (following the experimental graft conducted by Ky et al. 2013) and the duplicated grafts. All operations were performed on Rangiroa atoll (Tuamotu Archipelago).

Duplicate graft experiments: donor oyster ageing with pools of recipient oysters of fixed age

A bi-parental family named F616 (produced in hatchery system at the Ifremer facilities in Vairao) was used as the donor oyster family for two distinct experimental grafts: at 2 years old (G_{D2}) and 5 years old (G_{D5}) (Fig. 1). The two grafts were undertaken by the same professional technician. A total of 30 donors for G_{D2} and 25 donors for G_{D5} were used to perform 600 and 500 grafts, respectively (20 grafts per donor) at Gauguin’s Pearl Farm (Rangiroa atoll, Tuamotu Archipelago), under the same conditions as for a commercial graft (Ky, Molinari, Moe & Pommier 2014). The two batches of 600 and 500 recipient pearl oysters came from natural spat collection from the wild in the same geographic region (Ahe atoll) and were collected during the same spat collection seasonal period (at the end of each year). They were all aged around 2.5 years and were selected based on visible health status (colour of the visceral mass and gills), shell size appearance and muscle resistance when the shells were pried open. Each recipient was grafted using a 2.4 BU nucleus (7.304 mm diameter – Nucleus Bio, Hyakusyo). Following implantation, the recipient oysters were placed on chaplets in groups of 10 and were

protected with plastic mesh to avoid predation. During the culture time of 18 months, the pearl oysters were cleaned every 6 months using high-pressure seawater (Kärcher®).

Measurement of cultured pearl quality traits

The cultured pearls were cleaned in soapy water (hand washed) via ultrasonication, using LEO 801 laboratory cleaner (2-L capacity, 80 W, 46 kHz); they were then rinsed in distilled water. The surface defects, lustre, darkness, and colours of the cultured pearls were evaluated visually (without a loupe) by two operators working in cooperation with one another.

The surface defects and lustre (both components of the cultured pearl grading system) were determined separately so that they could be studied independently. Visible sample surface defects, including pits, bumps, scratches, deposits, and other surface flaws, were counted visually (without a magnifier), and each cultured pearl was then classified into one of four categories: (i) no defects, (ii) 1 to 5 defect(s), (iii) 6 to 10 defects and (iv) up to 10 defects (Fig. 2a). Pearl lustre was evaluated as follows: presence of lustre (glossy and shiny) and absence of lustre (matte appearance).

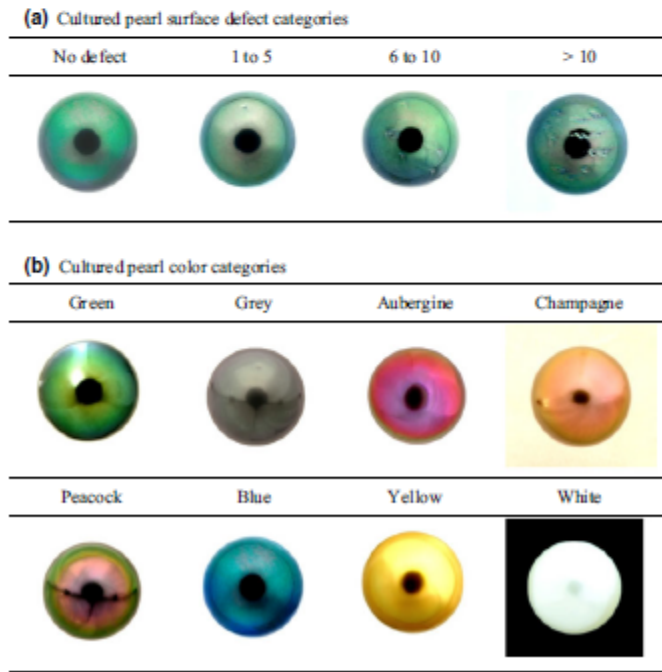


Figure 2 Cultured pearl surface defect (a) and visual colour (b) categories produced by *Pinctada margaritifera*. The picture samples indicate a round pearl shape.

The cultured pearl grade for each sample was determined by a single professional expert from *Maison de la Perle*, according to the official Tahitian grading system, from the most valuable to the least: A, B, C, D, and rejects (*rebut*s) (Journal Officiel de la Polynésie française, 2005). Rejects are cultured pearls that have too many defects to be graded. These pearls were discarded and, ultimately, destroyed.

Two kinds of colour evaluation (without loupe) were made in relation to the cultured pearls (Tayale *et al.* 2012): (i) the darkness of the colour, categorized into one of three groups, depending on the level: high, medium or low; and (ii) the visually perceived colour, caused by pigment (body colour) and secondary colour (overtone). Eight 'colour categories' (Fig. 2b) were detected, into which all the harvested pearls were classified (body colours: grey, white and yellow; secondary colours: green, aubergine, blue, champagne, and peacock – this last being a mixture of aubergine and green).

Statistical analysis

For the surgrefe experiment, the family effect among the 380 harvested cultured pearls obtained from the 10 families was analysed using a chi-squared test for all variables (Siegel & Castellan 1988; Winer, Brown & Michels 1991). Of the 380 cultured pearls harvested, 295 could be paired with pearls harvested from the same oysters in the graft experiment (Ky *et al.* 2013) to the present surgrefe experiment. These 295 paired samples made it possible to perform a comparison between graft and surgrefe methods in relation to: (i) lustre classes (presence/absence of lustre, using a McNemar chi-squared test) and (ii) surface defects, grade, darkness level, and colour categories (using a Friedman test) (Hutchinson 1996).

For the duplicated graft experiments, a comparison between G_{D2} ($N = 427$) and G_{D5} ($N = 329$) was made using: (i) a chi-squared test for the lustre classes and (ii) a logistic multinomial regression for surface defects, grade, darkness level and colour. The same tests were used to compare the quality traits of pearls obtained from G_{D2} and G_{D5} donors in the surgrefe experiment.

All the tests were performed using XLSTAT (version 2009.4.02), and P -values lower than 0.05 were considered significant (Dagnidie 2007).

Results

The effect of the surgrefe method on cultured pearl lustre, surface defects and grade

For the 380 harvested pearls produced using the surgrefe method, the overall rate of pearls with lustre was 45.8% ($N = 174$), with minimum and maximum values of 34.0% (Family A, $N = 18$ among 53 harvested pearls) and 57.1% (Family F, $N = 20$ among 35 harvested pearls) respectively. The corresponding average rate of pearls without lustre was 54.2% ($N = 206$). A comparison between the surgrefe and graft results ($N = 295$) revealed a highly significant difference ($P < 0.0001$) in average lustre pearl rates of 47.8% (surgrefe) versus 89.1% (initial graft) (Table 1). Thus, there was a –41.3% decrease

Table 1 Comparison between the graft and surgrefe experiments conducted with *Pinctada margaritifera* in terms of cultured pearl lustre (Yes: with lustre; No: without lustre), surface defects (0: no defects; 1: 1 to 5 defects; 2: 6 to 10 defects and 3: more than 10 defects), and classification grade (A, B, C, D, and R – reject)

Variables	Categories	Graft	Surgrefe	Significance
Lustre	Yes	89.1 (263)	47.8 (141)	***
	No	10.8 (32)	52.2 (154)	***
Surface defects	0	7.5 (22)	1.0 (3)	***
	1 to 5	52.2 (154)	9.5 (28)	***
	6 to 10	30.2 (89)	14.6 (43)	***
	>10	10.2 (30)	74.9 (221)	***
Grade	A	8.1 (24)	0.7 (2)	***
	B	24.1 (71)	2.0 (6)	***
	C	29.5 (87)	15.6 (46)	***
	D	27.8 (82)	50.5 (149)	***
	R	10.5 (31)	31.2 (92)	***

The first entry in each cell indicates the percentage contribution (%) for each of the quality trait categories in the graft or surgrefe operations. The second entry (in brackets) corresponds to the number of pearls observed in this category. The traits that were found to be significantly different in the graft and surgrefe operations ($P < 0.0001$) are denoted by ***.

in the lustre pearls obtained from the surgrefe method. Concerning the surface defect trait, the overall rate of pearls ($N = 380$) with no defects was 1.0% ($N = 4$), 1 to 5 defect(s) was 9.2% ($N = 36$), 6 to 10 defects was 18.2% ($N = 69$), and up to 10 defects was 71.6% ($N = 271$). For the 'up to 10 defects' category, family B showed the highest pearl rate (86.2%), whereas, family I gave the lowest rate (55.9%). A comparison of the surgrefe and graft results ($N = 295$) revealed a highly significant difference ($P < 0.0001$) between the rates of surface defects. In particular, there was as much as a sevenfold rise in pearls presenting up to 10 defects with the surgrefe method, in comparison with the graft method (Table 1). In addition, the rate of pearls with no defects was seven times smaller. Using surgrefe, the overall rate of Grade A pearls harvested was 0.5% ($N = 2$), with Grade B at 1.8% ($N = 7$), Grade C at 16.0% ($N = 61$) and Grade D at 47.6% ($N = 181$). The rate of reject pearls was 33.9% ($N = 129$). A comparison between the surgrefe and graft results ($N = 295$) revealed a highly significant difference ($P < 0.0001$) between all the grade categories. In particular, the average of the pearls categorized in classes A + B was nearly 12 times lower using surgrefe than in the corresponding graft experiment (Table 1).

No significant donor family effect was observed for the presence/absence of lustre in pearls obtained using the surgrefe method

($P = 0.372$). The family ranking, from the greatest to the smallest amount of pearls with lustre, was: F, E, I, C, H, D, J, B, G, A. Considering the families separately, significant differences were found in the numbers of lustre pearls obtained in the surgrefe and graft experiments in all families, except for E ($P = 0.206$) and G ($P = 0.102$). In addition, no significant donor family effect was observed for the surface defect category using surgrefe ($P = 0.484$). Considering the families separately, highly significant differences were found for surface defects between surgrefe and graft methods for all families. By contrast, a significant donor family effect was observed for the grade categories when surgrefe was used ($P = 0.005$). The family with the highest rate of Grade A pearls was Family F (2.9%), and Family A had the highest level of reject pearls (56.6%). In terms of the individual donor families, very highly significant differences were found between surgrefe and graft methods across all grade categories for all the families (Fig. 3).

Surgrefe effect on cultured pearl darkness level and colour

Looking at the pearl darkness level obtained using surgrefe, the overall rate among the 380 harvested pearls was 20.3% high darkness-level pearls ($N = 77$), 54.5% moderate darkness-level pearls ($N = 207$) and 25.3% low darkness-level pearls

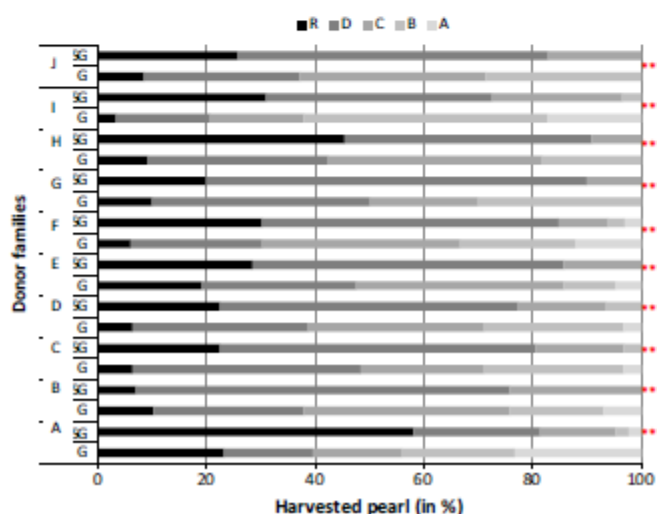


Figure 3 Comparison of culture pearl grade (A, B, C, D and R – reject) rate (in %) from graft (G) to surgrefe (SG), for each of the *Pinctada margaritifera* donor families (A–J). The difference between G and SG methods found to be very highly significant ($P < 0.0001$) are denoted by ***.

($N = 96$). Comparing the surgrefe and graft results ($N = 295$) revealed that there was a significant difference ($P = 0.04$) in high darkness levels, whereas no significant effect was observed for the moderate and low levels (Table 2). Thus, there was a -9% decrease in the high darkness levels achieved with the surgrefe method. In terms of the pearl colours obtained by surgrefe, the overall rates among the 380 harvested pearls were 40.8% green ($N = 155$), 33.7% grey ($N = 128$), 9.7% aubergine ($N = 37$), 7.9% champagne ($N = 30$), 5), 3.7% yellow ($N = 14$), 3.4% white ($N = 26$) and 0.8% peacock ($N = 3$). A comparison between the surgrefe and graft results ($N = 295$) revealed significant inter-family differences in the green ($P = 0.002$) and grey ($P = 0.042$) colour categories, whereas no significant effect was observed for the other colours. Thus, the trend exhibited was a decrease in green pearl from the graft to the surgrefe method (-12%) and an increase in grey pearls ($+7.5\%$) (Table 2).

A significant donor family effect was observed for pearl darkness levels obtained with the surgrefe method ($P < 0.0001$). Family G demonstrated the highest number of dark pearls on

average (nearly 40%), in comparison to Family I, where no dark pearls were found. The family ranking, from highest to lowest amount of dark pearls, was: G, H, A, F, J, C, D, B, E, I. Considering the families separately, no significant differences were found between surgrefe and graft methods for pearl darkness levels, except for Family B ($P = 0.040$) and Family J ($P = 0.013$). In addition, a significant donor family effect was observed for the pearl colours obtained using surgrefe ($P < 0.0001$). Family B had the highest average number of green pearls (65.5%), whereas Family H had the lowest number, with only 17.8% green pearls. Considering the families separately, no significant differences were found between the surgrefe and graft methods for the pearl colour statistics, except for in Families H, B and A (Fig. 4).

Duplicate graft effect on cultured pearl lustre, surface defects and grade

For the lustre pearl trait, a comparison between G_{D2} and G_{D5} revealed no significant difference ($P = 0.140$) between the average rate of pearls with lustre obtained in the two experiments

Table 2 Comparison between graft and surgrefe experiments for *Pinctada margaritifera* in terms of cultured pearl visual colours (body colours: grey, white and yellow; secondary colours: green, aubergine, champagne and peacock) and darkness levels (high, moderate and low)

Variables	Category	Graft	Surgrefe	Significance
Darkness	High	27.80 (82)	18.98 (56)	*
	Moderate	48.14 (142)	54.58 (161)	NS
	Low	24.06 (71)	26.44 (78)	NS
Colour	Green	56.27 (166)	43.73 (129)	**
	Grey	23.39 (69)	30.85 (91)	*
	Aubergine	5.42 (16)	8.14 (24)	NS
	Champagne	4.75 (14)	7.80 (23)	NS
	Peacock	4.07 (12)	2.37 (7)	NS
	White	4.07 (12)	5.08 (15)	NS
	Yellow	2.03 (6)	2.03 (6)	NS

The second entry (in brackets) corresponds to the number of pearls observed in the category. The traits that were significantly different between the two variants, at $0.01 < P \leq 0.05$, $0.001 < P \leq 0.01$, and $P \leq 0.001$, are indicated with 1, 2, or 3 asterisk(s) (*), respectively (and NS for 'not significant').

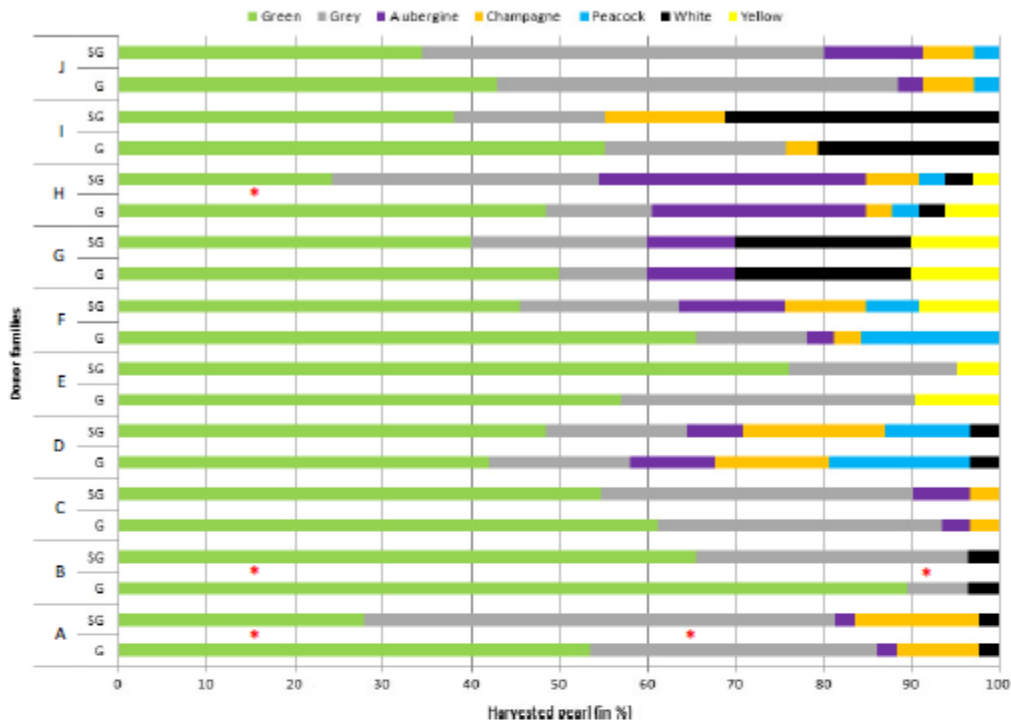


Figure 4 Comparison between the cultured pearl colour rates (green; grey; aubergine, champagne, peacock, white, and yellow) (in %) in the graft (G) and surgrefe (SG) experiments for each of the *Pinctada margaritifera* donor families (A–J). Significant differences between the graft and surgrefe methods in terms of green and grey colours are denoted by * ($P < 0.05$).

(Table 3). Thus, the age of the donor (2 years old versus 5 years old) does not seem to affect the luster trait.

In contrast, in terms of pearl surface defects, a comparison between pearls harvested from G_{D2} and G_{D5} revealed a very highly significant difference ($P < 0.0001$) for the ‘no defect’ category, where the rate was nearly double with a 2-year-old donor than with a 5-year-old donor. In addition, for the ‘6 to 10’ category, a very highly significant difference ($P < 0.0001$) was observed, with many more defects appearing in the 5-year-old group. However, no significant differences were observed for the ‘1 to 5’ ($P = 0.924$) and ‘>10’ ($P = 0.140$) defects categories.

With regard to the grades of the pearls, a comparison between the G_{D2} and G_{D5} groups revealed no significant difference ($P = 0.871$) for Grade A. The grade category B demonstrated a very highly significant difference ($P = 0.001$), with nearly three times more Grade B pearls being produced from 5-year-old donors than from 2-year-old

donors. Lastly, grade categories C, D, and R exhibited significant differences between 2-year-old donors and 5-year-old donors ($P = 0.011$, $P = 0.033$, and $P = 0.030$ respectively). Indeed, 2-year-old donors produced, on average, 10% more Grade C pearls and 10% fewer Grade D and Grade R pearls, than 5-year-old donors.

Duplicate graft effect on cultured pearl darkness level and colour

No difference was found between the pearls harvested from the G_{D2} and G_{D5} donors in terms of darkness level (Table 4): a similar range of darkness level was observed, with the most pearls in the moderate category, accounting for nearly 80%.

Where visual pearl colour is concerned, significant differences in pearls harvested from G_{D2} and G_{D5} donors were observed for the two most abundant colours, green and grey (Table 4). Indeed, the rate of green pearls obtained was 3.6 times

Table 3 Duplicate graft experiments comparing young (2-year-old) and old (5-year-old) *Pinctada margaritifera* donor oysters in terms of cultured pearl lustre (Yes: with lustre; No: without lustre), surface defects (0: no defects; 1: 1 to 5 defects; 2: 6 to 10 defects and 3: more than 10 defects), and classification grade (A, B, C, D, and R – reject)

Variables	Categories	2 years	5 years	Significance
Lustre	Yes	90.9 (388)	87.3 (288)	NS
	No	9.1 (39)	12.7 (41)	NS
Surface defects	0	34.2 (146)	16.1 (53)	***
	1 to 5	37.2 (159)	37.9 (124)	NS
	6 to 10	26.0 (111)	41.5 (137)	***
	>10	2.5 (11)	4.5 (15)	NS
Grade	A	0.7 (3)	0.6 (2)	NS
	B	3.3 (14)	9.1 (30)	***
	C	27.2 (116)	36.1 (118)	*
	D	35.8 (153)	25.5 (94)	*
	R	33.0 (141)	25.8 (85)	*

The second entry (in brackets) corresponds to the number of pearls observed in the category. The traits found to be significantly different between the two variants, at $0.01 < P \leq 0.05$, $0.001 < P \leq 0.01$, and $P \leq 0.001$, are indicated with 1, 2, or 3 asterisk(s) (*) respectively (and NS for 'not significant').

greater when using grafts from 2-year-old donor oysters than when using 5-year-old ones. For grey coloured pearls, the amount obtained was 1.5 times greater when using grafts from 5-year-old donor oysters than when using 2-year-old donors.

For the minority colours, no significance differences between the donor groups were observed for the peacock and white categories ($P = 0.702$ and $P = 0.618$, respectively). In contrast, a very highly significant difference was seen in relation to the aubergine and blue colour categories ($P < 0.0001$ and $P = 0.001$ respectively), with the average for aubergine pearls being 35 times lower and the average for blue harvested pearls being seven times higher for the 2-year-old donor than for the 5-year-old donor. Moreover, a highly significant difference was found between the 2-year-old donor and the 5-year-old donor for yellow pearls

Table 4 Duplicate graft experiments comparing young (2-year-old) and old (5-year-old) *Pinctada margaritifera* donor oysters in terms of cultured pearl visual colour (body colours: grey, white and yellow; secondary colours: green, aubergine, champagne and peacock) and darkness level (high, moderate and low)

Variables	Category	2 years	5 years	Significance
Darkness	High	9.4 (40)	8.2 (27)	NS
	Moderate	78.5 (335)	78.2 (257)	NS
	Low	12.2 (52)	13.6 (45)	NS
Colour	Green	42.6 (182)	11.8 (39)	***
	Grey	44.7 (191)	68.2 (225)	***
	Aubergine	0.2 (1)	7.3 (24)	***
	Blue	4.4 (19)	0.6 (2)	***
	Peacock	4.0 (17)	4.5 (15)	NS
	White	2.8 (12)	3.3 (11)	NS
	Yellow	1.2 (5)	4.2 (14)	**

The second entry (in brackets) corresponds to the number of pearls observed in the category. The traits found to be significantly different between the two variants, at $0.01 < P \leq 0.05$, $0.001 < P \leq 0.01$, and $P \leq 0.001$, are indicated with 1, 2, or 3 asterisk(s) (*) respectively (and NS for 'not significant').

($P = 0.007$), with the 5-year-old donor giving four times more yellow pearls on average.

Discussion

This study is the first to research the impact of pearl oyster age on pearl grade (including lustre and surface defects) and colour (in terms of darkness level and visual pigment) in *P. margaritifera*. As the cultured pearl production cycle in French Polynesia usually uses oysters of a common age (around 2.5 years) for graft operations, a better understanding of how age could modulate pearl quality traits is important for managing resource inputs (pearl oysters) and outcomes (cultured pearls). During the weeks following a graft operation, a pearl sac is formed in the gonad of the recipient oyster via cellular multiplication in the graft cells originating from the donor oyster (Machii 1968; Inoue, Ishibashi, Ishikawa, Atsumi, Aoki & Komaru 2011). Studies of the ultrastruc-

tures of pearl sacs from *Pinctada fucata martensii* have shown that they develop from the epithelial cells of mantle graft tissues (Du, Jiao, Deng, Wang & Huang 2010). Microsatellite analysis suggests that DNA originating from the donor oyster can still be detected in the pearl sac of pearl oysters (Arnaud-Haond, Goyard, Vonau, Herbaut, Prou & Saulnier 2007). The biomineralization process is the property of the epithelial cells of the external mantle of *P. margaritifera* (and by extent in mollusc with shells) to produced an organic matrix that controls nucleation, orientation, growth and the polymorphism of the calcium carbonate formed as aragonite and/or calcite from shell (Belcher, Wu, Christensen, Hansma, Stucky & Morse 1996). Matrix proteins play a major role in shell biomineralization process. Genes encoding some matrix proteins have been identified and are known to be specifically involved in the formation of the nacreous layer and/or the prismatic layer (Joubert, Piquemal, Marie, Manchon, Pierrat, Zanella-Cléon, Cochenec-Laureau, Gueguen & Montagnani 2010; Montagnani, Marie, Marin, Belliard, Riquet, Tayalé, Zanella-Cléon, Fleury, Gueguen & Piquemal 2011; Marie, Joubert, Tayalé, Zanella-Cléon, Belliard, Piquemal, Cochenec-Laureau, Marine, Gueguen & Montagnani 2012). Moreover, studies of the expression of two species-specific biomineralization genes (N66 and N44) in two pearl oyster species (*Pinctada maxima* and *P. margaritifera*) have revealed that donor oyster biomineralization genes are transcriptionally active in the pearl sac at the time of pearl harvest (McGinty, Zenger, Taylor, Evans & Jerry 2011). In the present study, the age of the cells derived from donor oysters was analysed in two ways: (i) through ageing together with the recipient oyster, studied via the surgrefe operation, and (ii) by using fixed-age recipients in two separate graft operations, where the ages of the donors were 2 and 5 years, respectively.

Overall, the results clearly demonstrate that there is a tendency for pearl quality traits to decrease in line with the ageing of the donor oysters. In the surgrefe experiment, and concerning pearl grade and its components, the grades A, B or C were obtained in up to three times fewer pearls than following the graft operation. This is consistent with most of the surgrefe harvests that have been observed in *P. margaritifera* production, where lustre, in particular, has been found to be reduced (farmers' com. pers.). This finding can

probably be attributed to the increase in the biological age of both the cells in the pearl sac, originating from the donor oyster (3.5 years old in the graft operation harvest and 5.5 years old in the surgrefe operation harvest), and the recipient oysters themselves (4 years old in the graft operation harvest and 6 years old in the surgrefe operation harvest). Indeed, ageing cells are characterized by several detrimental changes that cause differences in gene expression between younger and older individuals in the animal kingdom. For example, the more pronounced changes in expression of stress genes seen in younger individuals of the Antarctic bivalve *Laternula elliptica* as a response to injury in Husmann, Abele, Rosenstiel, Clark, Kraemer and Philip's (2014) study were in line with the age-dependent physiological differences witnessed elsewhere in marine bivalves (Philipp & Abele 2010). Potentially, this might indicate that the oysters are in better physical condition, which is corroborated by the higher mortality rates found in younger individuals following the graft operations in our study (15.9%), compared with older individuals in the surgrefe experiment (8.0%).

In the duplicate graft experiments that used recipient oysters of the same age, but donor oysters of two ages (2 and 5 years old) the grade classification rate was not clearly in favour of younger donor oysters. However, the statistics for surface defects showed that the younger individuals tended to be of a higher quality. This last trait has been shown previously to be influenced by donor oysters, as demonstrated by the xenograft experiments by McGinty *et al.* (2010) using *P. maxima* and *P. margaritifera*. Here, the effect of the donor species on pearl complexion was found to be highly significant, whereas the host species had no apparent influence on this trait. In fact, implantation with *P. maxima* mantle tissue produced pearls with smoother complexions (i.e. higher grades) than implantation with *P. margaritifera* tissue, regardless of the host oyster species. As grade classifications are based on lustre and surface-defect assessments, and a comparison between the two grafts in our study revealed similar rates of both pearl lustre and surface defects (in the '1 to 5' category), this may help to explain why effects of youth of the donor oyster in the duplicate experiment were not comparable to those in the graft-surgrefe experiment. This highlights the indirect role played by the age of the recipient oyster, which was fixed and young at harvest time in the

duplicate graft, in comparison with the graft-surgrefle experiment, in which the recipients were older at harvest time: 4 years old and 6 years old respectively.

Relations and interactions between recipient oysters and their rearing environments may also play a role, as the pearl lustre trait is known to be affected mostly by the environment (Ky, Nakasai, Molinari & Devaux 2015). Indeed, Snow, Pring, Self, Losic and Shapter (2004) hypothesized that pearls with a smooth surface and brilliant lustre are produced when consistent and regular crystal formation occurs, with their experiment confirming the hypothesis during winter, when nacre deposition is at its slowest. Nacre-based crystal formation is a complex biomineralization process in the mantle tissue, which involves numerous genes (Wang, Kinoshita, Riho, Maeyama, Nagai & Watabe 2009; Miyazaki, Nishida, Aoki & Samata 2010; Marie *et al.* 2012). Our results indicate that the lustre trait is not affected by the donor oyster's age, as no significant difference was observed in the duplicate graft. However, a difference in lustre incidence was observed in the graft-surgrefle experiment, where its rate was halved. This finding is supported by the similar pearl lustre rate found between the grafts realized before the surgrefle experiment and the average rate obtained in the duplicated graft (using 2 year-old donor oysters), which was 89.1%. Similarly, in McGinty *et al.* (2010) study, no significant differences in pearl lustre grades were evident among the various xenograft combinations, hosts and donor species used with *P. maxima* and *P. margaritifera*. We should therefore ask how a recipient oyster affects the rate of lustre in developing pearls. A reduction in the recipient's vigour with age could be an explanation, as the recipient regulates the metabolism of the pearl sac. Indeed, the pearl sac depends on the recipient oyster for the supply of nutrition throughout the period of pearl formation. A strong host oyster can provide sufficient nutrition and, potentially, a more suitable environment for the pearl sac, resulting in the greater vigour of the pearl sac, promoting nacre secretion rates (Yukihira, Klump & Lucas 1998). This has been seen with young oysters, where maximum shell growth was observed, in comparison with older individuals, where the growth rate slowed down (Pouvreau, Bacher & Héral 2000). In addition, the genomes of the recipient oysters may regulate the expression of biomineralization genes in the pearl

sac. In this way, the expression levels of these genes are controlled by the host oyster and are involved indirectly in pearl lustre expression.

Based on our results, the age of the mantle tissue derived from the donor oyster does not affect the pearl darkness level (except for in the graft-surgrefle experiment, where a correlation was found with high darkness). In terms of visual colour, the two most common colours were affected in the two experiments (graft-surgrefle and duplicated grafts) as follows: (i) the rate of green pearls was significantly higher with young donor oysters, and (ii) the rate of grey pearls was significantly higher in older donor oysters. In the existing literature, xenograft results have shown conclusively that the donor oyster is the primary determinant of pearl colour (McGinty *et al.* 2010). The results from a study of *Pinctada fucata martensii* has shown that the frequency of yellow pearls was significantly lower in a group produced by grafting mantle tissue from an inbred white line than from the brown lines (Wada & Komaru 1996). In addition, a study of the digital colour of *P. fucata martensii* has shown that the nacre colour of the donor oysters contributes to the resulting pearl colour (Gu, Huang, Wang, Gan, Zhan, Shi & Wang 2014). Recently, the pearl colour in *P. margaritifera* was demonstrated definitively to be related to the inner shell phenotype colouration of the donor (Ky, Le Pabic, Sham-Koua, Okura, Molinari, Nakasai & Devaux 2015). For the same wild spat collection location, frequency of colourful inner shells was higher in young individuals than in older ones. This was also confirmed with the older oysters used in successive surgrefle operations, which produced high frequencies of non-attractive, grey inner-shell phenotypes. Variations between inner shell colourations of juveniles and adults may suggest temporal variations in the fitness of the epithelial cells of the mantle, which was expressed as age-related green to grey pearl colour variation. The ageing of the mantle cells of the donor oysters seemed to alter the colouration in favour of the grey body colour. Dark tone is known to be linked to the deposition of black pigment, in which melanins have been implicated (Elen 2001; Landman, Mikkelsen, Bieler & Bronson 2001). Tyrosinases have been implicated in shell formation and pigmentation (Hofreiter & Schoneberg 2010; Cieslak, Rössmann, Hofreiter & Ludwig 2011) and catalyse melanin production (Sanchez-Ferrer, Rodriguez-Lopez, Garcia-Canovas & Garcia-Carmona 1995).

Thus, the alteration of colour in favour of the grey body colour observed could be caused by the high expression levels of tyrosinase genes, which are specific to the mantle tissues of pearl oysters (Aguilera, McDougall & Degnan 2014) and vary with age.

Conclusions

This 6-year-long study into the use of both graft-surgrefe and duplicated graft operations to examine the impact of the cellular age of the graft mantle derived from the donor oyster on pearl traits is the first that seeks to help us understand the complex processes involved in the realization of pearl quality in *P. margaritifera*. Here, the graft-surgrefe experiment demonstrated that ageing of the cells originating from the graft and recipient oysters leads to a significant decrease in lustre, pearls of grades A, B and C, darkness levels and rates of green pearls, all of which are traits of major importance in terms of demand in the Asian pearl market. The duplicated graft study highlighted the importance of the youth of both the donor and recipient oysters in relation to the prevalence of pearl surface defects and green and grey pearl colour rates. It can be concluded that, although both donor and recipient oysters may be involved in pearl formation in *P. margaritifera*, they probably play different roles. The present study has emphasized the role played by the donor oyster tissue, which influences the green/grey pearl colour ratio to a great extent in comparison with the recipient oyster. The latter may play a more significant role in regulating the rate of nacre secretion during pearl development, thus affecting lustre and grade in relation, predominantly, to the culture environment.

On a practical level, pearl farmers also appear to be aware of the impact of the age of the donor oyster being used for grafting in the pearl production process. Younger donor oysters, although they have limited mantle size for graft excision, must be considered for their quality. The genetic selection of larger individuals among hatchery progenies would, therefore, be of particular interest. Optimum shell-size selection would be also beneficial for recipient oysters, particularly in relation to surgrefe operations, where younger and larger recipient oysters could be used for initial graft in order to move the graft-surgrefe sequence forward in relation to recipient age and

thus increase the rates of lustre and colour in pearls produced by the surgrefe method. Fundamentally, further studies are needed to trace biomineralization gene expression patterns sequentially, for example, in mantle grafts and pearl sacs at harvest.

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ANNEXE 4 : CV

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 CNRS-EPHE-USR 3278, Ifremer /CRIOBE, Polynésie française/Perpignan
 Déterminisme génétique de caractères perlicoles d'intérêts chez l'huître perlière, *Pinctada margaritifera*
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- 2012-2014 (24 mois) **Volontaire du service civique : Ingénieur en génétique**
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 Amélioration de la qualité de la perle par la voie de la sélection génétique de l'huître perlière donneuse de greffon, *Pinctada margaritifera*
- Exploration des interactions génotypes * environnement
 - Expression des gènes associés aux variables de qualité/couleur
 - Gestion du cheptel (sexage, reproduction, élevage larvaire)
 - Planification des croisements et des campagnes de greffes/récolte
- 2011 (8 mois) **Ingénieur d'étude**
 IRD, UMR DIADE, Montpellier (34)
 Projet ARCAD : Crop adaptation to climate change : Genetic and evolutionary processes involded in phenological responses
- Génétique quantitative et des populations
 - Génotypage SNP sur le mil (Technique Veracode d'Illumina)
 - Gestion et caractérisation des ressources génétiques du Fonio
- 2010-2011 (6 mois) **Volontariat international à Niamey: Ingénieur en technique biologique**
 IRD, UMR DIADE, NIGER
 Compréhension de l'adaptation du mil à la variabilité temporelle et spatiale de la pluviométrie et étude de la domestication des plantes cultivées africaines (mil, fonio)

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LIVE-UNC (lab. insulaire du vivant et de l'environnement) NOUMEA (98)
 Valorisation de la biodiversité végétale : Stratégies d'adaptation aux sols ultrabasiques des plantes endémiques de la Nouvelle-Calédonie :
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FORMATION

- 2009 **Diplôme d'ingénieur** délivré par l'école de biologie industrielle (EBI)
 CERGY (95)
 Majeur Recherche et Développement équivalence Master 2
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COMPETENCES TECHNIQUES

- Langue Anglais : TOEIC 890/990 ; Allemand : base (LV2 : collège, lycée)
- Informatique Pack office, Statistica, R 2.14.1, Xlstat version 2012, GenMapper, Geneious, BioEdit, Genepop, Fstat
- Technique Biologie moléculaire (PCR, Q-PCR, extraction d'ADN et d'ARN, Génotypage SNP, microsatellite), sexage de *Pinctada margaritifera*, reproduction contrôlée et élevage larvaire

ENCADREMENT ET ENSEIGNEMENT

- Enseignement Université de Polynésie Française (2014/2015)
 Cours magistraux (sélection génétique) (8h)
 Travaux pratiques (Biologie moléculaire) (16H)
 Niveau master 2 module Aquaculture.
- Co-encadrements 2012 Stagiaire intechmer (4 mois)
 2013 Stagiaire Ecole sup de Biologie-Biochimie-Biotechnologie (6mois)
 2015 Stagiaire niveau Master 2 STEE spé océanographie (6 mois)

COMMUNICATIONS SCIENTIFIQUES

Publications

Barnaud A, Vignes H, Risterucci AM, Noyer JL, Pham JL, **Blay C**, Buiron M, Vigouroux Y, Billot C, **2012**, Development of nuclear microsatellite markers for the fonio, *Digitaria exilis* (Poaceae), an understudied West African cereal. *Am J Bot.*

Ky, C.L., **Blay, C.**, Sham-Koua, M., Vanaa, V., Lo, C., Cabral, P., **2013**, Family effect on cultured pearl quality in black-lipped pearl oyster *Pinctada margaritifera* and insights for genetic improvement. *Aquatic Living Resources* 26, 133-145.

Blay C., Sham-Koua M., Vonau V., Tetumu R., CabralP., Ky C.L.,**2014**, Influence of nacre deposition rate on cultured pearl grade and colour in the black-lipped pearl oyster *Pinctada margaritifera* using farmed donor families. *Aquaculture International* 22:937-953

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

Ky CL, **Blay C**, Lo C (**2015**) Half-sib families effect on cultured pearl quality traits in the black-lipped pearl oysters *Pinctada margaritifera*: testing for indirect benefits of polyandry and polygyny. *Aquaculture International*, 24(1), 171-182

Blay, C., Parrad S., Cabral, P., Aiho V., Ky, C.L., **2016**. Correlations between cultured pearl size parameters and PIF-177 biomarker expression in *Pinctada margaritifera* families reared in two contrasting environments. *ECSS (Estuarine Coastal and Shelf Science)*, 182, 254-260

Ky CL, **Blay C**, Aiho V, Cabral P, Le Moullac G, Lo C., **2017**. Macro-geographical differences influenced by family-based expression on cultured pearl grade, shape and colour in the black-lip pearl oyster *Pinctada margaritifera* : a preliminary case study in French Polynesia. *Aquaculture Research*, 48(1), 270-282

Ky CL, Demmer J, **Blay C**, Lo C, **2017**. Age-dependance of cultured pearl grade and colour in the black – lipped pearl oyster *Pinctada margaritifera*. *Aquaculture research* 48(3), 955-968.

Blay C, Planes S, & Chin-Long KY **2017**. Donor and recipient contribution to phenotypic traits and the expression of biomineralisation genes in the pearl oyster model *Pinctada margaritifera*. *Scientific Reports*, 7(1), 2696.

Congrès

Blay C, Planes S, Ky CL (2016). Combined interaction of size phenotype of donor and recipient pearl oyster and gene expression on final pearl growth in *Pinctada margaritifera*. 3rd international Conference Aquaculture and Fisheries, 29 September to 01 October 2016, London, UK

Blay C, Planes S, Ky CL (2016). Donneuse vs. receveuse, qui transmet les traits phénotypiques de la perle chez l'huître perlière *Pinctada margaritifera*? 37ème édition du Petit Pois Dérivé, 29-31 Août 2016, Amiens, France

Résumé

La production de perle de culture par l'huître perlière *Pinctada margaritifera* représente la seconde ressource économique après le tourisme en Polynésie Française. L'une des voies d'amélioration privilégiée de la qualité de production passe par la voie de la sélection génétique. Dans ce contexte, le déterminisme génétique des caractères d'intérêt perlicole et leurs variations à différentes échelles phénotypiques a été étudié. Les rôles respectifs de l'huître donneuse et de la receveuse, au travers de greffes expérimentales, ont révélées une corrélation positive entre les paramètres de croissance des coquilles de receveuses et la taille des perles, ainsi qu'un effet donneuse sur la qualité de la perle. Les analyses d'expressions de huit biomarqueurs de biominéralisation, codant des protéines des couches aragonitiques ou prismatiques, ont révélé une corrélation entre l'expression de ces gènes au niveau du sac perlier avec à la fois les paramètres de qualité des perles et de croissance des receveuses. L'âge de l'huître donneuse de greffon semble jouer un rôle déterminant aussi bien au niveau des phénotypes de la taille que pour le grade et les défauts de surface de la perle. Enfin, les valeurs d'héritabilité des phénotypes ont été estimées pour la première fois chez l'espèce, au travers de modèle animal utilisant des familles produites en éclosérie. Globalement, les résultats montrent une transmission génétique linéaire et schématiquement on peut dire que les receveuses contrôlent principalement la croissance et la taille des perles, alors que les donneuses influencent leur qualité.

Mots Clés

Pinctada margaritifera, génétique quantitative, héritabilité, expression de gène candidat, biominéralisation, perliculture

Abstract

Cultured pearl production in the pearl oyster *Pinctada margaritifera* represents the second largest source of revenue after tourism, and it is the top export industry in French Polynesia. One of pearl farming industry's greatest challenges is to "produce fewer but better pearls" through genetic improvement. To address this challenge, the genetic determinism of pearl quality traits and how they vary at different phenotypic scales was studied. The respective roles of donors and recipients was explored through uniform experimental grafts and revealed a positive correlation between recipient shell biometric parameters and pearl size, and a donor effect on pearl quality traits. Gene expression analyses of 8 biomineralisation biomarkers, encoding aragonitic and prismatic proteins, highlights a correlation between pearl sac gene expression with pearl quality traits and recipient shell biometric parameters. The age of the donor oyster also played a determining role with respect to the size phenotype and for grade and surface defects of the pearl. Finally, the heritability values of the phenotype were estimated for the first time for this species using an animal model on a family produced in a hatchery setting. Results show a linear genetic transmission and overall, suggest that the recipient oyster primarily controls the size of the pearls while the donor oyster influences the quality.

Keywords

Pinctada margaritifera, quantitative genetics, heritability, gene candidate expression, biomineralisation, pearl culture

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